Development of Fermentation Conditions for Baeyer-Villiger Monooxygenases Overexpressed in *E.coli* and

Approach to Synthesis of a New Chiral Ligand

by

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DEDICATION

To my beloved country
Libya

ABSTRACT

The Escherichia coli (*E.coli)* overexperssion systems for Baeyer-Villiger monooxygenases (BVMOs), cyclohexanone monooxygenase (CHMO), cyclopentanone monooxygenase (CPMO), and their mutants derived from directed evolution were used as catalysts in oxidations of 4-substituted cychexanones.

The proposal of this work is to show some advantage by using biocatalyst such as (BVMOs), and allows chemists to treat them as reagents in organic synthesis.

It was shown that the whole cell of Baeyer-Villiger monooxygenases carried out Baeyer-Villiger oxidation on a model compound with high volumetric productivity under non-grown conditions.

High enantioselectivity in the oxidation of 4-substituted cychexanones were ensured by choosing the most appropriate enzyme for a sepcific substrate. Suitable biocatalysts were obtained by screening whole cells under grown conditions. The highly enantioselectivities enzyme were used in scaled-up fermentations under non-growing conditions

Successful optimization and scale-up is a crucial part of the design and operation of industrial processes in order to increase production capacity. Two different fermentor scales were compared in order to optimize the fermentation conditions.

Based on the large scale optimized conditions, the yields of enantiopure lactones of all target substrates (4-substituted cychexanones) were sufficient to attach them to functionalized silica in an approuch to synthesis of a new chiral ligand.

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List of Abbreviations

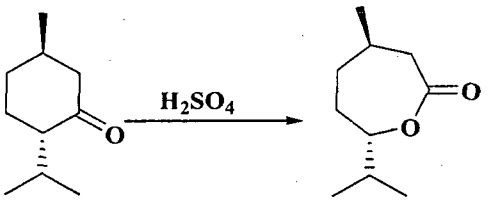
Ac	acetyl
Ben	Benzene
°C	degree of Celsius
δ	chemical shift
DO	dissolved oxygen
DMSO	dimethyl sulfoxide
E.Coli	Escherichia coli
ee	enantiomeric excess
Et	ethyl
GC	gas chromatography
h	hour
Hz	hertz
IPTG	isopropylthio-β-D-galactoside
IR	infrared, FT-RT Fourier transform-IR
J	coupling constant
LB	tryptone/yeast extract/NaCl medium
L	liter
LB-amp	LB medium + ampicillin
μ	micro
m-CPMA	*m*-chloroperoxybenzoic acid
Me	methyl

NAD(P)	nicotinamide adenine dinucleotide (phosophate)
NAD(P)H	reduced NAD(P)
OD	optical density
Ph	phenyl
ppm	parts per million
Pr	propyl
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
TsOH	*p*-toluenesulfonic acid
Tol	toluene

CHAPTER 1
Introduction

1.1 Baeyer-Villiger reaction

Baeyer-Villiger oxidation of cyclic ketones to lactones and linear ketones to esters is an important reaction that has been known to chemists for over a century.[1] The first example of this reaction used a mixture of sodium persulfate and concentrated sulfuric acid (Caro's acid)[1] to oxidize menthone to the corresponding lactone as shown in Scheme 1-1.



1-1 1-2

Scheme 1-1. Oxidation of menthone with Caro's acid

Later, persulfuric acid was replaced by an organic peracid, and the Baeyer -Villiger reaction became one of the best-known and widely applied reactions in organic synthesis.[2 (a, b)] Its success way largely because of its versatility: (i) a variety of carbonyl compounds can be oxidized; the acyclic ketones are converted to esters, cyclic ketones to lactones, benzaldehydes to phenols, and α-diketones to anhydrides; (ii) a large number of functional groups are tolerated; (iii) it is a stereoselective reaction, the migrating group retains its configuration; (iv) the regiochemistry is highly predictable, with the migratory

aptitude being tertiary alkyl >cyclohexyl >secondary alkyl >benzyl >phenyl >primary alkyl >CH_3;[3(a, b)] (v) a wide range of oxidants may be used, with their activity decreasing in the order CF_3CO_3H >monopermalic acid >monoperphthalic acid >3,5-dinitroperbenzoic acid >*p*-nitrobenzoic acid >*m*- CPBA-HCO_3H >$C_6H_5CO_3H$ > CH_3CO_3H >>H_2O_2 >*t*-BuOOH.[4]

1.1.1 Mechanism of the Baeyer-Villiger reaction

It is generally accepted that the mechanism proceeds by a two step process, as was initially proposed by Criegee (Figure 1-1).[5 (a, b)] In this mechanism a peroxy acid adds to a carbonyl group to form a so-called tetrahedral "Criegee intermediate", which undergoes rearrangement to the corresponding lactone or ester. It is important to note that, in general, the most substituted carbon centre migrates with retention of configuration. Steric, conformational, and electronic factors influence the rate of rearrangement and the migration preferences. Migration is also influenced by the type of peroxy acid used.[4]

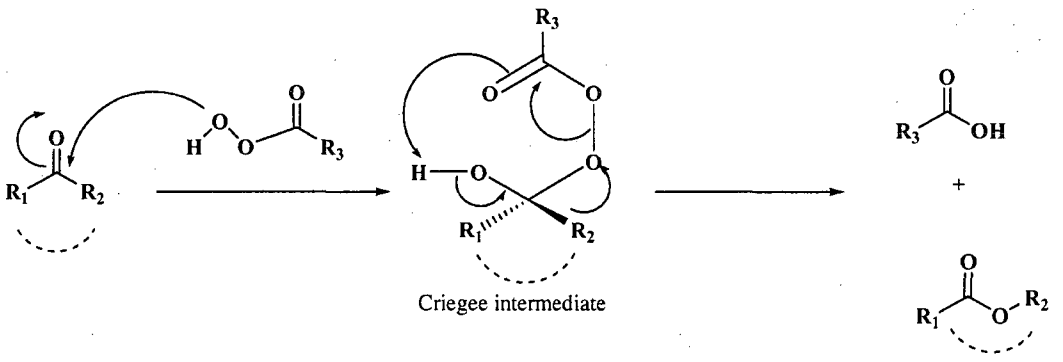


Figure 1-1. Mechanism of the Baeyer-Villiger reaction

An important property of the Baeyer-Villiger reaction is that migratory aptitude is highly predictable. This property has been attributed to a stereoelectronic effect

(Figure 1-2). According to stereoelectronic analysis of the Criegee intermediate, a proper alignment of orbitals is required for the rearrangement step to occur. The migrating group needs to be antiperiplanar to the O-O bond of the leaving group (primary stereoelectronic effect) and antiperiplanar to a lone pair of the hydroxyl group (secondary stereoelectronic effect).[6]

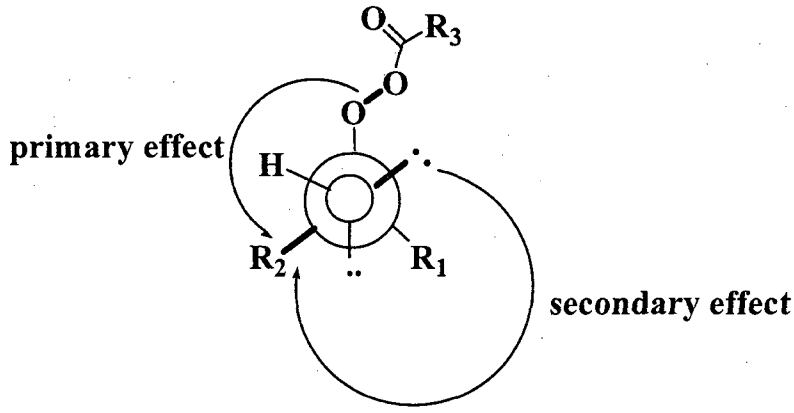


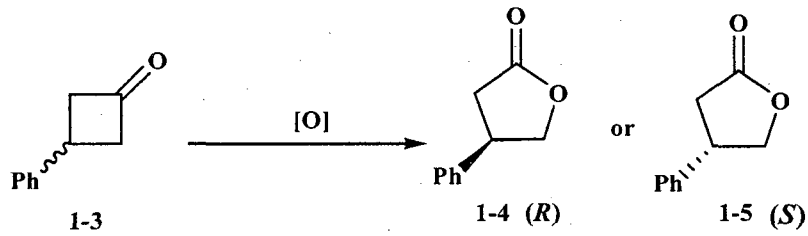
Figure 1-2. Stereoelectronic effects in Baeyer-Villiger reaction

1.1.2 Asymmetric Baeyer-Villiger oxidation of prochiral ketones

Baeyer-Villiger oxidation of prochiral cyclic ketones leads to the formation of a pair of enantiomeric lactones. Since enantiopure lactones are valuable intermediates for organic synthesis, the Baeyer -Villiger oxidation is an attractive target for modifications that would allow for an asymmetric version of the reaction. Asymmetry has been achieved when the Baeyer-Villiger reaction is carried out in the presence of chiral reagents or catalysts, be they organometallic compounds [7 (a-l)] or enzymes.[8(a,b)] As discussed above, Baeyer -Villiger oxidation is a two-step reaction: (i) nucleophilic addition of a peroxide, to give the Criegee adduct; and (ii) rearrangement of the adduct to

an ester or lactone. The stereochemistry of the Baeyer -Villiger reaction is therefore influenced by two factors: face selectivity in peroxide addition and selectivity in the bond migration. The migration factor is considered to have more influence on the stereochemistry of the Baeyer-Villiger reaction because rearrangement of the Criegee intermediate to lactones is irreversible.

Based on these considerations Watanabe and coworkers have tested a large number of organometallic catalysts in the Baeyer-Villiger reaction of racemic 3-phenylcyclobutanone (Scheme 1-2).[9] In this reaction the chiral catalyst imposes the required antiperiplanar configuration in the Criegee intermediate.

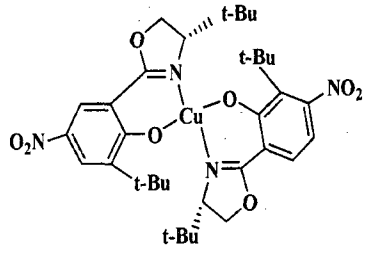
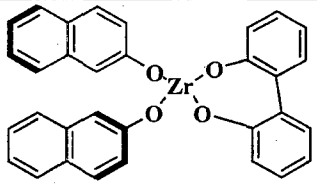
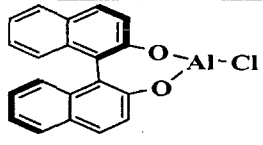
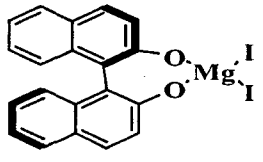
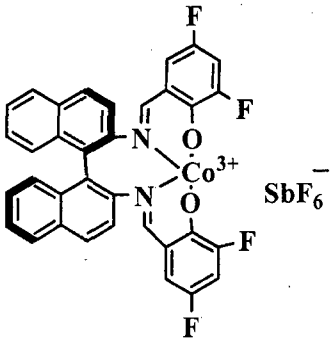


1-3 1-4 (*R*) 1-5 (*S*)

Scheme 1-2. Asymmetric Baeyer-Villiger reaction of racemic 3-phenylcyclobutanone.

Several reagents based on metal complexes and organocatalytic compounds have been used. However, so far, only moderate enantioselectivity has been achieved, and as the last entry in Table 1-1 shows, enzymes still out-perform organometallic catalysts.

Table 1-1. Baeyer-Villiger Oxidation of 3-phenylcyclobutanone

Catalyst	Conditions	ee (%), Yield(%)	Ref.
	1 mol% (*S,S*)-Cu-complex *t*-BuCHO	44 (*S*), 66	10
	1eq Zr-*S*-BINOL complex, 1.5eq TBHP, toluene, 25 °C	31 (*R*), 70	11
	15 mol% Al-*R*-BINOL complex CHP, CH_2Cl_2, 25 °C	68 (*R*), 100	12
	5 mol% *R*-BINOL-MgI_2, 1.5eq. CHP, CH_2Cl_2, 25 °C	65 (*R*), 91	13
	5 mol% Co-complex, 1.3 eq, 30% H_2O_2, EtOH, 0 °C	75 (*S*), 85	14
Cunninghamella echinulata	28°C, pH =8, cell conc: 5 g/L, 3.5 mL	>98 (*R*), 71	15

1.1.3 Disadvantages of the chemical methods

The protocols for Baeyer-Villiger oxidations using transition metal catalysts suffer from several disadvantages. Large amounts of catalysts are needed, adding to the cost and to the waste stream. The range of substrates tolerated is somewhat limited and the enantioselectivity obtained so far has been only moderate.

Furthermore, although several peroxy compounds can be used in the Baeyer - Villiger oxidation, toxic peracids like *m*-chloroperoxybenzoic acid (*m*-CPBA) and peroxytrifluoroacetic acid are most commonly used. Organic peracids are expensive and hazardous because of their shock-sensitivity and explosive character. Peracids are also powerful oxiditing agents, which react with other functional groups including alkenes, thioethers, and amines (which requires protection and deprotection steps in the synthetic strategies). The reaction also requires environmentally unfriendly halogenated solvents. Finally, because peracids are achiral, they afford racemic products.

There is a need therefore for "greener" versions of the Baeyer-Villiger oxidation reactions which would avoid the use of peracids and of transition metal catalysts. Good results have already been obtained with the fungus *Cunninghamella echinulata* (98% ee) (Table 1-1).[15]

1. 2 Enzyme-catalyzed Baeyer-Villiger reactions

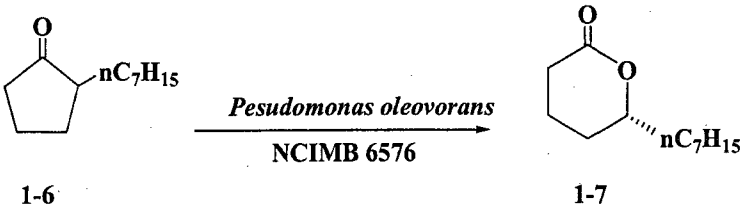
Asymmetric catalysis plays a profound role in modern organic chemistry.[16(a,b,c)] Numerous therapeutic drugs, plant-protecting agents, fragrances and most natural products are chiral, many exerting a specific biological effect only in one enantiomeric form. Many of these processes are successfully performed by enzymes.[17] The

advantages of biocatalytic reactions are: (i) high enantiopurity of product; (ii) potential to be environmentally friendly; (iii) capability for scale-up.

The Baeyer-Villiger reaction is a good candidate for biotransformation, since many microorganisms employ the Baeyer-Villiger reaction in the course of their metabolic pathways. The most extensively studied class of enzymes that perform such oxidations are referred to as Baeyer-Villiger monooxygenases (BVMOs). The fact that a number of these enzymes exhibit a broad substrate acceptability, in addition to high enantio- and regio-selectivity, make them particularly attractive to organic chemists.

1.2.1 Baeyer-Villiger monooxygenases

The first indication of a microbial Baeyer-Villiger process was discovered by Turfitt in 1948, in the microbiological degradation of steroid.[18] Baeyer-Villiger oxidation has since been encountered in the biosynthesis of several natural products, such as aflatoxins in fungi,[19] shellfish toxins,[20] and iridoids in plants.[21] Although many microorganisms produce enzymes that perform Baeyer -Villiger reactions, two bacterial species *Acinetobacter* sp. and *Pseudomona* sp. have received the most attention. In the first example of an asymmetric enzymatic Baeyer -Villiger oxidation, 2-heptylcyclopentanone was transformed into an optically active heptyl valerolactone by incubation with a whole-cell preparation of *Pseudomonas oleovorans* NCIMB 6576 **(Scheme 1-3)**.[22]



1-6 1-7

Scheme 1-3. Asymmetric enzymatic Baeyer-Villiger oxidation

Baeyer-Villiger monooxygenases have been found to catalyze *S*-oxidation of thiols,[24] dithioketals,[25] sulfites,[26] N- oxide formation,[27] boron oxidation,[28] selenium oxidation,[29] and epoxidation.[30]

1.2.2 Mechanism of the enzymatic Baeyer-Villiger reaction

The generally accepted mechanism for oxidations catalyzed by Baeyer-Villiger monooxygenases is shown in Figure 1-3.[31] Walsh and coworkers [32] first proposed this mechanism in their studies of cyclohexanone monooxygenase (CHMO) from *Acinetobacter calcoaceticus* NCIMB 9871.[33] This mechanism has been since re-investigated by Sheng, Ballou, and Massey.[31] They showed that the enzyme contains a tightly bound flavin adenine dinucleotide cofactor (FAD) that forms the key oxygenating intermediate. The biocatalytic process starts by reduction of this FAD by transfer of a hydride from a reversibly bound nicotinamide adenine dinucleotide phosphate (NADPH) and subsequent rapid oxidation by moleculer oxygen to produce the flavin 4-σ-peroxide anion. This intermediate constitutes the oxygenating species in the subsequent Baeyer-Villiger reaction. The terminal oxygen of this peroxide acts as a nucleophile and adds to the carbonyl of cyclohexanone to form a tetrahedral species analogous to the Criegee

intermediate. The catalytic cycle is completed with the elimination of water to reform FAD, and the release of the lactone and cofactor. Studies of the CHMO-catalyzed Baeyer-Villiger reactions using isotopic substitution and multinuclear NMR spectroscopy demonstrated that the mechanism of the enzymatic Baeyer-Villiger oxidation was similar to that of the non-enzymatic Baeyer-Villiger oxidation.[34] This implies that the enzymatic Baeyer-Villiger oxidation should obey the same stereoelectronic effects in the rearrangement of the Criegee intermediate.[35]

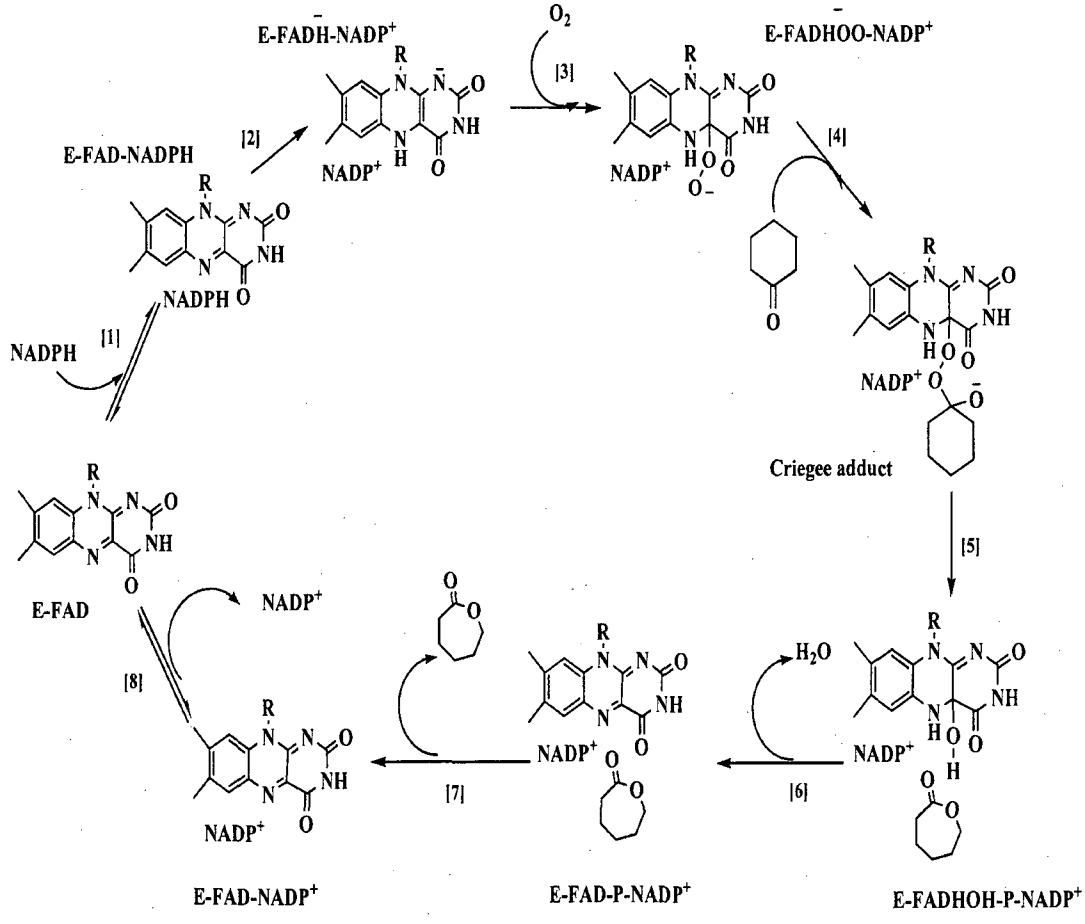


Figure 1-3. Mechanism of enzymatic (CHMO) Baeyer-Villiger oxidation.
Adapted from Sheng *et al.*[31]

1.2.3 Development of Baeyer-Villiger monooxygenases as bioreagents.

Baeyer-Villiger monooxygenases are reported to accept a multitude of non-natural substrates. Such biocatalysts are used in synthetic chemistry, either as isolated enzymes in combination with appropriate cofactor recycling systems, or as whole-cells in native or recombinant form.

I. Isolated enzymes strategy

The use of the isolated enzyme allows for the reaction to be performed at a higher substrate concentration and helps avoid side reactions. The major disadvantages of this method are the time and expertise required to isolate and purify the enzyme and the cost of the cofactor. Monooxygenases are dependent on two cofactors. The flavin prosthetic group is usually tightly bound to the enzyme and is regenerated in the catalytic cycle. The NADPH cofactor however, must be supplied. Because of the high price of NADPH it needs to be recycled to enable a cost-efficient biotransformation on a preparative scale. Recently, several economical systems for NADPH/NAD^+recycling have been developed. The most widely used method for NADH recycling utilizes formate dehydrogenase (FDH), which catalyses the oxidation of formate to carbon dioxide (Figure 1-4).[36] The formation of volatile CO_2 drives the recycling reaction in the desired direction. There are some advantages to this method: the substrate and the co-product can be easily removed from the reaction mixture; they do not cause problems such as inactivation or inhibition; and FDH is commercially available.

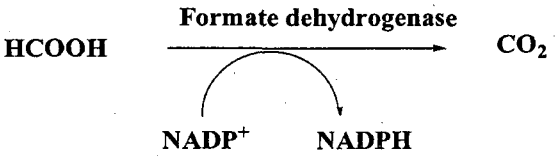


Figure 1-4. Cofactor recycling

The most efficient method for NADPH regeneration uses the glucose-6-phosphate (G6P) and glucose-6-phosphate dehydrogenase (G6PDH) system (Figure 1-5).[37] The advantages of this method are that G6PDH is inexpensive, stable, and accepts both NAD^+ and $NADP^+$. This system has been successfully used in biotransformations with isolated CHMO.[38 (a, b)] The major drawback to this procedure is the high cost of the G6P.

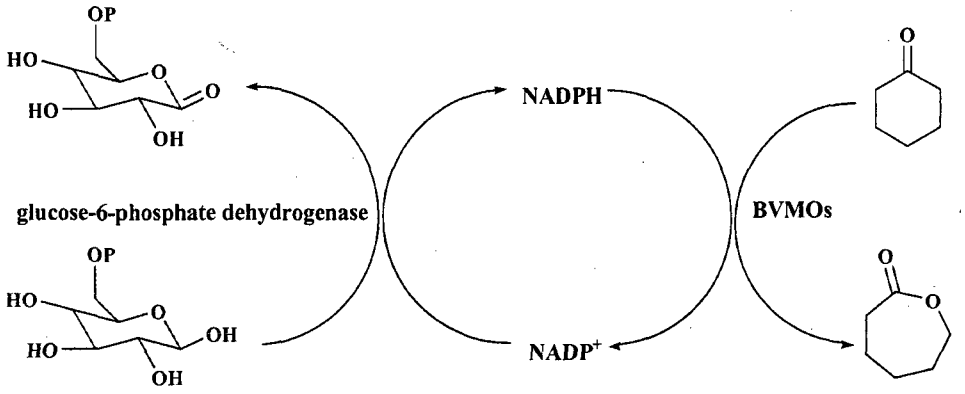


Figure 1-5. Glucose-6-phosphate dehydrogenase system

Willetts and coworkers have developed a closed-loop system to solve the cofactor problem.[39(a,b)] Purified BVMO and alcohol dehydrogenase from *Thermoanaerobium brockii* were coupled, the dehydrogenase oxidizes a secondary alcohol to a ketone that

then becomes the substrate for CHMO, with concomitant production of NADPH. This NADPH is then used by the CHMO during the transformation of the ketone into the lactone product and regenerating $NADP^+$(Figure 1-6).

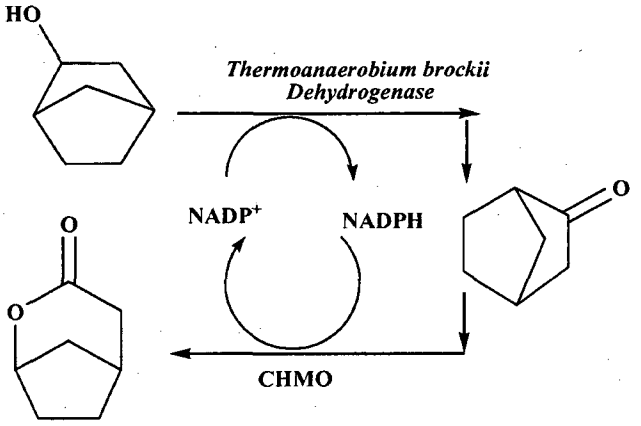


Figure 1-6. Substrate-coupled cofacter recycling

II. Whole-cells strategy

The simplest way to use BVMOs as reagents is as whole-cell transformations. This avoids the laborious process of enzyme isolation that is often complicated by limited protein stability. In addition, all cofactors required for biocatalysis are recycled by the living cells. In many cases, cells can be easily cultivated and stored, and such cultivation does not require special laboratory equipment or microbiological expertise. In addition, screening with the whole-cells allows for rapid identification of catalysts with the highest conversion and the best selectivity for a given substrate.

The problem with the whole-cell method is the increased potential for unwanted side-reactions since both substrate and product can be accepted by other enzymes present in the cell. This problem is minimized when the enzyme is overexpressed in a simple host organism, such as Escherichior coli (*E.coli)*.

Whole-cell transformations can be carried out using growing or non-growing cells. The growing-cell catalyzed reactions are suitable for small scale reactions and are often used in screening. They present a number of disadvantages in a larger scale transformation: (i) the reaction must be run in the same medium that is required for cell-growth, which may increase the cost; (ii) high volumes of the nutrient medium are used leading to low volumetric yields; (iii) the potential for unwanted side-reactions is increased. Non-growing cells are more suitable for large scale reactions since: (i) initial cell concentration can be made higher than for growing cells because the cells are resuspended in non-nutrient media; (ii) generally reactions are cleaner than with growing cells as side reactions can be more easily controlled; (iii) product isolation is easier; and (iv) the volumetric yields are higher.

In conclusion, although a variety of NADPH recycling systems are available it is still more convenient to use the whole-cells expressing BVMOs. This process already has been employed successfully for a number of enantioselective Baeyer-Villiger reactions.[40(a-d)]

1.3 BVMOs used in this project

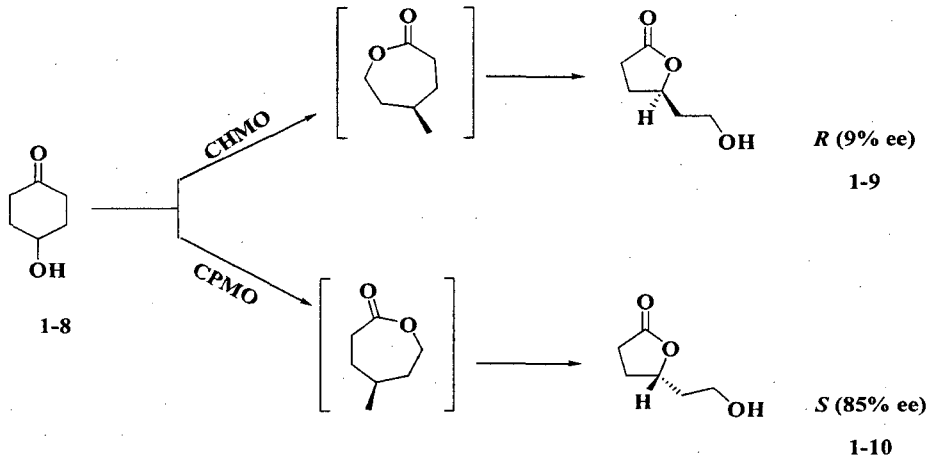
Although several BVMOs have been identified,[41] the best characterized is CHMO which was the first BVMO whose gene was cloned and overexpressed.[42] In addition, the

mechanism[43 (a, b)] and stereochemistry[44 (a, b)] of this enzyme have been extensively studied. Its ability to catalyze the Baeyer-Villiger oxidation of a wide range of ketones with high enantioselectivity makes it a potentially useful industrial catalyst.[45 (a,d)] Many synthetic applications have focused on this enzyme and numerous experiments, either with engineered whole cells or isolated enzymes, have shown that CHMO is a useful biocatalyst for the synthesis of interesting compounds such as bicyclic lactones,[46] various sulfoxides,[47(a,b)] cyclic sulfates,[48] and thiosulfinates.[49] In all, CHMO has been shown to transform more then 100 unnatural ketones to their corresponding lactones by both kinetic resolution[50] and desymmetrization[46] with good enantioselectivity. The production and application of CHMO from *Acinetobacter,* however, is limited by several problems. First, the *Acinetobacter* NCIMB 9871 is a class 2 pathogen and therefore unattractive for large scale fermentations. Secondly, production and use of the biocatalyst is cumbersome with low activities.[51] Finally, *Acinetobacter* NCIMB 9871 contains an active lactone hydrolase, and therefore either purification of the CHMO or selective inhibition of the hydrolase using a toxic inhibitor such as tetraethyl-pyrophosphate is required prior to the biotransformation. The manufacture of CHMO *via* the wild type host is thus unappealing, and an alternative route is required in order to implement catalyst production suitable for large scale biocatalytic processes. To overcome these generic problems, Kayser *et al.*, (1998-1999) and others (Stewart *et al.*, 1996; Chen *et al.*, 1999; Doig *et al.*, 2001) have constructed CHMO-overexpressing recombinant whole-cell biocatalysts using *E. coli* and *Saccharomyces cerevisiae* as hosts.[52 (a-d)]

Cyclopentanone Monooxygenase (CPMO)

CPMO was purified in 1976 by Griffin from *Comamonas* (previously *Pseudomonas*) NCIMB 9872 growing on cyclopentanone.[53] CPMO was cloned and expressed in *E. coli* by the Iwaki and Kayser groups in 2002.[54] CPMO was identified as an FAD-containing enzyme dependent on NADPH.[55] Most biocatalytic studies with CPMO have been performed with whole-cell overexpression systems.[56(a, b)]

Initial studies indicated that CPMO was less enantioselective than CHMO,[57] and for that reason it has been studied less than CHMO. More recently, however, it was shown to be quite versatile [58] and in some cases, highly enantioselective.[59(a-c)] In particular, several substrates can be converted by both CPMO and CHMO, but with the opposite enantioselectivities.[60(a, b)] An interesting case is illustrated in Scheme 1-4. Both CHMO and CPMO oxidized 4-hydroxycyclohexanone, CPMO gives the *S* lactone with 85% ee while CHMO gives the *R* enantiomer with lower enantioselectivity (9% ee).



Scheme 1-4. Baeyer-Villiger oxidation of 4-hydroxycyclohexanone

1.4 Directed evolution as a method to improve enantioselectivity

During the last decade directed evolution has become a key technology in the field of molecular enzyme engineering, in particular when neither the 3D structures nor the catalytic mechanisms of the enzymes are known. Even when the crystal structures are available and reaction mechanisms are well understood, however, directed evolution can provide alternative solutions in comparison to rational-design experiments.[61(a-f)] The combination of proper molecular biological methods for random mutagenesis and gene expression, coupled with screening systems for the rapid identification of enantioselective mutant enzymes, forms the basis of the concept. The idea is to start with a natural (wild-type) enzyme and create a library of mutants from which a more enantioselective variant is identified, and to repeat the process as often as necessary using, in each case, an improved mutant for the next round of mutagenesis. This is illustrated in Figure 1-7.

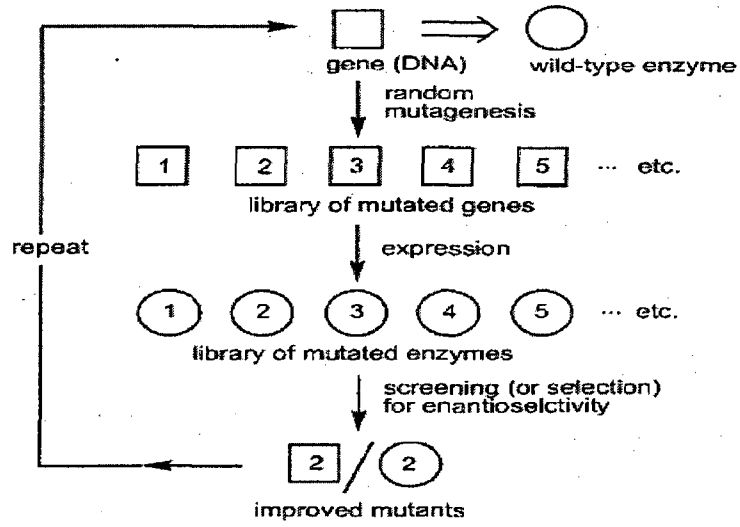


Figure 1-7. Directed evolution method

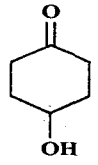
Directed evolution was applied for the first time in 1997 to improve the enantioselectivity of lipases.[62 (a-c)] Directed evolution has since been used to improve the enantioselectivity of cyclohexanone monooxygenase in Baeyer-Villiger reactions of several substrates.[63(a,b)] Recently, both CHMO and CPMO enzymes were evolved for higher enantioselectivity and increased substrate acceptance.[64(a,b)] In earlier work from the Kayser group[63(a)] several libraries of mutant CHMO genes were produced. Sequencing of several of the mutants displaying altered enantioselectivity identified the positions in the amino acid sequence of the wild-type WT-CHMO that seemed to be important in the selectivity control of Baeyer-Villiger reactions. Results are presented in Table 1-2.

The single mutation of Leu143Phe (1-F_1-F_5) resulted in enhanced (*R*) selectivity in the Baeyer-Villiger oxidation of **1-8.** Evolutionary optimization of *R* selectivity was then attempted by using the genes of some of the *R*-selective mutants as starting points for a second round of epPCR. This study was successful in producing I-F1-F5 which increased *R* selectivity up to 40% ee. A second round of epPCR led to a markedly improved mutant (II-D_{18}-C_8). Sequencing revealed that three new amino acid exchange events had occurred in II-D_{18}-C_8 (Glu292Gly, Leu435Glu, Thr464Ala), in addition to the already existing Leu143Phe mutation.

The most successful mutant identified in the directed evolution of CHMO carried a single mutation Phe432Ser (I- K_6-G_2). When its activity was compared to that of WT-CHMO, a switch from a low (*R*) to a high (*S*) selectivity was observed. These results demonstrate that directed evolution is a good strategy to improve the enantioselectivity of

CHMO. The mutants have already been applied successfully in the Baeyer -Villiger reaction of a wide range of cyclic and bicyclic ketones.[65(a,b)]

Table 1-2. Baeyer-Villiger oxidation of 4-hydroxycyclohexanone **1-8**

ketone	WT-CHMO	I- K_6-G_2 Phe432Ser	I-F_1-F_5 Leu143Phe	II-D_{18}-C_8 Glu292Gly, Leu435Glu, Thr464Ala
	%Con %ee	%Con %ee	%Con %ee	%Con %ee
 **1-8**	61(9 *R*)	100 (79 *S*)	80 (40 *R*)	73 (95 *R*)

1.5 Work presented in this thesis

In this project Baeyer-Villager transformations of several 4-substituted cyclohexanone substrates were performed using WT-CHMO, WT-CPMO and four of their mutants overexpressed in *E. coli.* The main step in the project was:

(i) The evaluation of methods for optimization of the synthesis of 1,4- cyclohexanedione monoketal and 4-substituted cyclohexanone substrates; (ii) Whole-cell screening for high enantioselectivity under growing cell conditions of Baeyer-Villiger reactions of various

4-substituted cyclohexanones was used to ascertain the most appropriate match between specific ketones and bioreagents; (iii) Scaling-up protocols for the preparation of enantiopure lactones using biocatalytic Baeyer-Villiger oxidation under non-growing cell conditions were investigated; (iv) Attachment of enantiopure lactones to functionalized silica in order to construct a new chiral ligand was investigated.

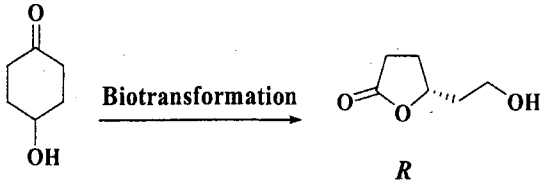
CHAPTER 2
Chemical Syntheses

2.1 Syntheses of 4-hydroxy-4-alkylcyclohexanone as prochiral substrates for enzymatic Baeyer-Villiger oxidations

2.1.1 Introduction

The Kayser group was investigated the use of BVMOs as catalysts for the Baeyer-Villiger oxidation of a variety of 4-substituted and 4,4-disubstituted cyclohexanones. Desymmetrization of prochiral substrates to homochiral lactones is a particularly attractive route to enantiopure lactones, which are important synthetic intermediates for the construction of a diverse range of natural products,[66(a-d)] pharmaceuticals and agrochemicals.[67(a-c)]

Two enzymes, CHMO and CPMO, have been evaluated in Baeyer-Villiger oxidations of 4-hydroxyl substituted cyclohexanones (Scheme 2.1). The objective of the present work was to extend this work to screen several other substrates having different R groups in the 4-position. The aim was to identify the most interesting substrate and microorganism matches from the point of view of activity and enantioselectivity.

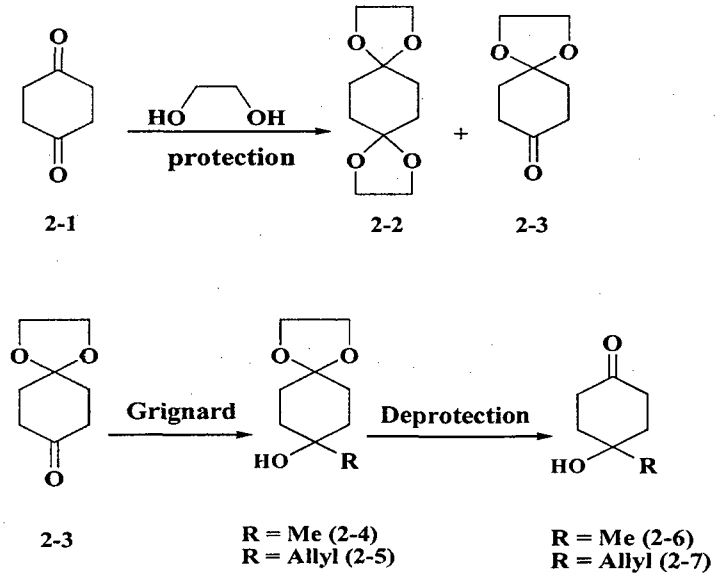


1-8 1-9

R

Scheme 2-1. Biotransformation using Bayer-Villiger monooxygenases

Thus, the first part of this work was the synthesis of the target substrates. The starting material for these reactions was commercially available 1,4-cyclohexanedione **2-1**. Grignard reactions were used to add the appropriate alkyl substituents to the mono-protected cyclohexanedione (Scheme 2-2). In multi-step syntheses, carbonyl groups are protected frequently as *O,O*-ketals or *S,S*-ketals.[68(a ,b)] For a diketone, however, the presence of two carbonyl groups complicates the reaction. Selective protection of only one carbonyl group can be problematic, especially in a symmetric molecule. Selective cleavage of the ketal group in the presence of other labile groups (such as lactone) in the products after reaction can also be difficult. Consequently, in the first part of this Chapter methods for the formation of the monoketal of cyclohexandione **2-3** and selective deprotection of the substituted products **2-4** and **2-5** after the Grignard reaction were examined.

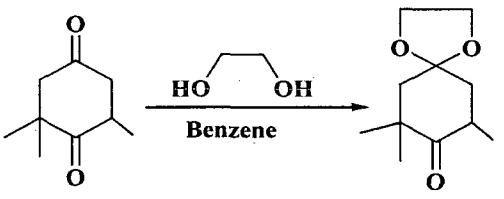


Scheme 2-2. Formation of the monoketal of 1,4-cyclohexandione and deprotection of the products

Results and Discussion

2.1.2 Optimization of protocols for the preparation of 1,4-cyclohexanedione monoketal

The major problem in this synthesis was the selective protection of only one of the two carbonyl groups. A molecule containing two ketone groups can be selectively protected at the less hindered carbonyl,[69 (a,b)] as shown in Scheme 2-3.



2-8 **2-9**

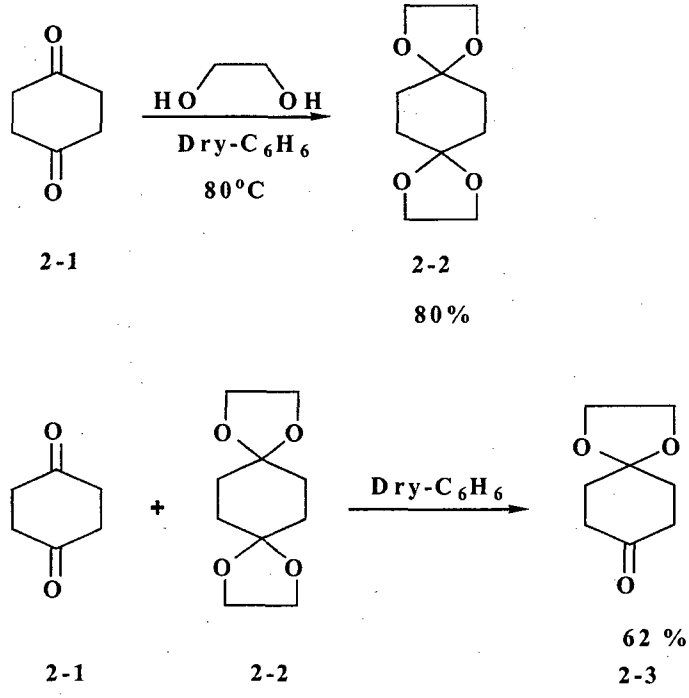
Scheme 2-3. Protection at the less hindered carbonyl group

In the case of 1,4-cyclohexanedione, however, the molecule is symmetrical, making mono-protection difficult.Three methods were tried to prepare **2-3,** the monoketal of 1,4-cyclohexanedione.

Method 1. From the diketal with azeotropic removal of water

1,4-Cyclohexandione **2-1** was reacted with two equivalents of 1,3-dioxolane (ethylene glycol) as the protective group under acid-catalyzed conditions in dry benzene with azeotropic removal of water using a Dean Stark trap (Scheme 2-4),[70] to gave the

diprotected ketone **2-2** (1,4-cyclohexanedione bisethylene ketal) in 80 % yield as white crystals.



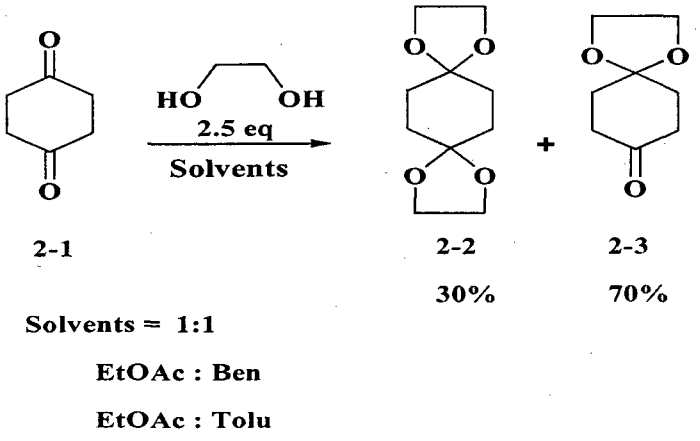
Scheme 2-4. Synthesis of **2-3** using a Dean Stark trap for azeotropic removal of water

In a second step, one equivalent of the isolated diketal **2-2** was mixed with one equivalent of the diketone **2-1** in dry benzene, this gave the desired monoketal as a white crystal in a modest yield (62%).

Method 2. Direct formation of monoketal

It is reported in the literature that by using the proper solvent system monoketals can be formed directly from diketones.[71] 2.5 equivalents of 1,3-dioxolane (ethylene glycol) were reacted with diketone **2-1** with subsequent azeotropic removal of water using 1:1

mixtures of EtOAc : benzene or EtOAc : toluene as the solvent mixture (Scheme 2-5). Results are summarized in Table 2-1.



Scheme 2-5. Direct preparation of the monoketal **2-3**

Table 2-1. Synthesis of **2-3** using different experimental conditions (temperature, and solvent)

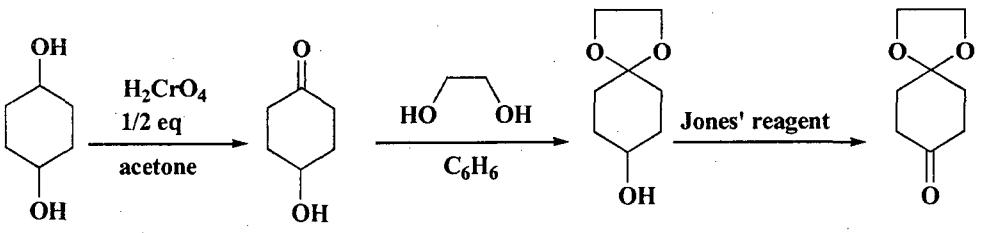
Temperature (°C)	Conversion (%)[(a)]	Solvent [(b)]	Time (h)
	2-2 / 2-3	50% ethyl acetate	
60	47 / 53	Benzene	3
60	50 / 50	Toluene	3
80	57 / 43	Benzene	3
80	68 / 32	Toluene	3
100	62 / 38	Benzene	3
100	70 / 30	Toluene	3

(a) Conversion based on GC analysis, (b) Solvents dried using $CaCl_2$; then distilled over Na, and stored over 4 °A molecular sieves

Optimization of the reaction conditions afforded a respectable 70% yield of monoketal in a single step.

Method 3. From 1,4-cyclohexanediol

Preparation of the monoketal starting from 1,4-cyclohexandiol **2-10** was also attempted.[72(a,b)] The diol **2-10** was first partially oxidized to 4-hydroxycyclohexanone **1-8** with chromic acid in acetone (Jones' reagent). **1-8** was then reacted with 1,3-dioxolane (ethylene glycol) to give the monoprotected alcohol **2-11** (4-hydroxycyclohexanone ethylene ketal). Oxidation of the protected alcohol again with Jones' reagent gave an 80% yield of the desired monoketal **2-3** (Scheme 2-6). This reaction was fast and gave a better yield than with the cylcohexandione and with no by-products. It was not, however, as convenient a synthesis since it was a multi-step process requiring a toxic reagent which would not be environmentally friendly on a large scale.

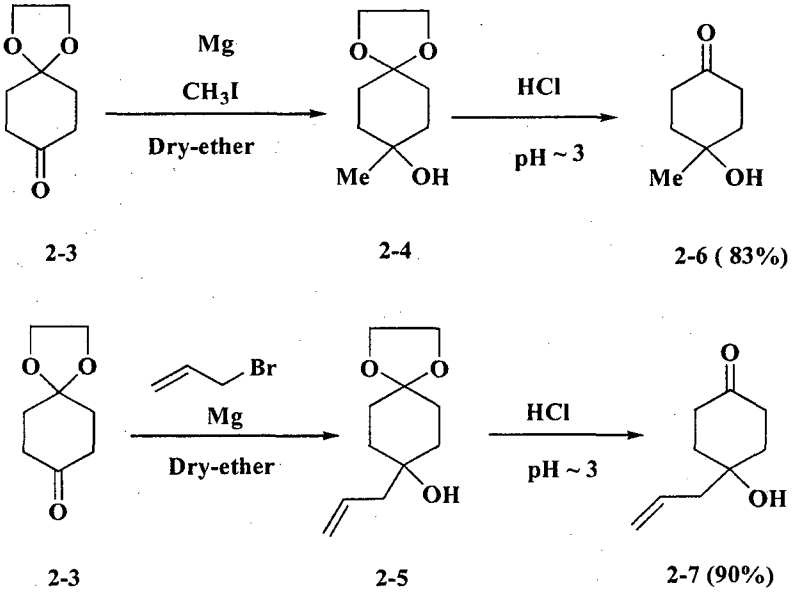


Scheme 2-6. Synthesis of **2-3** from 1,4-cyclohexanediol using Jones' reagent procedure

Thus, despite the modest yields, **Method 2** was adopted for the preparation of all the starting materials used in this work.

2.1.3 Introduction of the R groups using Grignard reactions

The monoketal **2-3** was converted to compounds **2-6** and **2-7** *via* Grignard reactions in good yields,[73] as shown in Scheme 2-7. In the first step of these reactions, an appropriate alkylmagnesium halide was added under argon to the ketal **2-3** dissolved in dry ether. After the reactions reached completion, as determined by TLC, they were quenched with water, the organic layer separated and the solvent removed by rotary evaporation. The crude reaction products were treated with aqueous HCl (pH ~3) to remove the protecting group.



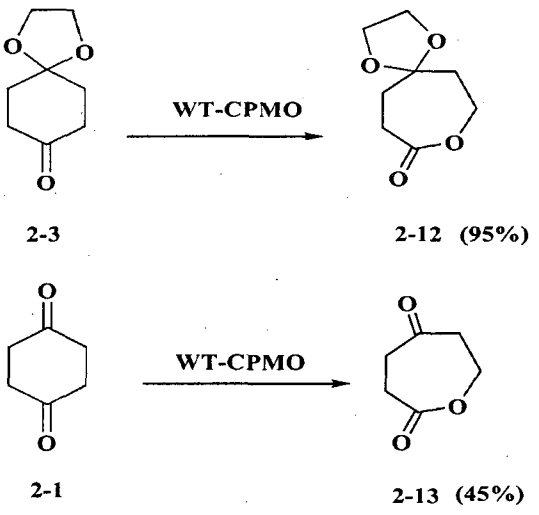
Scheme 2-7. Synthesis of **2-6** and **2-7** using a Grignard reaction

These two products, **2-6** and **2-7,** along with the unsubstituted 4-hydroxylcyclohexanone **1-8** were screened as target substrates for Baeyer-Villiger biotransformations in Chapter **3.** The unmodified 1,4-cyclohexanedione **2-1** and the monoprotected dione **2-3** were also screened.

2.2 Chemoselective deprotection of *O,O*-ketals

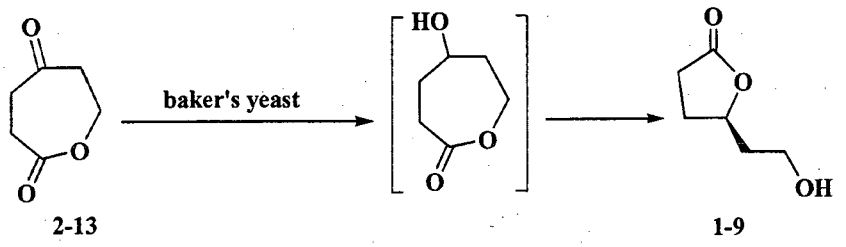
2.2.1 Introduction

The initial focus of this project was to prepare enantiopure lactones from the target substrates, using BVMOs (CPMO, CHMO and their mutants). However, in the course of this work it was found that CPMO would also oxidize the monoprotected ketal **2-3** (1,4-cyclohexanedione monoethylene ketal) and even 1,4 cyclohexanedione **2-1** to give seven-membered lactones (Scheme 2-8).



Scheme 2-8. Preparation of lactones **2-12** and **2-13** by Baeyer-Villiger oxidation using WT-CPMO

This was of interest because these seven-membered lactones can be tranformed efficiently to the desired five-membered ring enantiopure lactones by baker's yeast (Scheme 2-9).



2-13 1-9

Scheme 2-9. Synthesis of five-membered ring lactone from seven-membered ring using baker's yeast

Since lactone **2-12** was obtained in much higher yield (95%) than lactone **2-13** (45%), selective deprotection of **2-12** into **2-13** was of interest. However, the cleavage of the ketal group of lactone **2-12** into the ketone in the presence of other groups (such as lactone) is typically difficult. In this part of the Chapter are reported the results of a study of the deprotection of **2-12** using different kinds of deprotecting reagents.[74]

2.2.2 Results and Discussion

The deprotection of the ketal group by acid-catalysis is the most commonly used procedure.[75] However, very often this method is incompatible with the presence of some other functional groups such as a lactone. Several acidic cleaving methods were tried, such as treatment with an aqueous acid (HCl; pH ~3), however, no reaction was observed in any of these attempts (see Table 2-2)

Table 2-2. Synthesis of compounds **2-13, 2-14,** and **2-15**.

Substrate	Conditions	Product	Yield	Physical state
2-12	$FeCl_3.6H_2O$, RT[a]	**2-13**	73	brown powder
	CH_2Cl_2 : acetone (4:1)			
	TsOH, acetone, pH ~ 3, RT	**2-14**	76	yellow oil
	I_2, acetone, 50 °C	**2-15**	50	brown oil
	$FeCl_3.SiO_2$, RT	NR[b]	-	-
	HCl, H_2O, pH ~ 3, RT	NR	-	-
	HCl, acetone, pH ~ 3, RT	Unidentified materials	-	-

(a). RT= room temperature, (b). NR= no reaction

On the other hand, the non-aqueous method using acetone as a solvent caused complete decomposition of the product to several unidentified materials. When HCl was replaced with *p*-toluenesulfonic acid under the same conditions, both the ketal and the lactone groups were cleaved to give 6-hydroxy-4-oxohexanoic acid, **2-14** (Scheme 2-9).

Molecular iodine in acetone under neutral conditions cleaved the lactone rather than the ketal group to give **2-15**.[76] Cleavage of lactone in the conjugated system with the carbonyl group was faster than that of the saturated ketal group. Therefore, 3-(2-hydroxyethyl)-1,3-dioxolanyl)propanoic acid **(2-15)** was obtained.

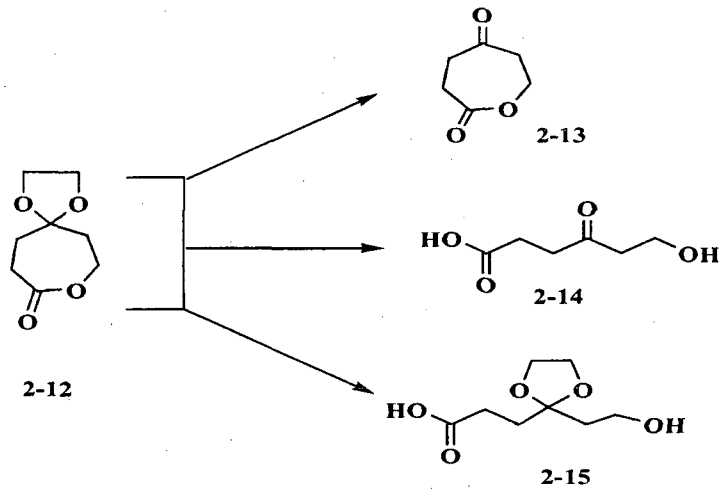


Figure 2-1. Products of the various attempts at chemoselective deprotection of **2-12**

Treatment of the ketal group with anhydrous $FeCl_3$ absorbed onto silica gel gave no reaction. The absorption of $FeCl_3$ on silica gel provided a weaker deprotection agent.[77] On the other hand, $FeCl_3.6H_2O$ in CH_2Cl_2 : acetone (4:1) gave the desired lactone **2-13**

(oxepane-2,5-dione) efficiently in a moderate yield. Acetone was needed to dissolve the Lewis acid and to participate in transketalization, resulting in both an increased yield and deketalization rate.[78]

2.3 Experimental

2.3.1 Preparation of monoketal 2-3

1- From the diketal with azeotropic removal of water

I. Preparation of 1,4-cyclohexanedione bisethylene ketal 2-2

A solution of 1,4-cyclohexanedione **2-1** (10 g) in benzene (200 mL), ethylene glycol (12 mL) and *p*-toluenesulfonic acid (0.8 g) was placed in a round-bottom flask fitted with a Dean Stark water separator and a reflux condenser. The reaction mixture was heated at 80 °C until no more water was collected. The cooled reaction mixture was washed with saturated $NaHCO_3$ solution, and the organic layer was dried with $MgSO_4$ and the solvent removed on a rotary evaporator. The crude product was purifed by flash chromatography with hexane : ethyl acetate (3:1) as eluent and gave **2-2** as white crystals (85%). IR ν_{max} 2959 (s), 2872 (s), 1463 (m), 1273 (m) 1100 (s, broad), 955 (s) cm^{-1}; ^1H NMR δ 3.98 (8H, s), (8H, s); GC (retention time 13 min).

II. 1,4-Cyclohexanedione monoethylene ketal 2-3

A solution of 1,4-cyclohexanedione bisethylene ketal **2-2** (11 g), 1,4-cyclohexanedione **2-1** (6 g) and *p*-toluenesulfonic acid (0.3 g) in benzene (150 mL) the reaction mixture was heated at reflux for 4 hours. The solution was cooled and the solvent was removed on a

rotary evaporator. The crude product was purified by chromatography on silica gel using petroleum ether : ether (1:1) to give the title compound **2-3** as white crystals (60%). IR ν_{max} 2959 (s), 2872 (s), 1729 (s, broad), 1463 (m), 1273 (m) 1100 (s, broad), 909 (m) cm^{-1}; ^1H NMR δ 3.98 (4H, m s), 2.49 (4H, t, J=1.83 Hz), 1.99 (4H, t, J=1.45 Hz); GC (retention time 10 min).

2- From the two-phase reaction

1,4-Cyclohexanedione monoethylene ketal 2-3

The synthesis was carried out according to the general procedure of the azeotropic removal of water using toluene : ethyl acetate (50 mL: 50 mL) with 1,4-cyclohexanedione **2-1** (6 g), ethylene glycol (9 mL) and *p*-toluenesulfonic acid (1 g). The crude product was dissolved in benzene and washed with water to remove any of the dione **2-1**. Then purification by chromatography on silica gel using petroleum ether : ether (1:1) gave **2-3** as white crystals (70%).

3- From 1,4-cyclohexanediol

I- Preparation of 4-hydroxylcyclohexanone (1-8) using Jones' reagent

1,4-Hydroxylcyclohexanediol **2-10** (4.8 g, 40 mmol) was suspended in 150 mL of acetone and stirred vigorously at room temperature. Jones' reagent (10 mL) was added dropwise over a period of 30 minutes. The mixture was stirred for an additional 5 minutes at room temperature. The dark green sticky oil at the bottom was discarded and the solution was neutralized with saturated $NaHCO_3$. The acetone was removed on a rotary evaporator and the residue was extracted with ethyl acetate (50 mL X 3). The

combined extracts were washed once with a small amount of brine and dried over anhydrous $MgSO_4$. The solvent was removed on a rotary evaporator and the residue was purified by flash chromatography using petroleum ether : ethyl acetate (1:1) as the eluent. Vanillin dip was used as the visualization method. 4-Hydroxycyclohexanone **1-8** was obtained as a colorless oil (63%): IR ν_{max} 3421 (br, vs), 2940 (vs), 3865 (vs), 1709 (vs), 1236 (vs), 951 (s) cm^{-1}; ^1H NMR δ 4.20 (1H, dd, J=12.42 Hz), 2.27-2,17 (4H, m), 2.09-1.91, (4H, m); GC (retention time 7 min).

II- Preparation of 4-hydroxycyclohexanone ethylene ketal (2-11) using the azeotropic removal of water

4-Hydroxycyclohexanone **1-8** (1.13 g, 0.01 mmol), ethylene glycol (0.62 mL, 0.01 mmol), *p*-toluenesulphonic acid (0.8 g) and anhydrous benzene (50 mL) were placed in a round-bottom flask fitted with a Dean Stark water separator. The reaction mixture was treated according to the procedure for the azeotropic removal of water described above. The solvent was removed on a rotary evaporator to give **2-11** as a yellow oil (83%). IR ν_{max} 3430 (s, broad), 2959 (s), 1453 (m), 1100 (s, broad), 950 (s) cm^{-1}; ^1H NMR δ 3.98 (4H, m), 3.83 (1H, m), 1.91 (2H, m), 1.84 (2H, m), 1.70 (4H, m), 1.37 (1H, br); GC (retention time 9.2 min).

III- 1,4-Cyclohexanedione monoethylene ketal 2-3 using Jones' reagent

4-Hydroxycyclohexanone monoethylene ketal **2-11** (1.7 g) was suspended in 100 mL of acetone and Jones' reagent was added (4 mL). The residue was purified by flash

chromatography with hexane : ethyl acetate (1:1) as eluent and gave **2-3** as white crystals (80%).

2.3.2 Grignard reaction

I- Preparation of 4-hydroxy-4-methylcyclohexanone 2-6

Magnesium metal (2.6 g, 110 mmol) and anhydrous ether (50 mL) were placed in a 250 mL 3-neck round-bottom flask equipped with a dropping funnel and a reflux condenser. Methyl iodide (5.3 mL) dissolved in anhydrous ether (50 mL) was added dropwise at a rate sufficient to maintain a gentle reflux; this was continued for an additional 5 hours at room temperature. 1,4-Cyclohexanedione monoethylene ketal **2-3** (2.4 g, 15 mmol) dissolved in anhydrous ether (40 mL) was added dropwise and stirring was continued overnight at room temperature. The reaction was quenched by adding 70 mL of water. The organic layer was separated, and the aqueous layer was extracted with ether (50 mL X 3); the combined organic layers were dried with $MgSO_4$, and the solvent was removed on a rotary evaporator. The crude product was dissolved in aqueous HCl (100 mL, pH ~3) and stirred at room temperature overnight. After being extracted with EtOAc (3 x 50 mL), the combined organic layers were dried with $MgSO_4$, and the solvent was removed on a rotary evaporator. The crude product was purified by chromatography on silica gel using hexane : ethyl acetate (1:1) to give **2-6** as brown crystals. IR ν_{max} 3415 (s,broad), 2966 (s), 2835 (m), 2900 (m), 1705 (vs), 1417 (m), 1251 (m), 1135 (s), 920 (s) cm^{-1}; ^1H NMR δ 2.75 (2H, ddd), 2.27 (2H, m), 1.97 (2H, m), 1.87 (2H, m), 1.38 (3H, s); GC (retention time 8.7 min).

II- Preparation of 4-hydroxy-allyl-cyclohexanone 2-7

The synthesis was carried out according to the Grignard reaction procedure using allyl bromide as the appropriate alkyl substituent to the mono-ketal **2-3**. The crude product was purified by chromatography on silica gel using petroleum ether : acetone (3:1) to give **2-7** as white crystals. IR ν_{max} 3439 (s, broad), 2966 (s), 3935 (m), 2885 (m), 1705 (vs), 1450 (m), 1334 (m), 1135 (s), 920 (s) cm^{-1}; ^1H NMR δ 5.89 (2H, m), 5.23 (1H, m), 2.72 (2H, m), 2.34 (2H, m), 2.25 (2H, dd), 1.95 (2H, m), 1.82 (2H, m); GC (retention time 9.2 min).

2.3.3 Deprotection of monoketal lactone 2-12

I. Acetone and *p*-toluenesulfonic acid catalyzed deprotection of ketals.

To a solution of ketal lactone **2-12** (1 g) in acetone was added $TsOH.H_2O$ (pTSA) (200 mg) and the resulting solution was refluxed for 4 hours. After removing the solvent under reduced pressure, the residue was diluted with EtOAc (200 mL). The organic layer was then dried over $MgSO_4$ and the solvent removed on a rotary evaporator. The residue was purified by column fluorescent chromatography using pure acetone to give compound **2-14**. ^1H NMR δ 1.8-2.0 (m, 4H), 2.3 (t, 2H, J=2.3 Hz), 3.6 (t, 2H, J=1.34 Hz), 3.98 (4H, m s).

II. Molecular iodine catalyzed deprotection of ketals in acetone.

A mixture of ketal lactone **2-12** (900 mg, 5 mmol) and iodine (125 mg, 0.5 mmol) in acetone (20 mL, reagent ACS, ≤0.5% H_2O) was stirred for 5 minutes at refluxing temperature (56 °C). Most of the acetone was then removed under vacuum, and the residue was diluted with dichloromethane (50 mL). The mixture was washed successively with 5% aqueous $Na_2S_2O_3$ (10 mL), H_2O (20 mL), and brine (20 mL).The organic layer was separated, dried over Na_2SO_4, and filtered. The solvent was removed on a rotary evaporator to give compound **2-15,** which was purified by short column fluorescent chromatography using pure ethyl acetate. ^{1}H NMR δ 2.5-2.7 (m, 6H), 3.98 (4H, m s), 3.7 (t, 2H, J=9.14 Hz)

III. Standard $FeCl_3.6H_2O$ catalyzed deprotection of ketals.

Ketal lactone **2-12** was added to 25 mL of a solvent mixture (CH_2Cl_2 : acetone = 4:1). To this solution $FeCl_3.6H_2O$ (90 mg, 3.5 eq) was added. The resulting yellow solution was stirred for 1 hour at room temperature. The reaction was quenched by the addition of saturated aqueous $NaHCO_3$. The aqueous layer was extracted three times with CH_2Cl_2, and the combined organic layers were washed with brine, dried over $MgSO_4$, and the solvent removed on a rotary evaporator. The crude product was redissolved in a minimum amount of CH_2Cl_2 and passed through a short silica gel column using pure hexane as solvent to remove any remaining iron impurities to give compound **2-13**. ^{1}H NMR δ 2.6-2.7 (m, 4H), 2.73 (t, 2H, J=1.43 Hz), 4.32 (t, 2H, J=1.205 Hz).

CHAPTER 3

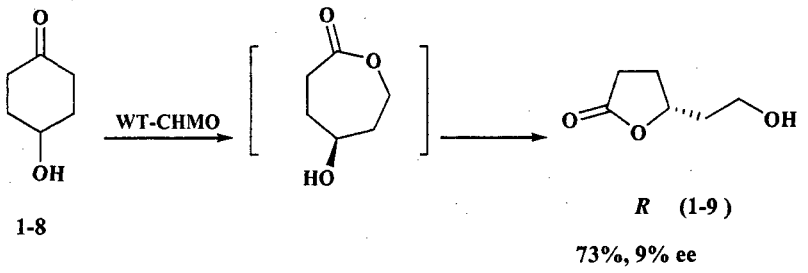
Screening of Baeyer-Villiger Oxidations of 4-substituted Cyclohexanones by BVMOs under Growing Conditions

3.1 Screening conditions using *E. coli* overexpession systems for WT-CHMO, WT-CPMO and three mutants

3.1.1 Introduction

A large number of microorganisms harbur enzymes that can perform Baeyer-Villiger oxidations.[79(a,b)] Such enzymes are referred to as Baeyer-Villiger monooxygenases (BVMOs).[80(a-f)] BVMOs have been shown to have many advantages as catalysts for Baeyer-Villiger oxidation reactions: (i) the product obtained can be of high enantiopurity; (ii) they are environmentally friendly; and (iii) the reaction can be scaled-up readily. Although several BVMOs have been identified,[81] the two most extensively studied BVMOs are cyclohexanone monooxygenase (CHMO) and cyclopentanone monooxygenase (CPMO). These enzymes have been shown to be highly selective bioreagents for a broad range of substrates.[82(a,b)] Recently, both enzymes were evolved for higher enantioselectivity and increased substrate acceptance using the directed evolution of enzymes method.[83(a-d)]

In 1988, Taschner and coworkers showed that Baeyer-Villiger oxidation of 4-hydroxylcyclohexanone **1-8** catalysed by WT-CHMO gave the rearranged valerolactone **1-9** rather than the expected caprolactone but with only a small (9 %) enantiomeric excess of the *R* isomer (Scheme 3-1).[84]



Scheme 3-1. The rearrangement of caprolactone to valerolactone

The availability of compound **1-9** in optically pure form would be of considerable synthetic interest. Both enantiomers of 5-(2-hydroxyethyl) oxolane-2-one [*R* (**1-9**), *S* (**1-10**)] are useful building blocks for the synthesis of natural products.[85] For example, the *S* enantiomer (**1-10**) was used in the synthesis of Aspinolide B; part of the retrosynthesis is illustrated in Figure 3-1.[86]

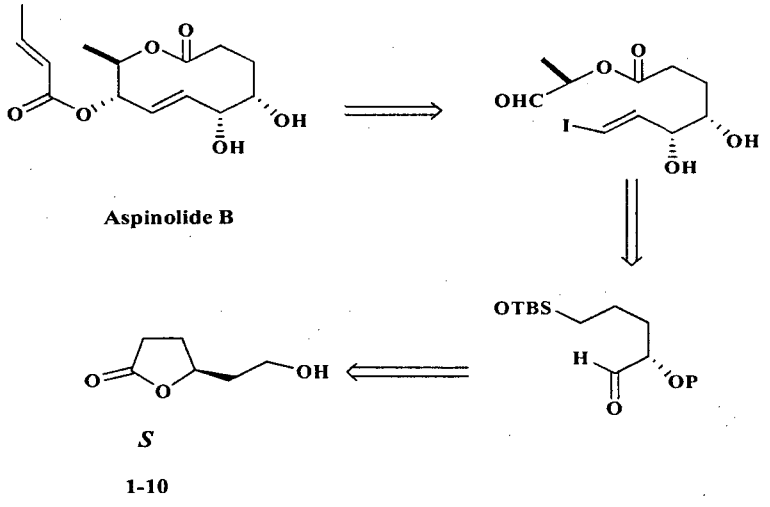


Figure 3-1. The use of the ***S*** enantiomer (**1-10**) in the synthesis of Aspinolide B

Some potential uses of the *R* enantiomer (**1-9**) are for the asymmetric synthesis of antibiotics such as vermiculine,[87(a,b)] decarestrictine D,[88] the marine polycyclic ether toxin (-)-gambierol,[89] and pheromones such as 6-hydroxyalkan-4-olide (a male pheromone of a large family of butterflies, Idealeucone) as shown in Figure 3-2. [90(a-c)]

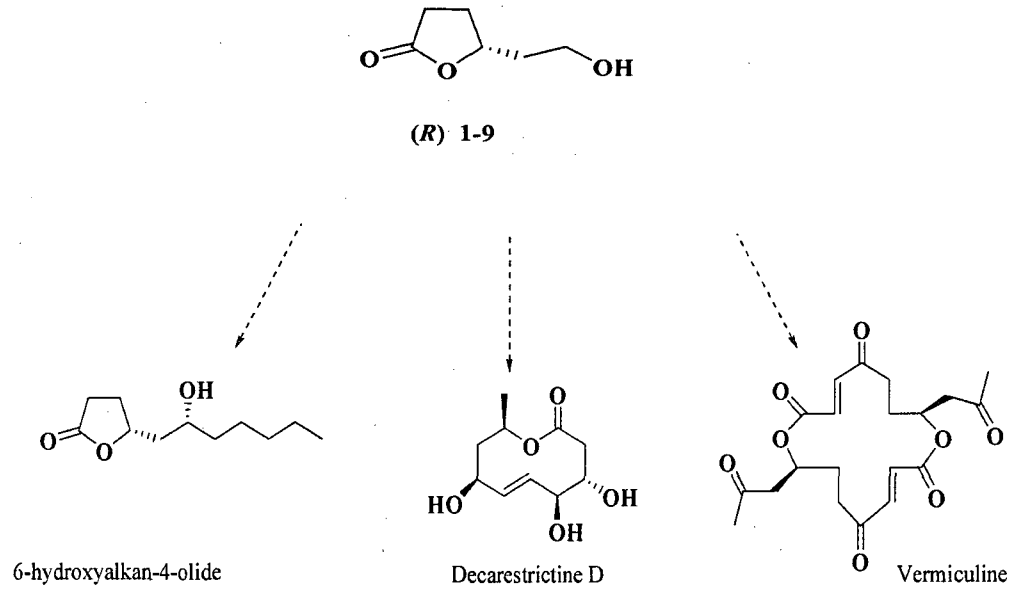


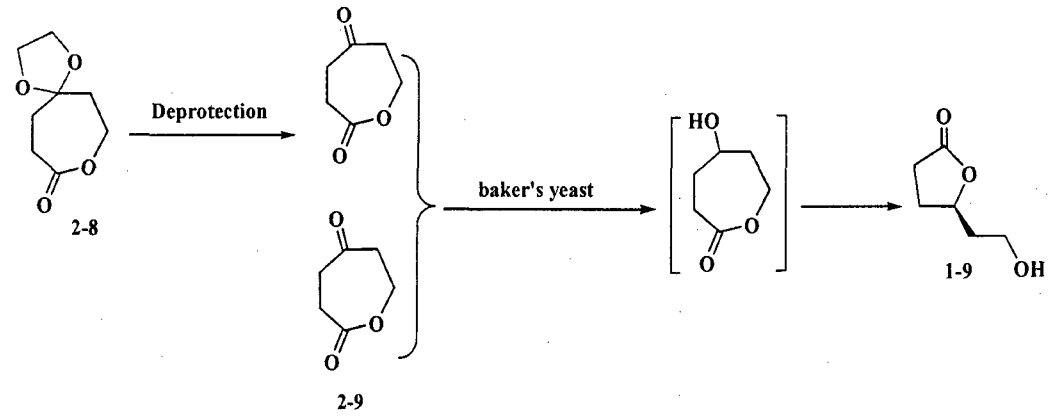
Figure 3-2. Versatile chiral synthon for the synthesis of natural products

Figure adapted from Wang's thesis.[73]

Preliminary studies by us and by the Reetz's group on the directed evolution of a CHMO as a biocatalyst in the Baeyer–Villiger reaction of 4-hydroxycyclohexanone **1-8** show that the wild-type CHMO from *Acinetobacter sp.* NCIMB 9871 leads to poor enantiomeric excess (9%) values in favour of (*R*)-**1-9**.[84] The enantioselectivity of this synthetically interesting transformation could be increased substantially to 85% ee by

using a CHMO mutant [I-K_2-G_6 (Phe432Ser)], and to 95% ee by using a second mutant [II- D_{18}-C_8 (Glu292Gly, Leu435Glu, Thr464Ala)].[83 (a-d)] It was of practical and theoretical interest to evolve both (***S***) and (***R***) selective CHMOs in this project, because this would allow for enantiodivergence on an optional basis. The evolution of both ***S*** and ***R*** selective CHMOs was investigated in this project.

The activity and selectivity results were impressive when substrate **1-8** was oxidized to the corresponding lactone **1-9** using a CHMO second generation mutant **(II-D_{18}-C_8).** However, because of the instability of this mutant, and the difficulty in isolating the lactone, it is easy for this mutant to lose its activity during the reaction, and the potential for unwanted side-reactions is increased. Therefore another method was attempted to synthesize this lactone **1-9** using baker's yeast (Scheme 3-2). It should be pointed out that there is no previous attempt to synthesize of lactone **1-9** using the baker's yeast procedure.



Scheme 3-2. Synthesis of lactone **1-9** using baker's yeast.

In this project enzyme-catalyzed reactions were performed using WT-CHMO, WT-CPMO enzymes overexpressed in *E. coli*, for Baeyer-Villiger oxidations of the five ketones listed in Figure 3-3. The substrates **1-8, 2-6, 2-7, 2-3,** and **2-1** were selected on the basis of the usefulness of their lactonic products **1-9, 3-1, 3-2, 2-12,** and **2-13** respectively.

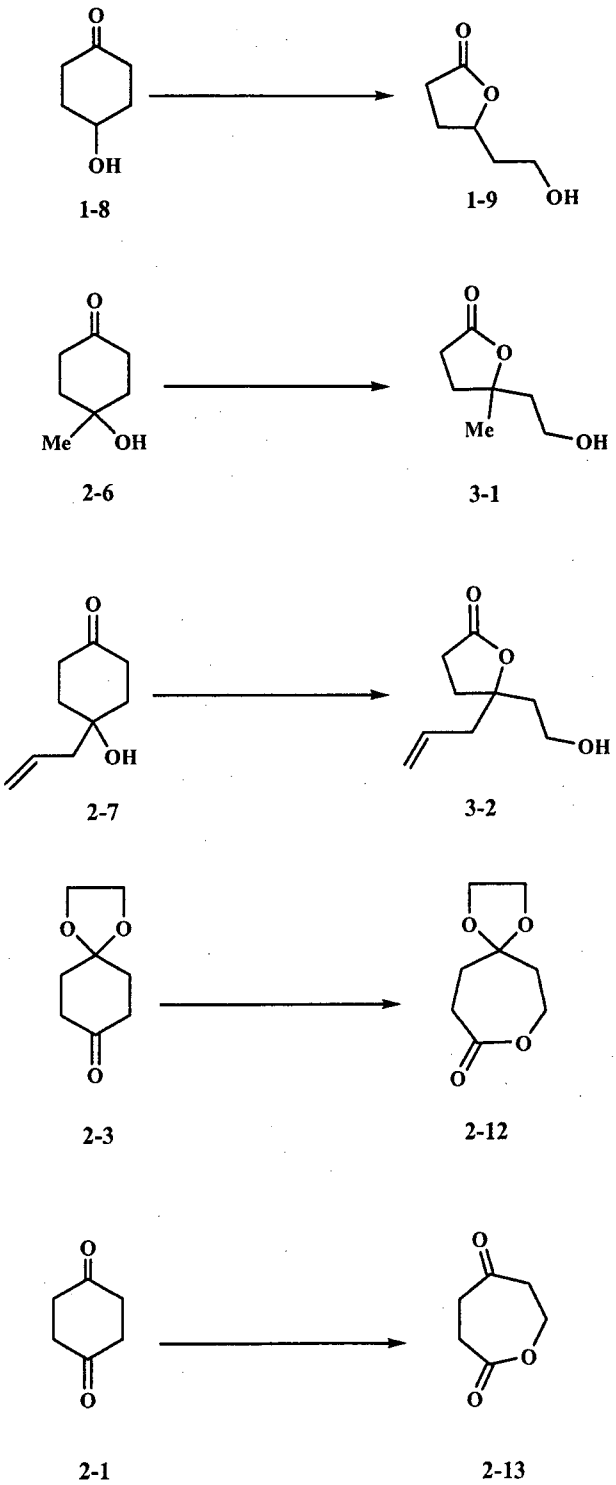


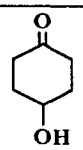
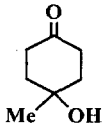
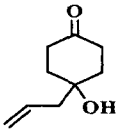
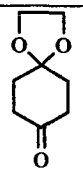
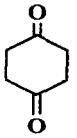
Figure 3-3. Enzyme catalyzed Baeyer-Villiger oxidations that were screened

3.1.2 Results and Discussion

The ketone substrates were prepared as described in **Chapter 2**. The ketones **2-(1, 3, 6, 7)** and **1-8** were oxidized under screening conditions using *E. coli* overexpession systems for WT-CHMO, WT-CPMO and three mutants. The screening was carried out in baffled Erlennmeyer flasks on a very small scale (20 mg of substrate) under growing conditions and all biotransformation reactions were monitored by achiral and chiral GC as described in the experimental section.These intitial experiments were designed to establish whether the substrate base and the enantionselectivity of these enzymes were sufficiently distinct in order to merit a more extensive effort to develop CPMO and CHMO or some of their mutants as "bioreagents".

The results are presented in **Table 3-1**. The yields of lactonic products reported refer to the ioslated yields of chromatographically purified samples. The chemical and optical purities were established by comparing the chiral GC anaylses of the lactonic products from biotransformations with those from chemical oxidations. Protein production was induced by addition of isopropyl-β-D-galactopyranoside **(IPTG)**, and the biotransformations were usually carried out in the presence of β-cyclodextrin to improve the solubility of ketones and in order to limit possible toxicity of the whole-cells method.

Table 3-1. Initial screening of ketones **1-8** and **2-(6, 7, 3, 1)**

Ketone	WT-CHMO	WT-CPMO	II- D_{18}-C_8 Glu292Gly, Leu435Glu, Thr464Ala	I-K_6-G_2 Phe432Ser	A_1- A_{10} Phe156Gly, Leu157Phe
	Conv % (% ee)	Conv % (% ee)	Conv % (% ee)	Conv % (% ee)	Conv % (% ee)
 **1-8**	61 (9 *R*)	>98 (85 *S*)	73 (95 *R*)	98 (85 *S*)	98 (49 *S*)
 **2-6**	100 (96 *R*)	80 (78 *S*)	NR	100 (87 *R*)	NR
 **2-7**	100 (27 R)	80 (25 S)	NR	100 (96 *R*)	NR
 **2-3**	NR	95 (NA)	NR	NR	NR
 **2-1**	NR	49 (NA)	Trace	NR	NR

NR =no reaction; NA =not applicable

1. Baeyer-Villiger oxidation of 4-hydroxy-disubstituted substrates

All three 4-hydroxy-disubstituted substrates, **1-8, 2-6,** and **2-7** were accepted by WT-CHMO. However, only substrate **2-6** was oxidized with both high conversion and high enantioselectivity (96% ee, *R*). WT-CPMO gave respectable conversions for all three 4-hydroxy-disubstituted substrates **1-8, 2-6,** and **2-7**. Furthermore, the reverse enantiomeric preference *S* rather than *R* was observed. Note also that WT-CPMO was the only enzyme tested that accepted the diketone **2-1** and the monketal **2-3** substrates (more on this below).

The mutant that performed best was the first generation CHMO mutant **I-K_6-G_2**. It converted all three hydroxy substrates **1-8**, **2-6**, and **2-7** to their lactones in high yield and good ee. In the oxidation of **1-8** it gave the *S* isomer of lactone **1-9** but the *R* isomer of lactones **3-1** and **3-2**. Also, lactone **3-2** was difficult to obtain in high ee by chemical oxidation of **2-7**.

The second generation CHMO mutant **II-D_{18}-C_8** accepted only **1-8** in modrate yields but with good enantimeric excess of the *R* isomer. For this substate only this mutant performed better than WT-CHMO.

The CPMO mutant **A_1-A_{10}** performed poorly, accepting only the substrate **1-8** and giving only the lactone **1-9** in low ee of the *S* isomer.

The preliminary results described above allowed identification of the most interesting substrate and microorganism matches from the point of view of activity and

enantioselectivity. In the next part of this project, these reactions were scaled-up under controlled (optimized) fermentation conditions (see Section 3.2 below).

2. Baeyer-Villiger oxidation of ketone substrates

As mentioned above, WT-CPMO was the only enzyme tested that catalysed the Baeyer-Villigaer oxidation of both 1,4 cyclohexanedione **2-1** and the monoprotected ketone **2-3**. Although conversion was low (45%), biocatalysed Baeyer-Villiger oxidations of the symmetrical diketone **2-1** in particular merit further investigation, since the corresponding chemical Baeyer-Villiger oxidation with *m*-chloroperbenzoic acid and trifluoroperoxyacetic acid gave **2-13** in less than 20% conversion even when long reaction times were used. In addition, oxidation of the monoketal **2-3** occurred in high yield (95%). The ability of WT-CPMO to oxidize the ketones **2-1** and **2-3** is of considerable interest, because these ketones are apparently not a suitable substrate for CHMO and their mutants. Furthermore, lactone **2-13** is desirable substrates for biotransfomation to the corresponding enantiopure five-membered lactone (such as **1-9**) *via* baker's yeast reductation. The reaction of baker's yeast is inexpensive and fast (Scheme 3-2). The lactone **1-9** was obtained in good isolated yield (60%).

3.2 Screening of Saturation Mutants of CHMO

3.2.1 Introduction

Saturation mutagenesis technology is a method for rapid laboratory evolution of proteins whereby a specific amino acid in a protein is replaced with each of the other 19 naturally occuring amino acids.[91] Usually an excess of 300-400 clones are needed in order to ascertain that the 20 enzyme variants are, in fact, in the library. Researchers have successfully used saturation mutagenesis to improve such enzymatic properties as thermostability,[92] substrate specificity,[93] and enantioselectivity.[94] For example, systematic saturation mutagenesis was used by scientists at Diversa (San Diego) [95] in their efforts to improve the enantioselectivity of a nitrilase in the desymmetrization of the prochiral dinitrile **3-3**. This reaction is of special industrial interest because the ethyl ester of **(*R*)-3-4** is an intermediate in the synthesis of the cholesterol-lowering drug Lipitor.[96]

3-3 **(*R*)-3-4**

Figure 3-4. Synthesis of (*R*)-4-cyano-3-hydroxybutyric acid

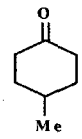
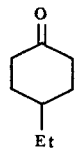
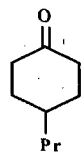
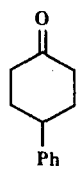
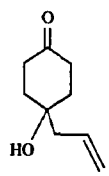
The problem of choosing the appropriate position in the enzyme must to be solved first.[97] The current work was conducted in order to obtain a saturation CHMO mutant with greater stability and enantioselectivity,[98] and also to obtain a greater understanding

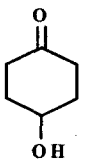
of the function of this enzyme. A site-directed mutagenesis study revealed that **Ph432** is an attractive target for mutagenesis to change the CHMO into a first generation mutant [I-K_6-G_2 (Ph432Ser)]. This mutant was shown to be the more active and selective mutant when compared to WT-CHMO. Therefore, saturation mutagenesis was performed to create every possible amino acid substitution at position 432 of this particular mutant. [98] Phenylalanine had the greatest positive influence on enantioselectivity at this position.[99]

3.2.2 Results and Discussion

Baeyer-Villager transformations were performed using four saturation CHMO mutants as enzyme-catalysts. The test substrates were selected in view of the usefulness of their lactonic products and their potential as probes in the study of the CHMO's active site. Preparation of ketones **1-8** and **2-7** was described in **Chapter 2.** Ketones **3-5, 3-6, 3-7,** and **3-8** were purchased from Sigma-Aldrich. Screening results are presented in **Table 3-2.**

Table 3-2. Initial screening of 4-substituted ketones

4-substituted ketones	WT-CHMO Conv % (% ee)	I-K_6-G_2 **Phe**432Sr Conv % (% ee)	$SAT_{22}B_6$ **Phe**432Leu Conv % (% ee)	$SAT_{21}A_5$ **Phe**432Gly Conv % (% ee)	$SAT_{21}D_{10}$ **Phe**432Val Conv % (% ee)	$SAT_{24}D_{11}$ **Phe**432Pro Conv % (% ee)
 **3-5**	100 (95 *S*)	100 (99 *S*)	99 (97 *S*)	34 (39 *S*)	98 (95 *S*)	54 (46 *S*)
 **3-6**	100 (95 *S*)	100 (99 *S*)	99 (97 *S*)	49 (45 *S*)	95 (95 *S*)	39 (37 *S*)
 **3-7**	100 (92 *S*)	100 (99 *S*)	55 (57 *S*)	79 (78 *S*)	34 (31 *S*)	41 (40 *S*)
 **3-8**	NR	NR	NR	Trace	NR	NR
 **2-7**	100 (27 *R*)	100 (97 *R*)	NR	53 (34 *R*)	NR	NR

 **1-8**	61 (9 *R*)	100 (79 *S*)	50 (48 *R*)	17 (20 *S*)	64 (17 *R*)	7 (72 *R*)

Ketones with relatively small, nonpolar groups in the 4-position (**3-5, 3-6, and 3-7**) were oxidized rapidly and with high (*S*) selectivity by WT-CHMO and a single mutant (I-K_6-G_2). Low yield and enanatioselectivity were obtained when these ketones were oxidized by the four saturation WT-CHMO mutants ($SAT_{21}A_5$, $SAT_{21}B_6$, $SAT_{21}D_{10}$, and $SAT_{24}D_{11}$).

4-Phenyl-cyclohexanone **3-8** was a poor substrate for either WT-CHMO or any of its mutants. The substrate **2-7**, which was effectively oxidized by WT-CHMO with low enantioselectivity (27% ee *R*), was converted only by a single mutant (I-K_6-G_2) with an efficient enhancement in enantioselectivity (97% ee). Also, substrate **2-7** was a poor substrate for saturation WT-CHMO mutants ($SAT_{21}A_5$, $SAT_{21}B_6$, $SAT_{21}D_{10}$, and $SAT_{24}D_{11}$).

All the used enzymes accepted the substrate **1-8**; however, I-K_6-G_2 and $SAT_{21}A_5$ reverse preference for the *S* an antiomer over the *R*. Only the single mutant (I-K_6-G_2) resulted in both good yield and ee.

These results suggest the existence of a midsize hydrophobic group, which has not been significantly affected by the mutations. The saturation mutagenesis on Phe432 did not improve the mutants' capacity to accept 4-substituted cyclohexanones.

Baeyer-Villiger oxidations of 4-alkyl-substituted cyclohexanones were straightforward reactions that afforded only single lactone products. We were unable to identify monooxygenases that provided (*R*)-lactones from ketones with acceptable optical purities, although we were able to produce (*S*) enantiomers with uniformly high % ee values using biocatalysts identified in this study. Discovering (*R*)-selective Baeyer-Villiger monooxygenases remains an important goal that will require a further expanded set (evolution) of enzymes.

3.3 Experimental

3.3.1 General procedure for the biotransformation with growing cells (Screening).

Fresh plates were streaked from a frozen stock of an appropriate organism on a LB-agar plate supplemented with ampicillin. A single colony was used to inoculate 10 mL LB medium containing 50 μL ampicillin stock solution and the preculture was incubated overnight with shaking (200 rpm) at 37 ℃. The following morning, the preculture (200 μL) and 2 mL of 20% glucose were added to 20 mL of fresh LB in a 250 mL baffled flask. The reaction was incubated at 30 ℃ with shaking at 240 rpm until the culture reached OD_{600} =0.04; at that point the flask was allowed to equilibrate to room temperature (approximately 20 minutes) and 20 μL of IPTG was added. If cyclodextrin was necessary to alleviate the solubility problem, it was introduced at this stage. The culture was shaken another 30 minutes at room temperature and then 10 mg of substrate

was added. The biotransformation was shaken at room temperature for 24 hours and was monitored by GC.

3.3.2 General procedure for preparation of reference samples of racemic lactones.

Baeyer-Villiger oxidation of 4-substituted cyclohexanones using *m*-CPBA / TFA was carried out in a general procedure: 60 mg of *m*-CPBA with 200 μL TFA in 10 mL CH_2Cl_2 were cooled to 0 °C for 30 minutes, then kept at 25 °C overnight. The target product was isolated and separated by micro-column silica gel. Lactone **3-2** could not be obtained by this method; it was difficult to obtain lactone **2-9** as well.

3.3.3 The baker's yeast procedure

Commercial baker's yeast (0.5 g) was added to a solution of sucrose (2 g) in sterile water (25 mL). The mixture was stirred at 30 °C over 30 minutes to activate the yeast. The lactone (25 mg) was added to the reaction. The conversion was monitored by GC; after the reaction reached completion, the yeast was harvested by centrifugation. The aqueous layer was extracted three times with CH_2Cl_2; after the solvent was removed, the crude product was purified by column chromatography using hexane : ethyl acetate =2:1 to give lactone **1-9**.

CHAPTER 4

Scale-up Preparation of Enantiopure Lactones Using Baeyer-Villiger Monooxygenases

4.1 Introduction

The focus of the work presented in this Chapter were the development of a bench-scale Baeyer-Villiger biotransformations of 4-substituted cyclohexanones to the corresponding lactones. The work was based on an original protocol developed by Dr. Stewart's group at the University of Florida.[100] The main objectives were to maximize the concentration of product in the reaction mixtures and the yield of lactone in order to simplify subsequent isolation of the products. It was hoped to demonstrate the viability of the biocatalytic Baeyer-Villiger oxidations for the synthesis of many enantiopure lactones on preparative scales.

Bioprocesses are usually developed in three stages: [102] (i) laboratory scale, where basic screening procedures are carried out; (ii) pilot plant scale, where the optimal operating conditions are ascertained; and (iii) industrial plant scale, where the process is brought to economic fruition. Effective scale enlargement is essential for successful industrial bioprocessing.

Large scale biotransformations are preformed in fermentors that provide stable and optimal environments for large scale operations.[103] In a fermentor it is possible to control many of the physiological factors that can affect the biotransformation to an extent not feasible in flasks and tubes.

Scale-up requires the optimization of all relevant physiological factors that have been established previously in small scale studies.[104] This requires control of the enviromental conditions. Physical factors that have been found to be important in scale-up include agitation, aeration (air flow), mixing time, mass transfer, and shear stress. Many of these factors are interrelated. For example, in reactor design, mixing time, shear stress, and mass transfer aspects are coupled, while heat transfer can be controlled separately. Chemical factors, such as pH, medium composition, and concentration, are related by economic considerations. Optimization of processing factors, such as number of precultures needed, sterilization conditions, and foam control can also require repeated multiple tests to achieve optimal operating conditions.

4.2 Physiological factors

Determining scale increase criteria that are optimal for a particular microorganism used for metabolite production is a difficult task. For this reason, the characteristics of a microorganism should be well-known before scale-up is attempted. Several physiological factors that depend on the characteristics of the microorganism must be considered.[105(a,b)]

1. Physical factors

1. (a) Mass Transfer.

For almost all microorganisms, oxygen is a very important factor in growth and productivity. Lack of oxygen often causes the death or at least a reduction in the yield in the microorgansim. In scale-up it is therefore critical to control oxygen transport in order

to maintain the dissolved oxygen (DO) concentration at a suitable level.[106] DO concentration is the most commonly used indicator for monitoring conditions in a fermentor. It is controlled by changing the agitation speed and the shape of the impellers.[107] When the strength of the motor of the existing fermentor is insufficient to increase the DO concentration for cultivation of a microorganism, a decrease in the volume of the fermentor is sometimes required as a means to maintain the DO concentration.[108]

1. (b) Shear Stress.

When microorganisms such as fungi are cultured in a large fermentor, cells can be damaged by shear stress.[109] Shear stress is considered to be proportional to the speed of the agitation impeller.[110] This agitation must not be so fast as to damage microorganisms. Organisms that are sensitive to shear stress must be cultured with large–diameter impellers in order to maintain a suitable DO level at low agitation speed.[111]

1. (c) Mixing Time.

Mixing time is one of the criteria most widely used to characterize the mixing intensity in bioprocesses. It is defined as the time needed to obtain a specific degree of homogeneity in the reaction mixture. In bioreactors, the mixing time depends mainly on the amount of energy consumed, reactor and stirrer shapes, rate of air flow, and the concentration of the medium. Mixing time is often a key factor in the bioprocesses in which pH control or substrate feeding is needed.[112]

For example, when a feed substrate inhibits cell growth, or when substrate concentration has to be controlled at a low level, a long mixing time in a large fermentor can result in low production yield. To shorten mixing time, one can use large diameter or sloped impellers or feeding from mutiple points in the fermentor.[113]

2. Chemical Factors

For economic reasons, low grade or cheap natural ingredients are often used for medium preparation in large scale fermentations. Different microorganisms have special requirements for optimal growth; the ratio and the amounts of carbon, nitrogen, phosphorus, and special growth factors are important in proper nutrition in the media. The *E.coli* used in this work was grown in nutrient medium (LB-Broth) containing bacto troptone, and bacto yeast extract. Sodium chloride is the most common and useful additive to enhance the cell growth. Controlling the nutrient concentration to a suitable level is an effective way to scale-up the chemical conditions of the culture broth.[114]

3. Process Factors

As the size of the fermentor increases, scale enlargement processes have to change. For example, to increase cell mass, the number of precultures must be increased. A large amount of a natural medium may be needed. Complete sterilization of a large fermentor also becomes more difficult.

3. (a) Number of Precultures.

The mass of cell is defined as the amount of cells that were obtained from the preculture medium at the time in the growth cycle when enzyme activities are highest. To increase the mass of cell that is prepared for a production culture in a large scale fermentor, several repeated precultures may be required. The condition of the cells, that are inoculated into the production culture is extremely important, and succcessful microorganism propagation protocols should be able to synchronize the cell growth, minimize any possible contamination, maximize the mass yield from a given culture, and maximize the microorganism activity.[115]

3. (b) Sterilization.

Sterilization time in a large scale fermentor can be considerably longer than that which is required in a small fermentor. The long sterilization often causes caramelization of the media and degradation of important components in the media.[116] These chemically changed media often inhibit the growth rate of cells and negatively affect the production yield.[117]

3. (c) Foam Control.

Many media will develop a foam-head when mixed vigorously in the fermentor. A stable foam constitutes an additional potential barrier to gas exchange between the vessel's headspace and the liquid phase and thus adversely alter the DO concentration.[118] Foam can be controlled by the addition of antifoams. Common criteria for selection of an antifoam are:[119]

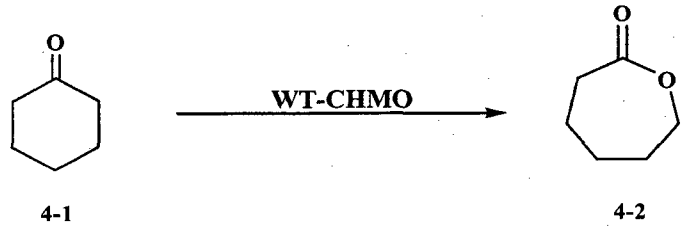
- Effectiveness in controlling foam
- Absence of toxicity
- Compatibility with harvest or scale enlargement procedures
- Positive (or minimal negative) effect on gas-liquid oxygen transfer
- Cost

Silicon based antifoams are most often used, followed by those based on polyglycols and oils. In this study poly-dimethyl siloxane was used.

In this chapter the optimization of scale-up Baeyer-Villiger biotransformations was investigated. The optimization of the fermentation procedure for Baeyer-Villiger oxidations in non-growing conditions was carried out with WT-CHMO and some of its mutants. Non-growing cells are preferred for the scale-up biotransformations since they offer extended reaction times between addition of the substrate portions and the time at which the best volumetric yield of the product is achieved. This can help avoid problems of substrate and product toxicity.

4.3 Results and discussion of the optimization conditions

In the first series of measurements, cyclohexanone was used as a test substrate and WT-CHMO was the enzyme catalyst (Scheme 4-1). The substrate was added in three portions at one hour intervals, since this was experimentally the most convenient.



4-1 4-2

Scheme 4-1. Oxidation of cyclohexanone by Baeyer-Villiger oxidation

In the second part of this work, the conditions established for cyclohexanone and WT-CHMO systems were used to scale-up the Baeyer-Villiger oxidation of five 4-substituted cyclohexanones using WT-CHMO, WT-CPMO, and their mutants from the 20 mg scale used in the screening studies (Chapter 3) up to the 350 mg scale using the 500 mL fermentor at UNBSJ (working volume 250 mL). In the last part of this work, the cyclohexanone-WT-CHMO-system was further scaled up to the 3 g scale using the 2 L fermentor volume (working volume 1 L) at the Centre of Excellence in Agriculture and Biotechnological Sciences, Grand Falls, NB.

4.3.1 Scale up of Baeyer-Villiger oxidation of cyclohexanone, and 4-substituted cyclohexanone to the 350 mg Scale

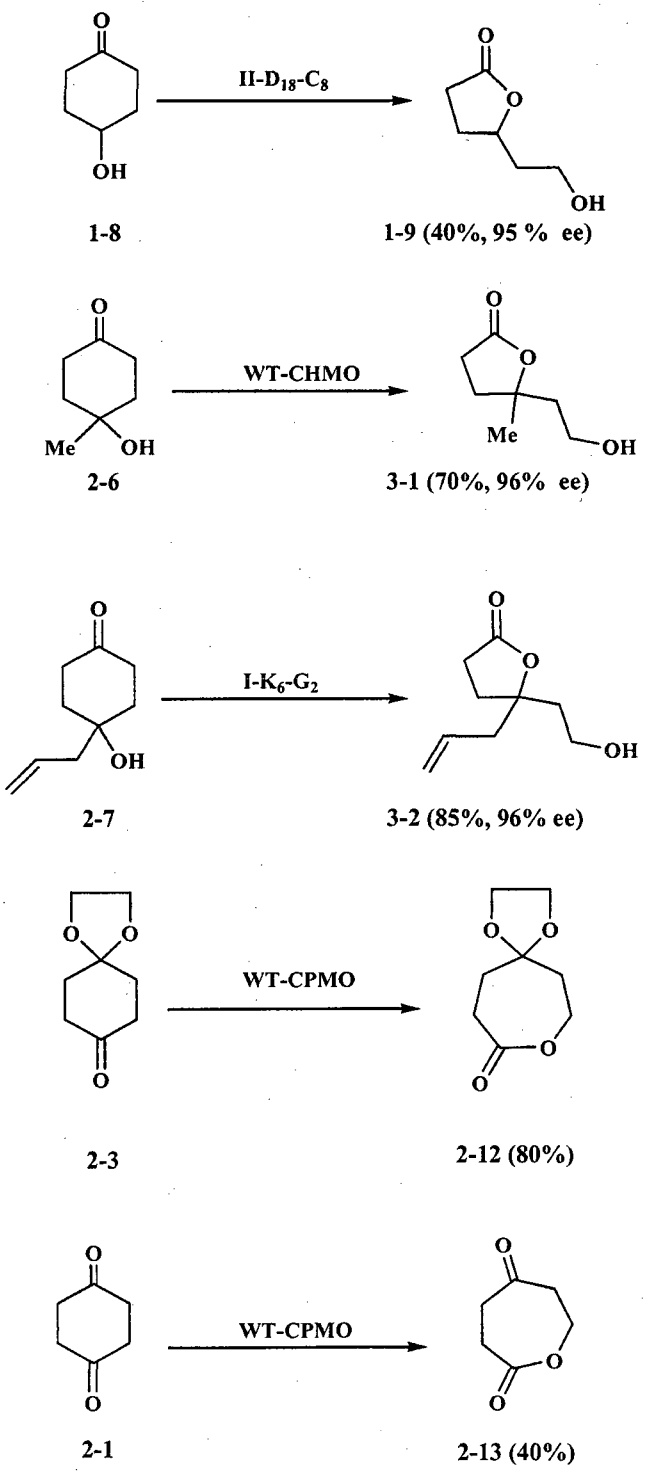
The Baeyer-Villiger oxidation of cyclohexanone catalyzed by WT-CHMO was investigated first. Results are summarized in Table 4-1. As can be seen, no conversion occured in the 24-hour reaction time when small amounts of cells were used (2.022 g and 3.905 g). For an efficient conversion, more than a 6.990 g biomass was needed. To achieve this biomass the products of three precultures had to be combined.

Table 4-1. Scale-up of biocatalytic Baeyer-Villiger oxidations to 350 mg scale in a 500 mL fermentor

Wet -weight Cells (g)[1]	Air (L/min)	pH	Agitation (rpm)	Time (h)	Conversion[2] (%)
2.022	2	7	200	24	NR[3]
3.905	2	7	200	24	NR
6.990	2	7	200	24	90

(1)The cells were grown in LB medium, and harvested by centrifugation.
(2) Conversion was determined by GC.(3) NR= no reaction

The scale-up conditions for the Baeyer-Villiger oxidation of cyclohexanones were used for the oxidation of other target substrates to their corresponding lactones. The five best results from the screening reactions described in Chapter 3 (see table 3-1) were chosen for a scale-up to the 350 mg level under non-growing conditions. The results are shown in Scheme 4-2.



Scheme 4-2. Scaled-up preparation of lactones **1-9, 3-1, 3-2, 2-12**, and **2-13**

The yields of the scale-up process were obtained by the isolating of the products from the aqueous layers using time-consuming continuous extraction. Based on the large scale optimized conditions, the yields of enantiopure lactones of all target substrates were sufficient to attach them to functionalized silica.

4.3.2 Scale-up to the 3 g scale in a 2 L fermentor

The optimized reaction conditions obtained for the 500 mL fermentor at UNBSJ were adapted for use in a 2 L fermentor with a working volume of 1 L at the Centre of Excellence in Agriculture and Biotechnological Sciences, Grand Falls, NB.

Again it was found that a large cell mass was needed. Table 4-2 shows that no reaction was obtained for cell masses of 6.304 g and 9.225 g. A large cell mass of 13.993 g was needed for efficient conversion in the 24-hour reaction time used. To reach this cell mass, three preculture had to be combined.

Table 4-2. Effect of cell mass on biocatalytic Baeyer-Villiger oxidations of cyclohexanone on 3 g scale

Wet-weight Cells (g)	Air (L/min)	pH	Agitation (rpm)	Time (h)	Conversion (%)
6.304	4	7	450	24	NR
9.225	4	7	450	24	NR
13.993	4	7	450	24	99

The effect of aeration was investigated. As expected, decreasing the air flow decreased the conversion (Table 4-3). Also as expected, pH =7 was optimum. Increasing the pH to 8 decreased the percent conversion after 24 hours of reaction. In contrast, there was no substantial effect observed when changing the agitation factor.

Table 4-3. The factors (aeration and pH) affecting the scale-up conditions

Wet-weight Cells (g)	Air (L/min)	pH	Agitation (rpm)	Time (h)	Conversion (%)
~13.993	2	7	450	24	77
	3	7	450	24	89
	4	7	450	24	99
	4	8	450	24	69

Finally, two CHMO mutants, I-K_6-G_2 and (II-D_{18}-C_8) were tested using the optimum condition determined for WT-CHMO. The first mutant (I-K6-G2) gave very comparable results to that of WT-CHMO. The second mutation gave significantly lower conversion, however, and the results are shown in Table 4-4.

Table 4-4. The results of the scale-up using CHMO mutants

	Wet-weight Cells (g)	Air (L/min)	pH	Time (h)	Agitation (rpm)	Conversion (%)
WT-CHMO	13.993	4	7	24	450	99
I-K_6-G_2	13.823	4	7	24	450	98
II-D_{18}-C_8	13.980	4	7	24	450	79

4.4 Experimental

4.4.1 Procedure for biotransformtions with non-growing cells in a 250 mL fermentor

Fresh plates were streaked from a frozen stock of an appropriate organism on an LB-agar plate supplemented with ampicillin. A single colony was used to inoculate 10 mL LB medium containing 50 μL of ampicillin. Preculture (8 mL) was added to 800 mL of fresh LB-ampicillin (500 μL) medium in a sterile 2 L baffled flask and incubated at 37 °C with shaking at 240 rpm until the culture reached OD_{600} = 0.04. At that point IPTG (100 μL) was added. The culture continued to grow at 30 ^{0}C until the OD_{600} was 0.4. Then, cells were harvested by centrifugation; the cell pellet was suspended in 250 mL of M9 minimal salt medium and was placed in a 500 mL fermentor. The substrate was added

continuously to the bioreactor each hour until a total of 400 mg of substrate had been added; fermentation was carried out at room temperature with the stirrer speed set at 200 rpm, and the air flow set at 2 mL/ min. The pH was maintained at 7 manually with 3M NaOH, and 25 mL of 20% glucose was added each hour during the fermentation process. The reaction mixture was sampled periodically and analyzed by GC. Samples (200 μL) were diluted with the same amount of ethyl acetate and were centrifuged for 1 minute; 0.1 μL of the supernatant solution was analyzed by GC. When the reaction reached completion, as determined by GC spectra conversion or after 24 hours (whichever occurred first), the fermentation mixture was saturated with NaCl and the cells were removed by centrifugation.The aqueous layer was then extracted with ethyl acetate by using a liquid / liquid continous extractor. The organic layers were combined, washed with brine, and dried with $MgSO_4$. The solvent was removed under vacuum. All the products were purified by chromatography on silica gel.

4.4.2 Procedure for biotransformations with non-growing cells in 1 L fermentor

The reaction occurred over a period of four days

Day 1: An appropriate *E.coli* strain was streaked on a fresh LB-agar plate supplemented with ampicillin and incubated at 37 ^{0}C until the colonies were 1-2 mm.

Day 2: A single colony was used to inoculate a 10 mL LB-medium with 50 μL ampicillin. The culture was incubated overnight with shaking (200 rpm) at 37 °C. The LB-medium (1 L) was autoclaved in a 2 L fermentor.

Day 3: The fermentor was set up for growing the culture. This involved:

- Inoculation of the medium with 10 mL of a preculture containing 1 mL of ampicillin using the inoculation bottle
- Growth of the culture in the fermentor at 37 ℃ with an air flow of 2 L /min. The pH was adjusted with 3M NaOH and 1 M HCl; the agitation speed was set at 300 rpm.
- Sampling of the culture to check the OD (optical density), when the OD_{600} value reached 0.06, IPTG (100 μL) was added and the growth was continued until the D_{600} was 0.4.
- Harvesting of the cells by centrifugation.
- Cleaning the vessel after the fermentation (see the fermentation protocol- appendix I)
- Assembly of the fermentor (see the fermentation protocol- appendix I)
- Preparation of the fermentor for the autoclave; 1 L of M9 minimal salt medium was placed in the 2 L fermentor and autoclaved using a special autoclave. (see the fermentation protocol- appendix I)

Day 4: The fermentor was set up for the biotransformation reaction by inoculation of the autoclaved M9 minimal salt medium (1 L) with wet-weight cells (13.7993 g); 30 mL of 20% glucose was added with a substrate (1 mL).

- The biotransformation reaction was carried out at 32 °C with a stirrer speed of 450 rpm and an air flow of 4 L/ min. The pH was maintained at 7 by automatic addition of 3 M NaOH.
- 10 mL of 20% glucose was added each hour during the fermentation process.
- The reaction mixture was sampled periodically and analyzed by GC every hour.

4.5 The enantiopure lactones scaled-up

The isolated yields of these materials were obtained after 24-hour extractions using a liquid / liquid continuous extractor.

5-(2-Hydroxyethyl)-dihydrofuran-2-one (1-9) was synthesized according to the general procedure for non-growing cells using **mutant II-D_{18}-C_8**. The reaction was complete after 24 hours. The product was purified by column chromatography on silica gel by eluting with petroleum ether : ethyl acetate (1:3) to give a colorless oil (40 %). IR ν_{max}: 3434 (br, s), 2960 (s), 2894 (m), 1775 (vs), 1190 (s), 1052 (s), 959 (m) cm^{-1}; ^1H NMR δ: 4.75-4.68 (1H, m), 3.84 (2H, t, J=8.8 Hz), 2.56 (2H, m), 2.41-2.35 (2H, m), 2.16 (s), 1.98-1.89 (3H, m); GC (retention time 10.6 min)

5-(2-Hydroxyethyl)-5-methyl-dihydrofuran-2-one (3-1) was synthesized according to the general procedure for non-growing cells using **WT-CHMO**. The reaction was complete after 7 hours. The product was purified by column chromatography on silica gel by eluting with petroleum ether : acetone (2:1) to give a colorless oil (70 %); IR ν_{max}: 3434 (br, m), 2970 (s), 2835 (m), 2900 (m), 1773 (vs), 1432 (m), 1200 (m), 1135 (s), 920

(s) cm^{-1}. ^1H NMR δ: 3.91 (2H, t, J=9.6 Hz), 2.68 (2H, m), 2.25 (2H, m), 2.03-1.98 (3H, m); 1.64 (2H, t, J=1.09 Hz), 1.48 (3H, s). GC (retention time 11.0 min).

5-Allyl-dihydro-5-(2-hydroxyethyl)furan-2(3H)-one (3-2) was synthesized according to the general procedure for non-growing cells using **CHMO mutant I- K_6-G_2**. The reaction was complete after 24 hours. The product was purified by column chromatography on silica gel by eluting with hexane : ethyl acetate (1:1) to give a yellow oil (85 %); IR ν_{max}: 3436 (br, m), 3077 (s), 2970 (s), 2835 (m), 1767 (vs), 1641 (s), 1434 (m), 1278 (m), 1189 (s), 1048 (s), 928 (s) cm^{-1}. ^1H NMR δ: 5.82 (1H, m), 5.22 (2H, t), 3.86 (2H, dd, J_1=3.3 Hz, J_2=7.56 Hz), 2.66 (2H, m), 2.47 (2H, m), 2.16 (2H, m), 1.99 (3H, m). GC (retention time 9.6 min).

1,4,8-Trioxaspiro[4.6]undecan-9-one (2-12) was synthesized according to the general procedure for non growing cells using **WT-CPMO**. The reaction was complete after 7 hours. The product was purified by column chromatography on silica gel by eluting with petroleum ether : acetone (4:1) to give a brown powder (80 %); IR ν_{max}: 2962 (s), 2890 (s), 1731 (vs), 1478 (m), 1293 (m), 1157-1027 (s, m), 942 (m) cm^{-1}. ^1H NMR δ: 4.22 (2H, dm), 3.98 (4H, m), 2.71 (2H, td, J_1=5.3 Hz, J_2=1.4 Hz), 2.01 (2H, m), 1.91 (2H, m). GC (retention time 12.5 min).

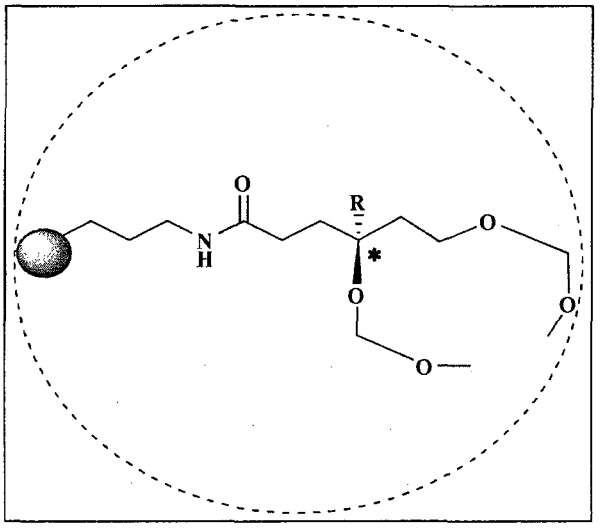
Oxopane-2,5-dione (2-13) was synthesized according to the general procedure for non-growing cells using **WT-CPMO**. The reaction was complete after 24 hours. The product was purified by column chromatography on silica gel by eluting with petroleum ether : acetone (3:1) to give a brown crystal (40 %); IR ν_{max}: 2962 (s), 2890 (s), 1738-1717 (s, m), 1437 (m), 1240 (m), 1116 (s), 900 (m) cm^{-1}. ^1H NMR δ 2.6-2.7 (m, 4H), 2.73 (t, 2H, J=1.43 Hz), 4.32 (t, 2H, J=1.205 Hz); GC (retention time 9.6 min).

CHAPTER 5

Attachment of Enantiopure Lactones to Functionalized Silica. A New Class of Chiral Ligands

5.1 Introduction

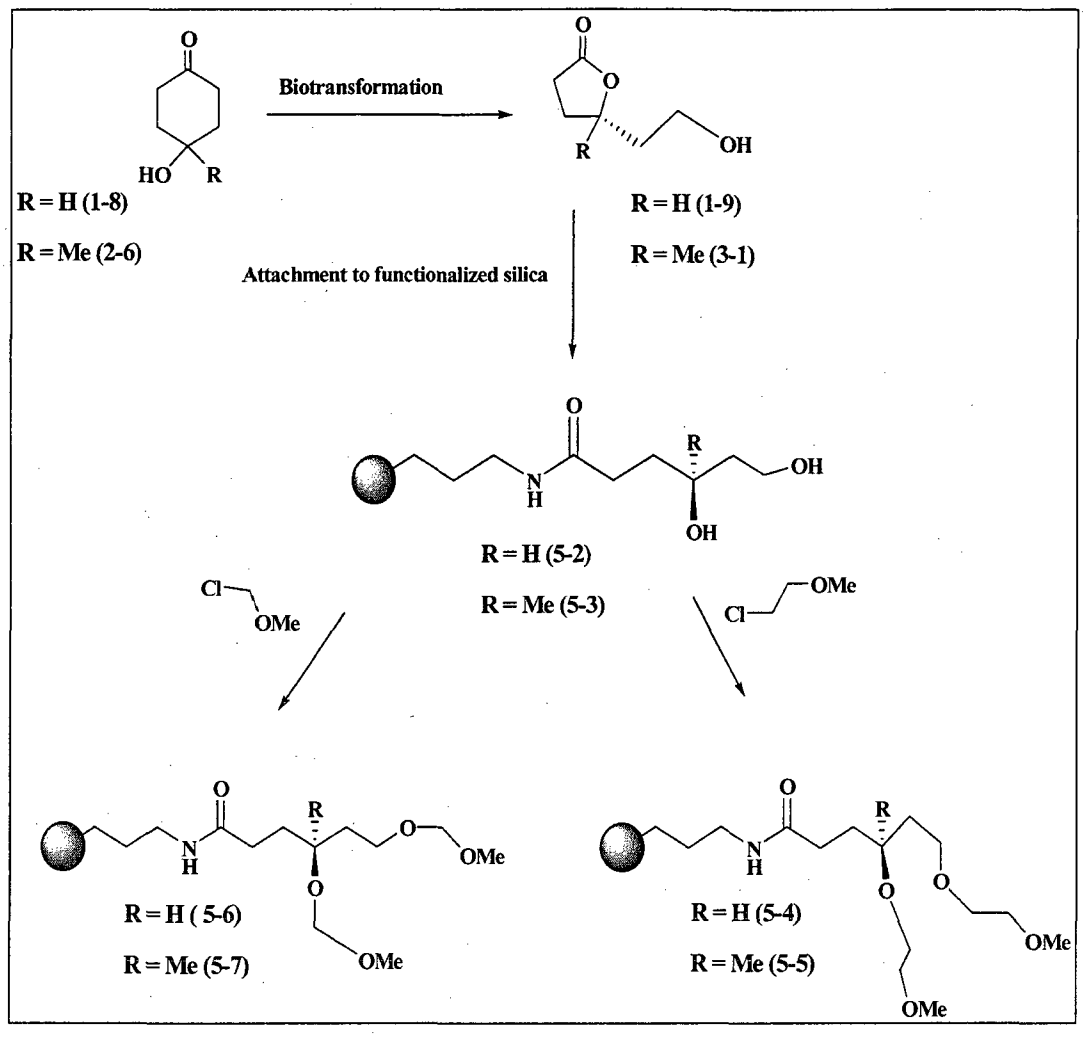
The goal of the project presented in this chapter was the synthesis of a new solid supported chiral ligand shown in Figure 5-1.



5-1

Figure 5-1. A new chiral ligand

The aim was to attach some of the enantiopure lactone produced by enzymatic Baeyer-Villiger oxidation of 4-substituted cyclohexanones described in Chapters 2, 3, and 4 to amine functionalized silica supports as shown in Scheme 5-1.



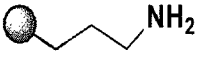
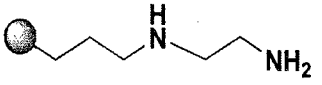
Scheme 5-1. Attachment of enantiopure lactones to functionalized silica

In Chapter 4, it was shown that *E. coli* cells overexpressing BVMOs could carry out model Baeyer-Villiger oxidations of cylcohexanones to enantiopure lactones with high volumetric productivity. Here, is described attempts to attach two of these

enantiopure lactones, **1-9** and **3-1**, to solid silica supports *via* formation of amides with NH_2-groups grafted to the silica surface. The amide products **5-2** and **5-3** could have the potential to be used as scavenger reagents in organic synthesis.[119(a-c)] Furthermore, they can be converted easily to chiral ligands **5-4, 5-5, 5-6,** and **5-7** that for example, could be used for the coordination of metals such as zinc[120] to generate new chiral catalysts.

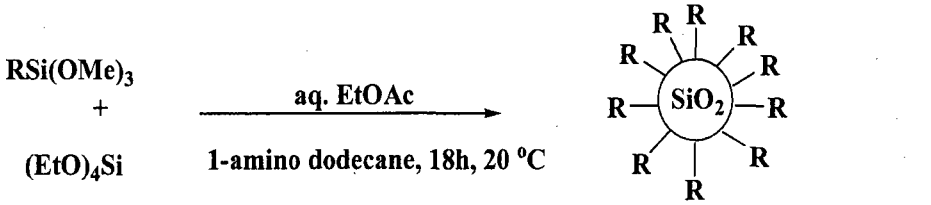
The attachment reactions were carried out using two functionalized silicas **5-1** and **5-2** purchased from SiliCycle® Inc. Company. The characteristics of these functionalized silicas as provided by SiliCycle® are summarized in Table 5-1.

Table 5-1. The characteristics of silica supported amine **5-1** and **5-2.**

Silica	Mesh size (μm)	Analysis		Loading (mmol/g)
		%C	%N	
 **5-1**	40-63	7.27	2.18	1.54
 **5-2**	40-63	9.55	4.26	1.52

Functionalized silicas have proven to be effective solid supports. Which have been shown to have several advantages:[121 (a,b)] (i) supported reactions are easy to monitor using simple analytical techniques; (ii) excess reagent can be used to drive reactions to completion and can be removed by simple washing of the silica without compromising the purity of the products; and (iii) functionalized silica shows broad solvent

compatibilities; they do not swell and are stable over a range of temperature and pH conditions. The literature on silica and amine derivatized silica gel interactions is vast and reflects the versatility that can be obtained by manipulation of the initial nature of the silica and of the silylation reaction conditions. This versatility has led to many opportunities to create designed silane-silica structures.[122] Silica supported amines are easily prepared. For example Macquarrie *et al.* reported the preparation of amine and diamine functionalized silica gels in high loading by the condensation of 3-aminopropyltrimethoxysilane or 2-aminoethyl-3- aminopropyltrimethoxysilan)[123] and their use as efficient scavengers for acid chlorides, sulfonyl chlorides, isocyanates and other electrophiles (Scheme 5-2).[119(a-c)]



Scheme 5-2. Synthesis of the high loading functionalized silicas

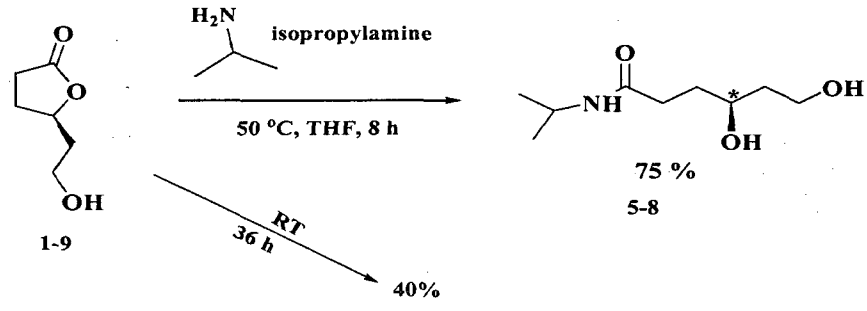
The loading capacity is of considerable importance when choosing a scavenger for purification since higher loading allows the use of less reagent and lower expenses. Therefore, the search for higher loading alternatives that are inexpensive and easily handled has become increasingly important.

5.2 Results and Discussion

5.2.1 Solution phase reaction

Before attempting the reaction with solid support amines, the coupling reaction was first attempted in the solution phase to show that formation of the desired amide by reaction of the lactone with an amino group would proceed as anticipatied.

In a typical experiment, lactone **1-9** was dissolved in anhydrous THF and treated with one equivalent (1eq) of isopropylamine. When the reaction was performed at room temperature only 40% conversion was obtained after 36 hours of reaction. Raising the temperature to 50 °C gave a better conversion (75%) in a more reasonable 8-hour reaction period (Scheme 5-3).



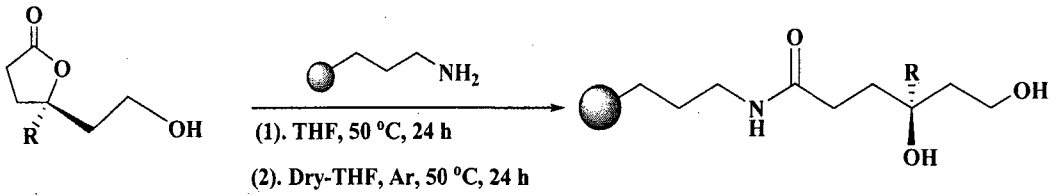
Scheme 5-3. Attachment of enantiopure lactone **1-9** to isopropylamine in solution

5.2.2 Solid phase reaction

Having shown that an enantiopure lactone would react with an amine, the conditions that gave the best results in the solution phase were then tried with the two supported amines.

1. Attachment to 3-aminopropyltrimethoxysilane (5-1)

The reactions of the lactones **1-9** and **3-1** with silica **5-1** were attempted in THF as the solvent (commercially available THF and dry THF). The procedures are shown in Scheme 5-4.



1-9 (R = H)
3-1 (R = Me)

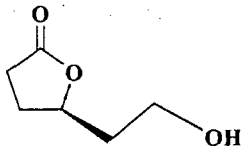
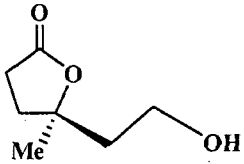
5-2 (R = H)
5-3 (R = Me)

Scheme 5-4. The attachment procedures of lactones **1-9** and **3-1** to silica **5-1**

Reaction in commercially available THF as a solvent gave a granular white powder. Elemental analysis of the product gave nitrogen contents of 1.65% and 1.67% for reaction with **1-9** and **3-1** respectively (Table 5-2). By assuming that all this nitrogen was part of organic chains attached to the silica surface, amide loadings of 1.18 and 1.19 mmol/g were calculated. This would mean that more than three-quarters of all the amine groups initially present in **5-1** were converted to amides **5-2** and **5- 3** (76% and 78% respectively).

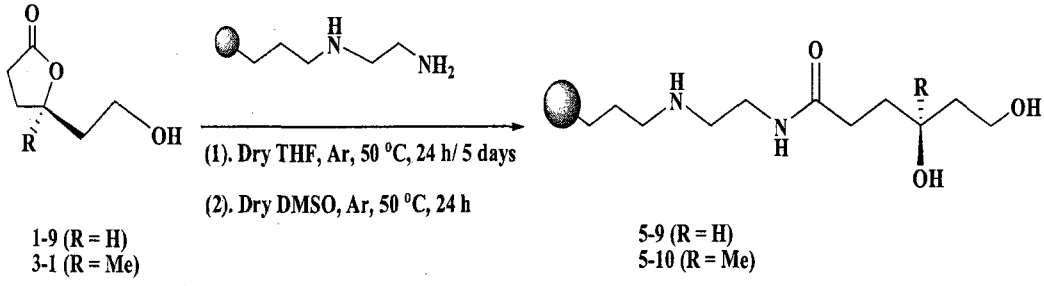
Using THF that had been previously dried with Na metal gave yellow granules that had lower nitrogen contents, corresponding to slightly lower loadings than with the commercial THF (Table 5-2).

Table 5-2. Attachment results of the lactones **1-9** and **3-1** with silica **5-1**

Lactone	Solvent	Analysis		Loading
		%C	%N	mmol/g
	THF	11.82	1.65	1.18
	Dry THF	14.03	1.49	1.08
	THF	10.39	1.67	1.19
	Dry THF	13.24	1.51	1.08

2. Attachment to 2-aminoethyl-3-aminopropyltrimethoxysilane (5-2)

The reactions of the lactones **1-9** and **3-1** with silica **5-2** were attempted in two different solvents (dry THF and dry DMSO) (Scheme 5-5).

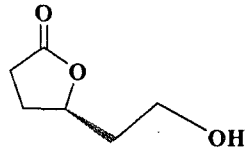
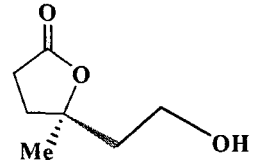


Scheme 5-5. The attachment procedures of lactones **1-9** and **3-1** to silica **5-2**

The product using dry THF produced yellow granules. Elemental analysis again showed high nitrogen contents that correspond to amide loadings of 1.16 mmol/g for both **5-9** and **5-10** after 24 hours of reaction. This did not change significantly when the reaction time was extended to 5 days. Results are shown in Table 5-3.

Reactions in DMSO gave brown granules. Elemental analysis of the products gave lower nitrogen contents corresponding to a much lower loading and also a significant sulfur content, indicating that not all the DMSO solvent could be washed out of the products (Table 5-3).

Table 5-3. Attachment results of the lactones **1-9** and **3-1** with silica **5-2**

Lactone	Solvent	Time	Analysis			Loading
			%C	%N	%S	mmol/g
	Dry THF	24 h	19.09	3.24		1.16
	Dry THF	5 days	18.15	3.34		1.19
	Dry DMSO	24 h	11.07	1.59	0.33	0.57
	Dry THF	24 h	17.39	3.15		1.16
	Dry THF	5 days	17.26	3.27		1.19
	Dry DMOS	24 h	17.36	2.74	0.23	0.98

5.3 Details of the solid state NMR experiments

The sample lactone **3-1** reacted with silica **5-2** was sent to Halifax for solid state ^{13}C NMR to see if any signals were attributable to supported amide **5-10**.

The spectra obtained after 2 hours of acquisition is given in Figure 5-2. The middle trace is the spectrum acquired with 5400 scans. The bottom trace is the spectrum obtained after 7200 scans and the top trace is the sum of the two. Three small peaks at 42, 22, and 9 ppm were obtained. Figure 5-3 shows a representation of the ^{13}C NMR spectra that was expected for amide **5-10**. It was noted that these three signals could be assigned to carbons **X, Y,** and **Z** Figure 5-3. Close exmanination of the spectra in Figure 5-2 also shows the presence of a very small signal at 172 ppm that would correspond to the amide carbon. However, to definitely establish that the desired amide did form would require a much longer acquisition time (see future work section at the end of the thesis).

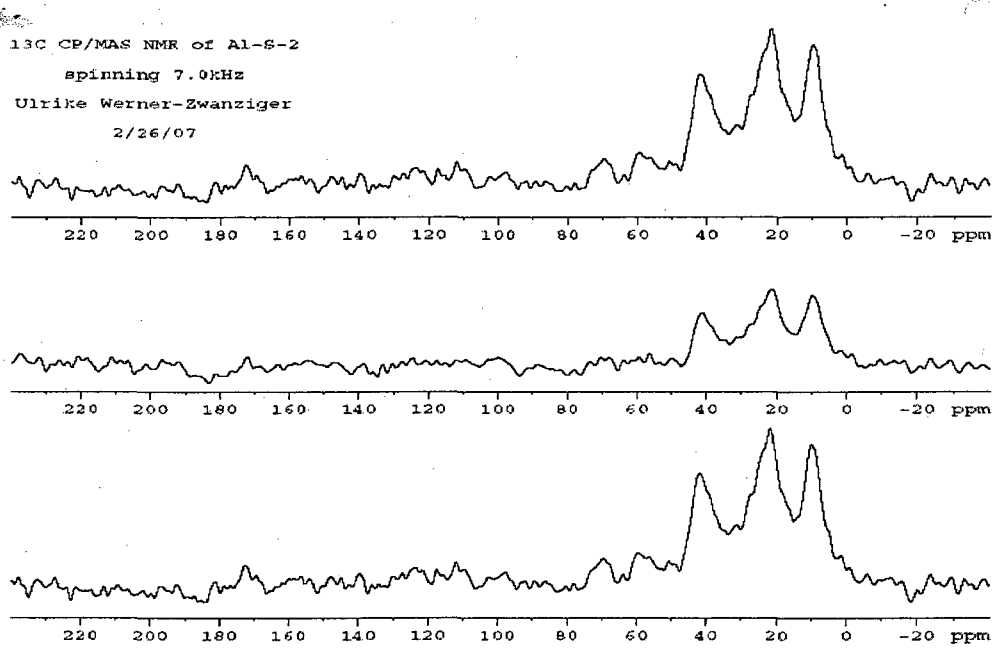


Figure 5-2. The solid state 13C NMR spectra

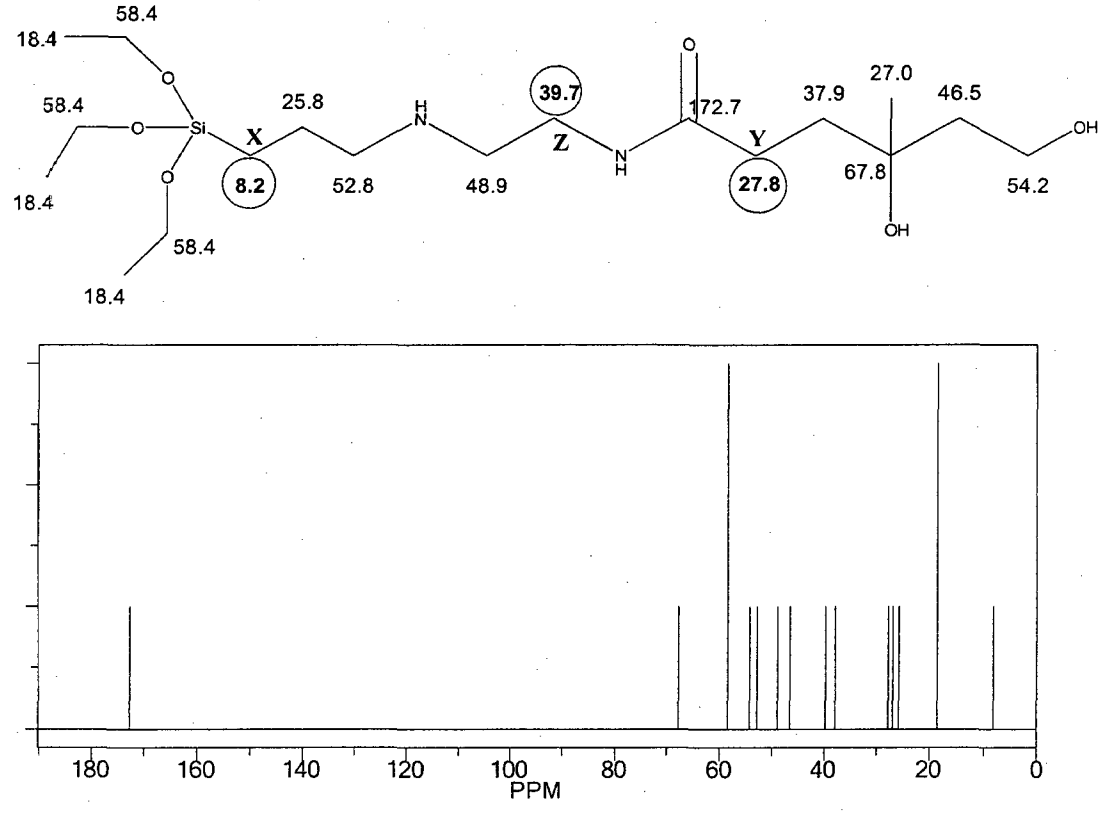


Figure 5-3. ^{13}C NMR estimation for the initial test sample

The ^{13}C NMR estimation for the initial test sample was adapted from chemistry office drawing, modeling and information (ChemDraw Ultra 8.0)

5.4 Experimental

5.4.1 Procedure for solution reaction

1. Preparation of *S*-4,6-dihydroxy-isopropylhexanamide (5-8)

In a typical experiment, lactone **1-9** (1.3 g) dissolved in anhydrous dry THF (30 mL) was treated with isopropylamine (0.6 mL). The reaction was heated at reflux with stirring for 8 hours. The progress of the reaction was monitored by TLC; when complete, THF was removed under reduced pressure and the crude product was purified by column chromatography using (hexane : ethyl acetate =3:1) to yield compound **5-8**. ^{1}H NMR δ 1.19 (6H, s), 1.61-173 (4H, m), 2.44 (2H, t), 3.80 (2H, t), 4.00 (1H, m), 4.2 (1H, m), 7.6 (1H, s)

1.2.4 Procedure for solid reactions

The attachment reactions were set up under argon when dry THF or DMSO was used as the solvent, and under air when commercial THF was used. All the reactions were carried out at 50 °C. The reactions were heated at reflux with stirring for 24 hours or 5 days (in the case of **3-1** with dry THF, Table 5-4). The progress of the reactions was monitored by TLC. When complete the silica was filtered, and washed sequentially with THF, ether, acetone, and ethanol.

CHAPTER 6

Conclusion and Future Work

Conclusion

Many methods were examined in this project for both the formation and the removal of the ketal group.

To further screen the substrate base and the selectivity of CHMO, CPMO, and their mutants, several ketone precursors of potentially useful lactones were tested, and excellent catalytic matches were identified.

Biotransformations performed under non-growing conditions gave higher conversions and consequently higher isolated yields for all reactions studied.

Non-growing cells are preferred for biotransformations carried out in a fermentor and give the best volumetric yield of product.

Scale-up methods were carried out using fermentors of different volume: the small-scale fermentor had a working volume of 250 mL, and the large-scale fermentor had a working volume of 1 L. The scale-up strategy was improved by optimization of fermentation conditions for these fermentors, since effective scale-up is essential for successful biotransformations.

The development of a larger-scale process for economic production of the intermediate was plagued by small-scale fermentor work; isolation of the products from the aqueous layers using continuous extraction was more time-consuming.

The current screening of saturation mutants of CHMO demonstrated that the first generation CHMO mutant enzymes [I-K_6-G_2 (F432S)] showed the highest enantioselectivities, and activity, as compared to its saturation mutants in the oxidations of 4-substituted cyclohexanones..

Future Work

The adaptation of diverse organic reactions to solid supports requires significant reaction optimization efforts.Therefore, several enantiopure lactones have to be attached to fuctionalized silica in order to construct more stable and selective catalysts.

These results would be accurate if the reaction had occurred at 100% (which is difficult to evaluate because the loading is measured according to the nitrogen analysis). Usually, the loading should be lower than for the original product because many of the atoms on the molecule were added, which would make the nitrogen percentage diminish.But how much lower? From the loading results, it can be seen that they are lower than for the original; to be sure of the percentage of reactivity, a hydrogen analysis should be carried out.

It would be intersting to determine whether amide groups can be detected in the samples by solid state NMR. A future goal would be to see whether any ^{13}C signal can be obtained in a reasonable length of time. In order to improve the signals, the sample would have to acquire 16 times as many scans because of the root dependence. In other words, rather than accumulating for the two hours invested into the top spectrum, the sample has to develop for approximating 32 hours. Other samples would require even more scans and time. It seems that ^{13}C solid state NMR may be a suitable technique to answer the question of whether there are amide linkages in the samples.

CHAPTER 7

References

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Appendix I

Description and Operation of the Equipment

1. The large fermentor (New Brunswick Scientific Bioflo 2000) has a capacity of 2-L (working volume 1-L); and is capable of controlling the pH, dissolved oxygen concentration, temperature, and agitation rate.

- Agitation speed is adjustable from 50 to 1000 rpm for a wide range of applications.
- Precise temperature control is provided by an electrical heater and large – capacity cooling coil.
- The vessel is constructed of heavy duty borosilicate glass with a stainless steel headplate; the vessel uncouples easily to facilitate cleaning and autoclaving.
- Baffles provide better oxygen transfer for cultures of aerobic organisms.
- Resterilizable 0.2 μ L inlet and exhaust-gas filter cartridges minimize the risk of contamination.
- Two six-blade Rushton impellers provide vigorous agitation and efficient mixing.
- An optional exhaust-gas condenser minimizes evaporation.
- The pH controller is an Optional pH-2000, designed for precise regulation of pH from 2-12; two peristaltic pumps are mounted on the module for automatic addition of acid and /or base to the culture medium.

- The dissolved oxygen controller is an Optional DO-2000, used to satisfy increased DO saturation levels needed to meet the demand of a wide range of microorganisms.
- The foam control module, an Optional FA-2000, is equipped with a panel mounted pump for the addition of antifoaming agent.

2. The small fermentor (New Brunswick Scientific Bioflo C 30) has a capacity of 500 mL (working volume 250 mL); and is not capable of controlling the pH, dissolved oxygen concentration, temperature, and agitation rate.

3. Optical density was measured on a spectronic 20 D spectrophotometer (Milton Roy Co).

4. Centrifugation (Jouan Centrifuge CR 4.22- CT 4.22) was used to harvest the cells.

5. Biofuge 13 microcentrifuge (Baxter Canlab) and a Genie-2 Vortexer (VWR Scientific) were used in the preparation of samples for analysis.

6. A low temperature freezer (SANYO Electric Co. Ltd) operating at -82 °C was used to store the yeast and *E.coli* strains.

7. Cell culturing and biotranformation reactions were performed on an Innova 4000 orbital shaker (New Brunsick Scientific).

8. Melting points were determined on a Fisher-Johns melting point apparatus.

9. IR spectra were recorded as thin films on a Mattson Satellite FTIR spectrometer.

10. ^{1}H NMR spectra were recorded in $CDCl_3$ solution, chemical shifts (δ) are reported in ppm using Me_4Si as internal standard.

11. The solid state ^{13}C NMR experiments were conducted on a Bruker Avance DSX NMR spectrometer with a 9.4 T magnet (proton Larmor frequency 400 MHz) using a

HXY probe head with rotors of 4 mm diameter. The solid samples were studied by ^{13}C cross polarization magic angle spinning (CP/MAS) NMR using TPPM proton decoupling.

12. Thin layer chromatography was performed on Sigma-Aldrich 0.2 mm aluminum-backed silica gel plates. Flash chromatography was performed on 230-400 mesh silica gel (SiliCycle® Inc. Company). The functionazed silicas (amine-containing silicas, based on the condensation of 3-aminopropyltrimethoxysilane or 2-aminoethyl-3-aminopropyltrimethoxysilane) were generous gifts from SiliCycle® Inc. Company.

13. Elemental analyses were performed by SiliCycle® Inc in Quebec City using Carlo Erba Instruments Na1500 Nitrogen-Carbon-Sulphur analyzer.

14. The GC instrument used a flame ionization detector (FID) with helium as the carrier gas. Typical operating parameters for **GC HP-5890**

Carrier gas	Helium 60mL/min	
Oven temperature:	initial temperature	:100 °C
	initial time	: 4 min
	final temperature	: 180 °C
	final time	: 10 min
	rate	: 10 °C/min
Injector temeprature	250 °C	
Detector temperature	300 °C	
Range	2	
Attenuation		

Appendix II

Protocols for Using Fermentor

1. Cleaning of the fermentor vessel

Cleaning is an important step in the biofermentation process. Mistakes will reveal themselves as lost time, money, and poor results. The desired cleaning of the equipment is essential for the completion of a proper fermentation. The equipment to be used will have been exposed to acids, bases, bacteria, as well as other chemicals; the removal of all of these contaminates is crucial in order to obtain reliable results.

Initial and final cleaning technique:

Prior to the termination of the fermentation, the air flow is discontinued.

At the control panel:

The agitator, the temperature control, and the pH control are turned off. (The temperature probe is removed; without first turning off the temperature control the heat blanket will overheat the vessel). The water supply to the condenser and cooler circuit are turned off. The heater blanket is disconnected and removed. All tubes, wires, probes, and cables are disconnected from the vessel.The agitator motor is removed from the head plate (HP) by turning the brass agitator one quarter turn, and lifting it from the vessel. The temperature probe is removed from the thermowell. The air and water tubes are disconnected (water inlet tubes first). The port adapters are loosened; then the pH probe

(with adapter attached) is removed , the probe is washed with water, wiped with 70% ethanol, rinsed with water, gently dried with tissue, and stored. After the port adapters are loosened, the dissolved oxygen (DO) probe with adapter attached is removed, cleaned (in a manner similar to that of the pH probe), and stored. The level probes are removed and cleaned and dried in a similar manner. The condenser is removed, as well as the filter from the sparger line. The NaOH tubing is removed from the peristaltic pump, the tubing is disconnected from the triport inlet, as well as other tubes from the inlet (and the small guard screw from each arm of the vessel), and the tubing from the sample line to the sample vial. The liquid addition bottles (Inoculation bottle) are emptied, washed, and dried. All ports are removed from the vessel and washed in a solution of soapy water, as well as all seals from the HP. The HP is slowly lifted, the two tabs on the baffle are pressed together, then the HP is removed completely. The baffle is removed from the vessel. All surfaces of the HP and baffle are scrubbed with a brush using soapy water, and then rinsed with 70% ethanol. Any remaining culture is poured into a container of disinfectant (bleach); the vessel is carefully rinsed with 70% ethanol, and then all the surfaces are scrubbed with a brush. Any remaining port adapters, inlet ports, and injection ports are removed, wiped with 70% ethanol, and dried. The O-rings are inspected for wear and a small amount of grease is applied if necessary. The underside of the HP is washed carefully without allowing the agitator housing on the top of the HP to become wet. The impellers, cooling coil, and sparge line are washed with water to remove any debris, and dried with tissue. After all the parts of the vessel are completely dry, the vessel is reassembled.

2. Assembly of the fermentor:

After verifying that all hoses, ports, baffles, and the vessel have been cleaned and dried, the baffle is inserted into the vessel. The unsterilized medium is poured into the vessel. The head plate is lowered into the vessel. A large O-ring and the thermometer housing are placed in the thermowell port and tightened. An O-ring and the DO hose are placed in the DO port and tightened. An O-ring and the condenser housing are placed in the condenser port. The condenser is added to its housing (and the flow of water into the cooling coil is started). A filter is attached to the end of the condenser by meaner of silicon tubing. An O-ring, the pH housing, and the pH probe are placed in the pH port and tightened. A filter is attached to the air intake port by means of silicon tubing; similarly a piece of silicon tubing is attached to the air out port. An O-ring, a small long stem, and silicon tubing connected with the inoculation bottle are placed in the inoculation port, and tightened. The sample bottle is connected to the sample port in a similar manner. The acid and base hoses are placed in their ports on the fermentor and secured with a pull tie.

3. Prepartion of the Fermentor for the Autoclave

The medium is added to the fermentor vessel. The HP is attached with the baffle ends hold together (with care). The motor housing is covered with foil. The cap is screwed onto the pH probe connector and covered with foil. The cap is pushed onto the DO probe connector and covered with cotton wool and foil.

All the filters are covered with cotton wool and foil. (Extra room is allowed for air to escape from the condenser filter. The clips between the sample port and inlet, inoculate port and bottle, and air inlet filter are closed using metal clips.

All holding nuts and port adapters are tightened, all unused ports are sealed, and all O-ring are checked for damage prior to autoclaving. The fermentor and attached bottles are placed in the autoclave, and the autoclave is set to the liquid media cycle (121 ^{0}C,15 min).

4. Calibration of the pH probe

The pH cable is attached to the biofermentor control panel. The pH controller and the biofermentor controller are turned on.The pH electrode is rinsed with distilled water, and dried with tissue. The probe is placed in a pH 7 buffer for several minutes prior to calibration. After pressing the function select key, and the mode select key, the display is adjusted to the 0-7 range. The probe is removed from the buffer and rinsed with distilled water, and dried with a tissue. The probe is placed in a pH 4 buffer and calibrated in a similar manner. (The pH probe is allow to sit in the buffer while the fermentor is being assembled).

5. Calibration of the dissolve oxygen probe

The DO probe is allowed to polarize in the fermentor medium for 4-6 hours before calibration. On the first panel of the fermentor the function select key, and the mode select key are pressed and released until the display is adjusted to zero. The DO probe cable is disconnected from the fermentor control; the function select key, and the mode select key are calibrated to zero again. After allowing the DO probe to be disconnected for 1 hour, the DO probe cable is reconnected to the controller. The air flow is increased to 2 L/min for 10 minutes, and the function select key is set to 100.

6. Connecting the acid/ base

After verifying that the pH probe is connected to the pH cable and the pH cable is connected to the pH controller, the hose for the base is connected to the glass rod on the base solution container, and as well the hose for the acid is connected. The hose of the base is fed to the base pump in a clockwise motion by turning the pump by hand gently. The hose for the acid is fed in a similar manner using the acid pump. The acid and the base are moved from their flasks into the fermentor by pressing the toggle switch to the prime position and monitoring the flow of liquid.

Appendix III

Recipes

Ampicillin stock solution

Sodium ampicillin	100 mg
Autoclaved water	1 mL

Store at 4 ^{0}C in an autoclaved bottle

IPTG

Isopropylthio-β-D-galactoside	100 mg
Autoclaved water	1 mL

Store at 4 ^{0}C in an autoclaved bottle

M9 minimal salts medium

Disodium phosphate (Na_2HPO_4)	30 g
Monopotassium phosphate (KH_2PO_4)	15 g
Sodium chloride (NaCl)	2.5 g
Deionized water	500 mL

Add 10 mL of a sterile 20% aqueous glucose solution after the other materials have been autoclaved.

LB-aqueous medium

Bacto Troptone	10 g
BactoYeast Extract	5 g
NaCl	10 g
Deionized water	1 L

Autoctave, then mix with 4 mL ampicillin stock solution.

Agar plate

LB-medium	6.2 g
Agar	2 g
Deionized water	250 mL

Autoctave, then mix with 1mL ampicillin stock solution.

Dipping solutions for TLC

Vanillin solution

Vanillin	5 g
EtOH	100 mL
H_2SO_4	0.5 mL

Anisaldehyde solution

Anisaldehyde	10.7 mL
EtOH	200 mL
H_2SO_4	2.0 mL

Permanganate solution

$KMnO_4$	1.5 g
K_2CO_3	10 g
Deionized water	150 mL
NaOH	2.5 mL

Appendix IV

^{1}H NMR spectra

1. 1,4-Cyclohexanedione monoethylene ketal **(2-3)**

2. 1,4,8-Trioxaspiro[4.6]undecan-9-one **(2-12)**

3. 4-Hydroxycyclohexanone **(1-8)**

4. 5-(2-Hydroxyethyl)-dihydrofuran-2-one **(1-9)**

5. 4-hydroxy-4-methylcyclohexanone **(2-6)**

6. 5-(2-Hydroxyethyl)-5-methyl-dihydrofuran-2-one **(3-1)**

7. 4-Hydroxy-allyl-cyclohexanone **(2-7)**

8. 5-Allyl-dihydro-5-(2-hydroxyethyl)furan-2(3H)-one **(3-2)**

9. Oxepane-2,5-dione **(2-13)**

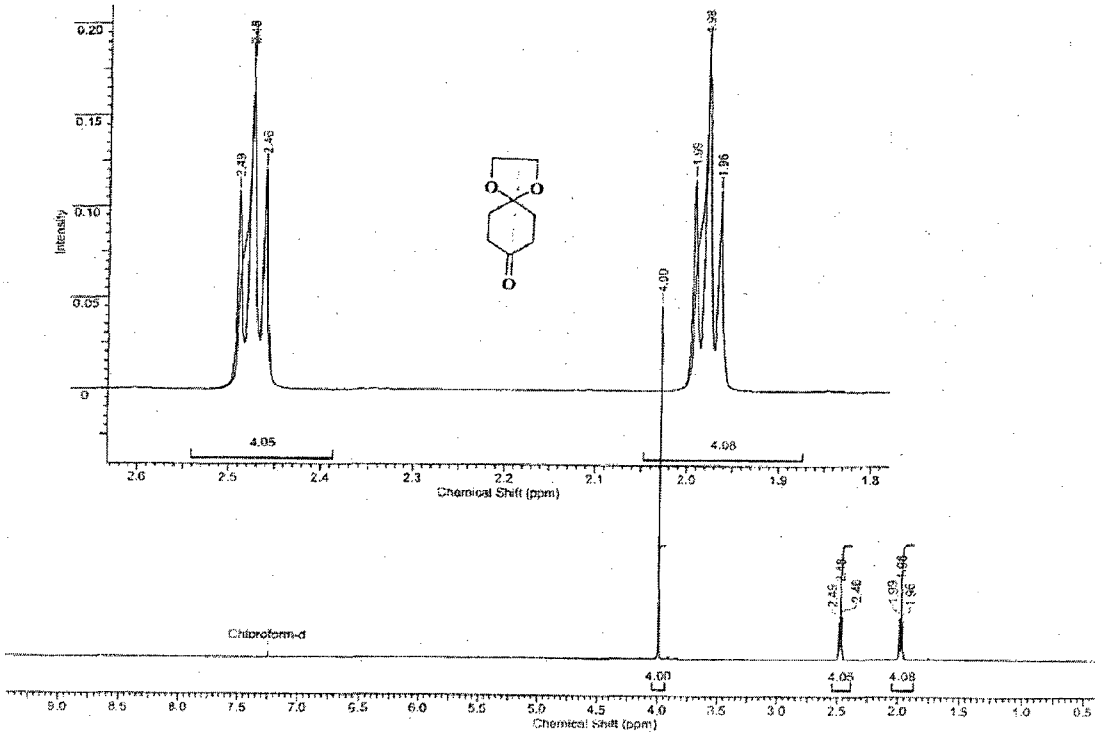
10. 6-Hydroxy-4-oxohexanoic acid **(2-14)**

11. 3-(2-Hydroxyethyl)-1,3-dioxolanyl)propanoic acid **(2-15)**

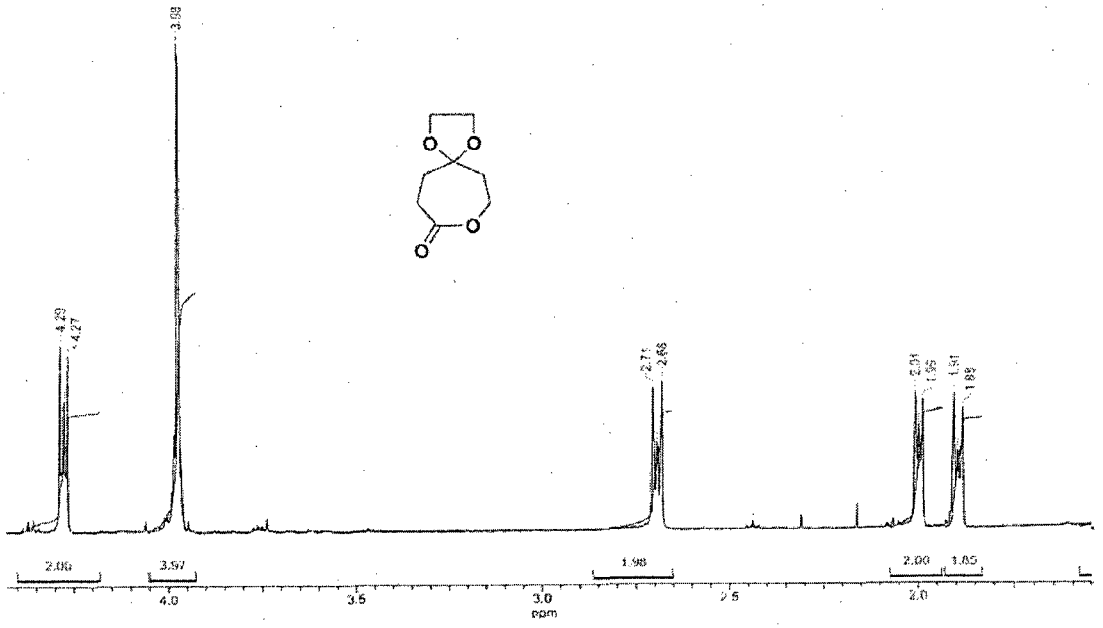
12. (*S*)-4,6-dihydroxy-isopropylhexanamide **(5-8)**

13. Solid State ^{13}C NMR

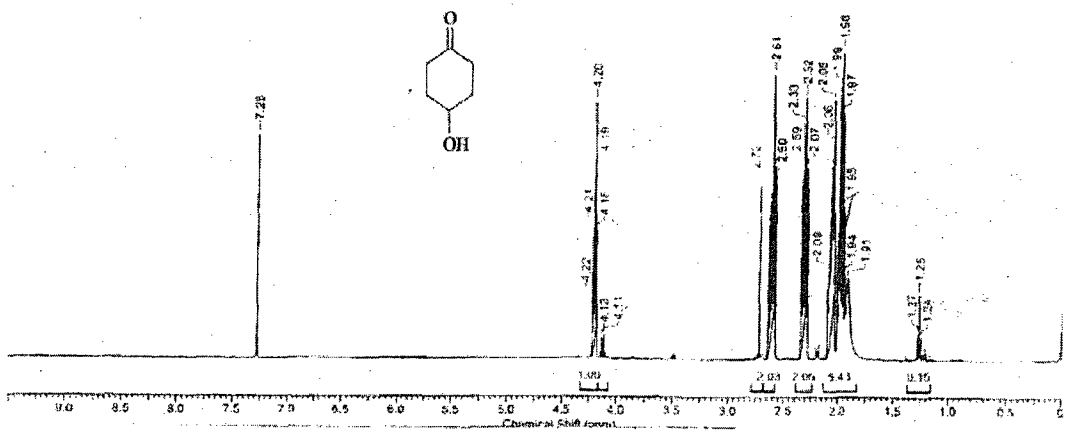
1,4-Cyclohexanedione monoethylene ketal (2-3)



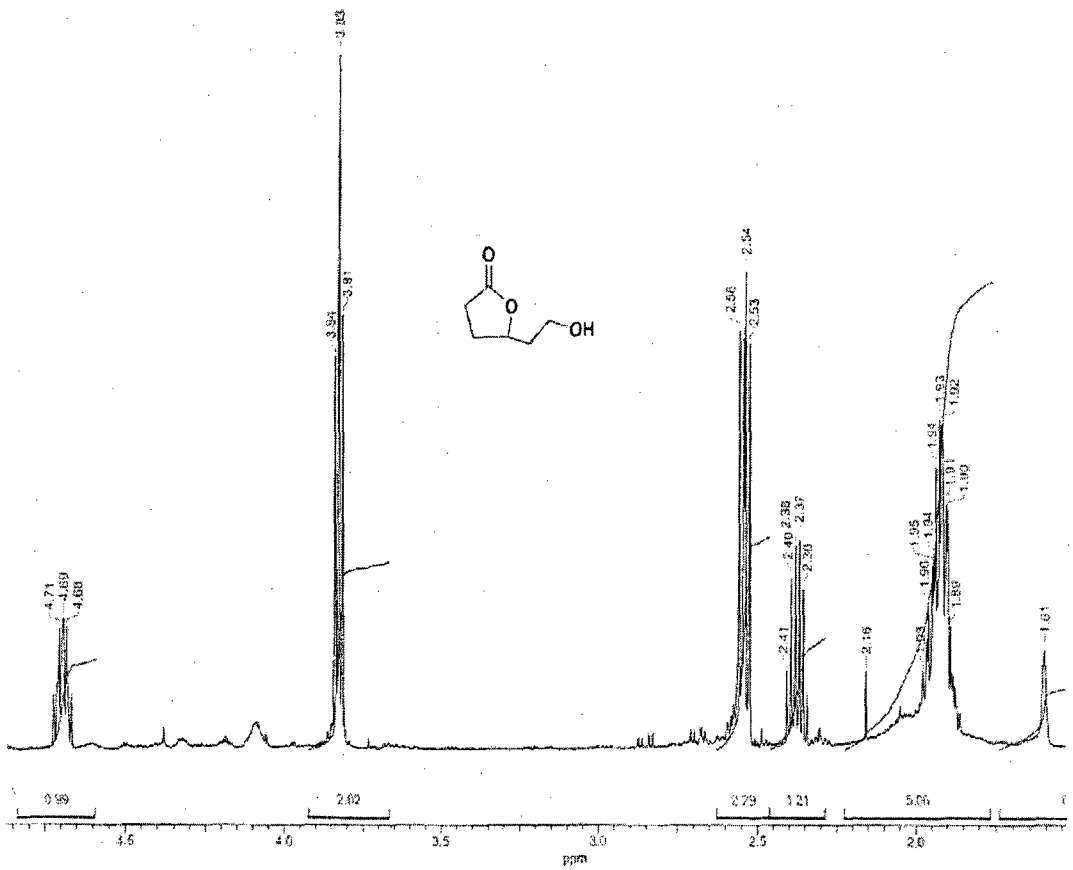
1,4,8-Trioxaspiro[4.6]undecan-9-one (2-12)



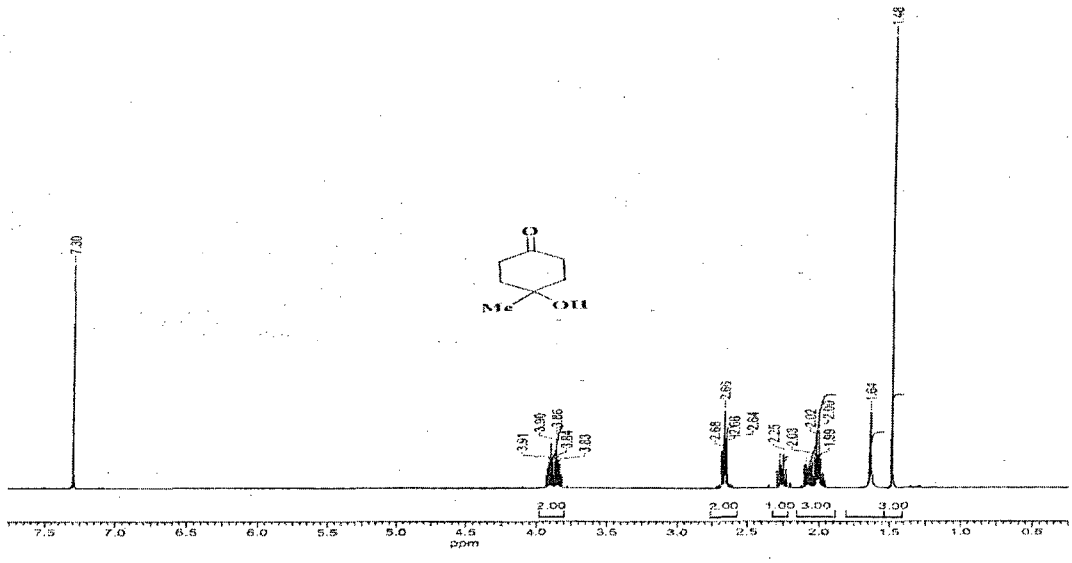
4-Hydroxycyclohexanone (1-8)



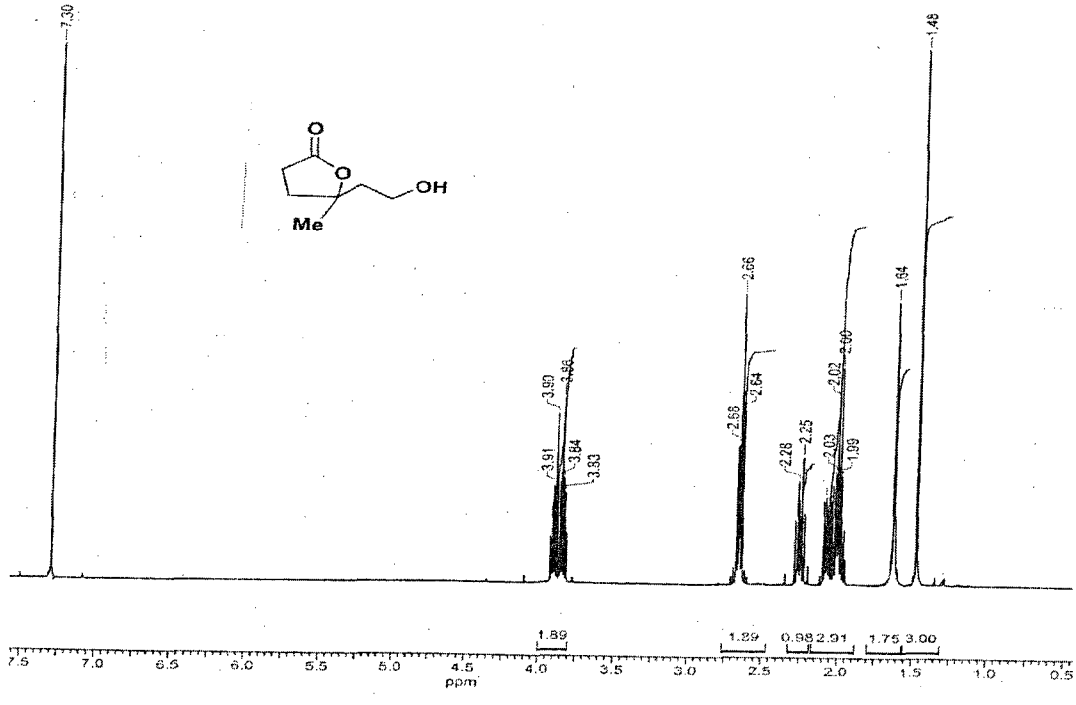
5-(2-Hydroxyethyl)-dihydrofuran-2-one (1-9)



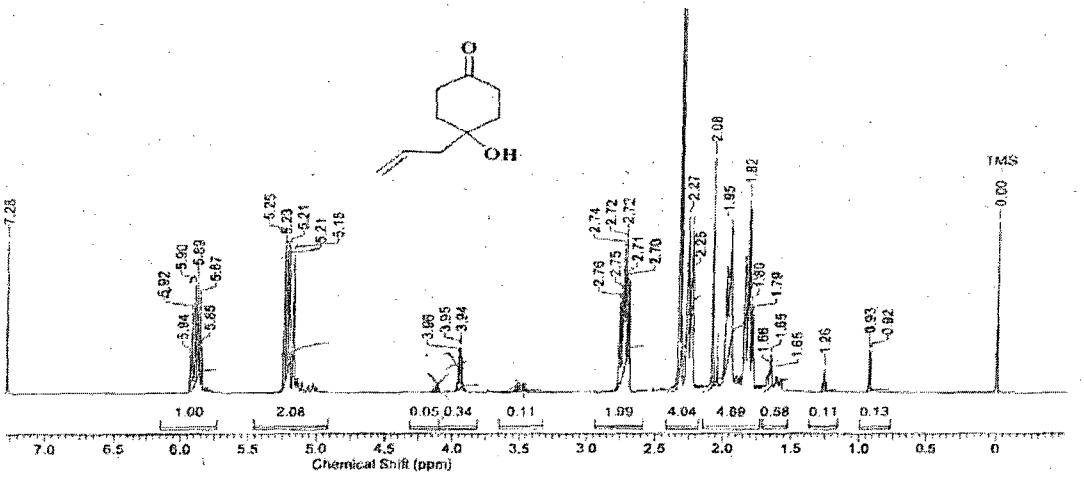
4-hydroxy-4-methylcyclohexanone (2-6)



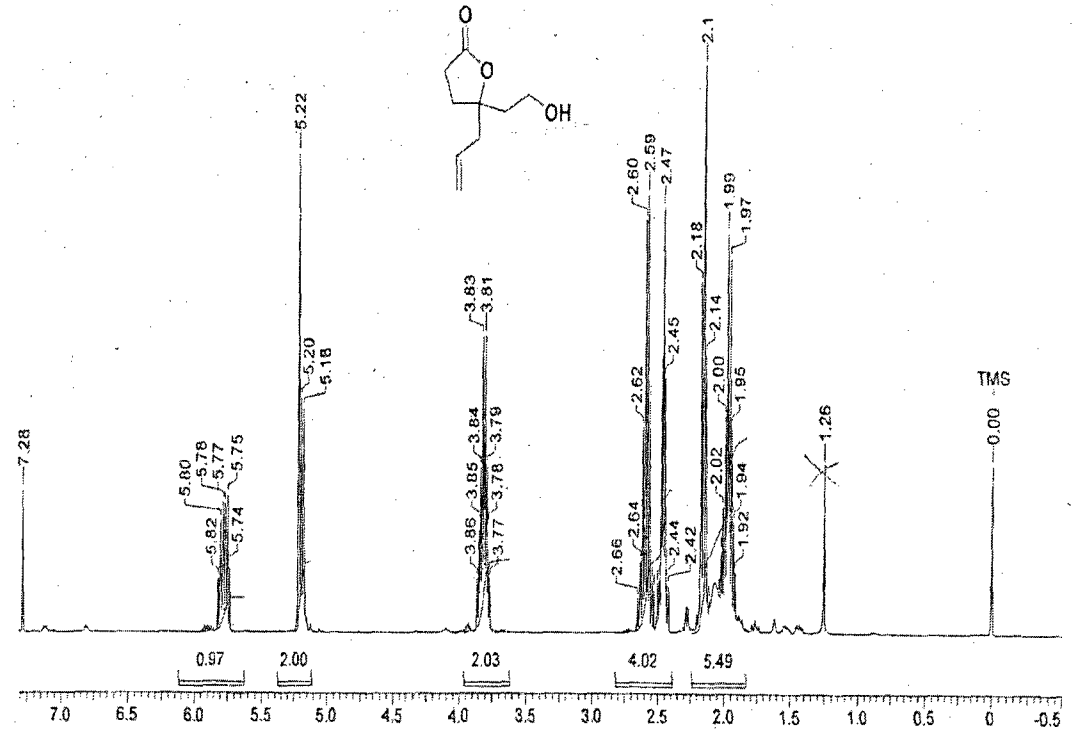
5-(2-Hydroxyethyl)-5-methyl-dihydrofuran-2-one (3-1)



4-hydroxy-allyl-cyclohexanone (2-7)

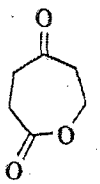


5-Allyl-dihydro-5-(2-hydroxyethyl)furan-2(3H)-one (3-2)

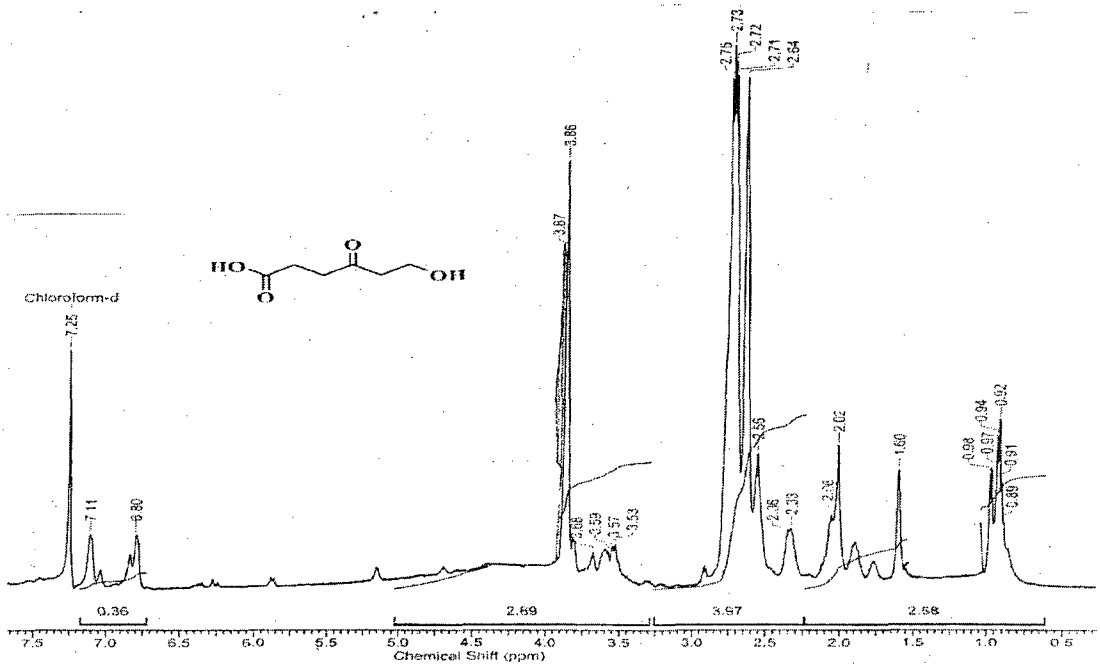


Oxepane-2,5-dion (2-13)

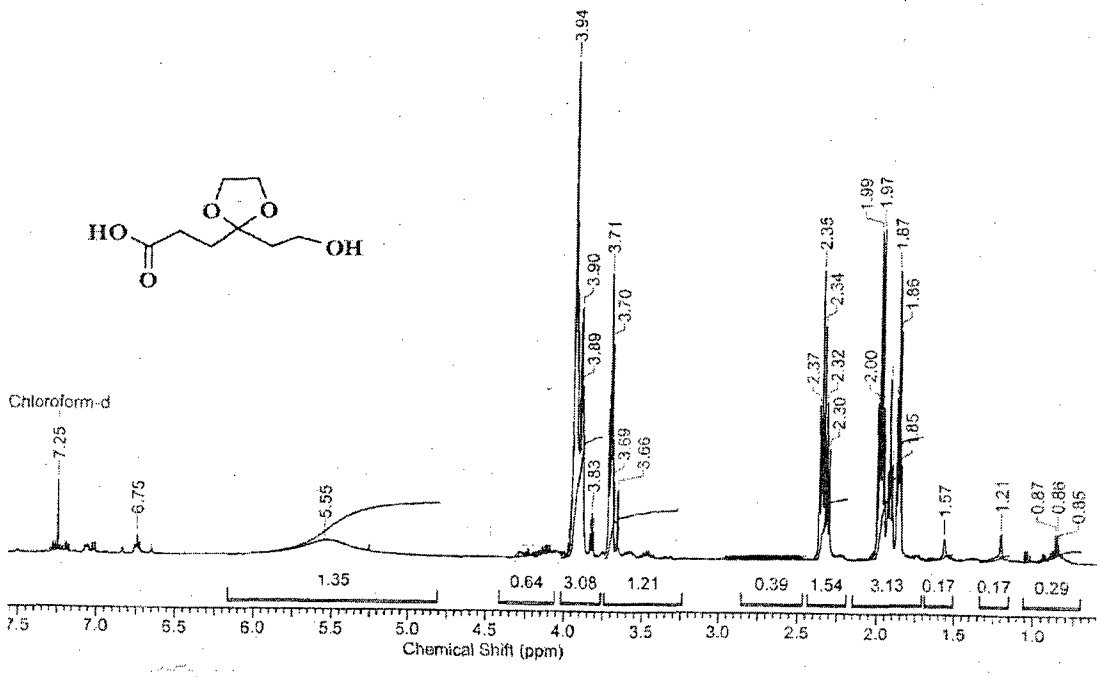
No.	(ppm)	Value	Absolute Value
1	[1.11 .. 1.36]	0.255	2.37846e+9
2	[1.36 .. 1.45]	0.119	1.11273e+9
3	[1.50 .. 1.90]	3.292	3.07233e+10
4	[1.93 .. 2.16]	4.313	4.02515e+10
5	[2.18 .. 2.29]	0.217	2.02552e+9
6	[2.29 .. 2.41]	2.008	1.87431e+10
7	[2.58 .. 2.73]	2.198	2.05182e+10
8	[4.12 .. 4.32]	1.000	9.33282e+9



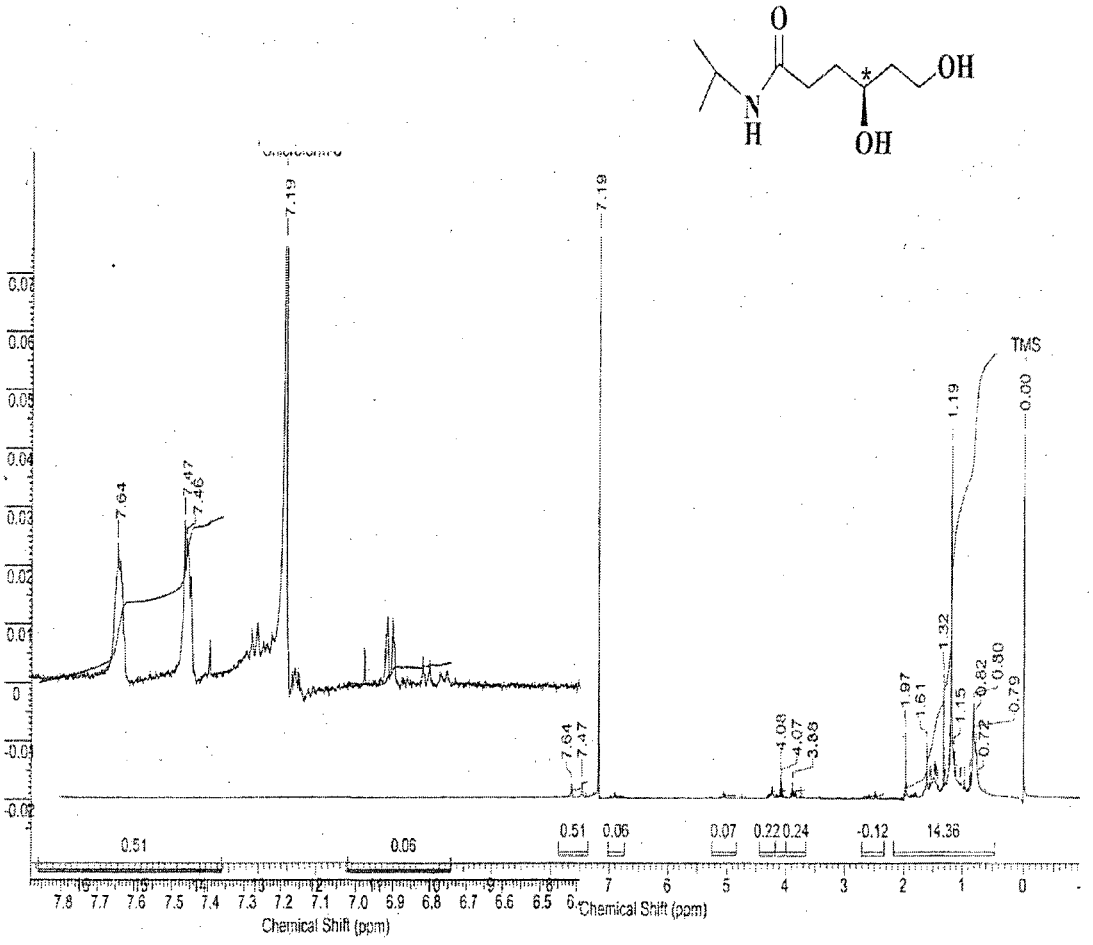
6-Hydroxy-4-oxohexanoic acid (2-14)



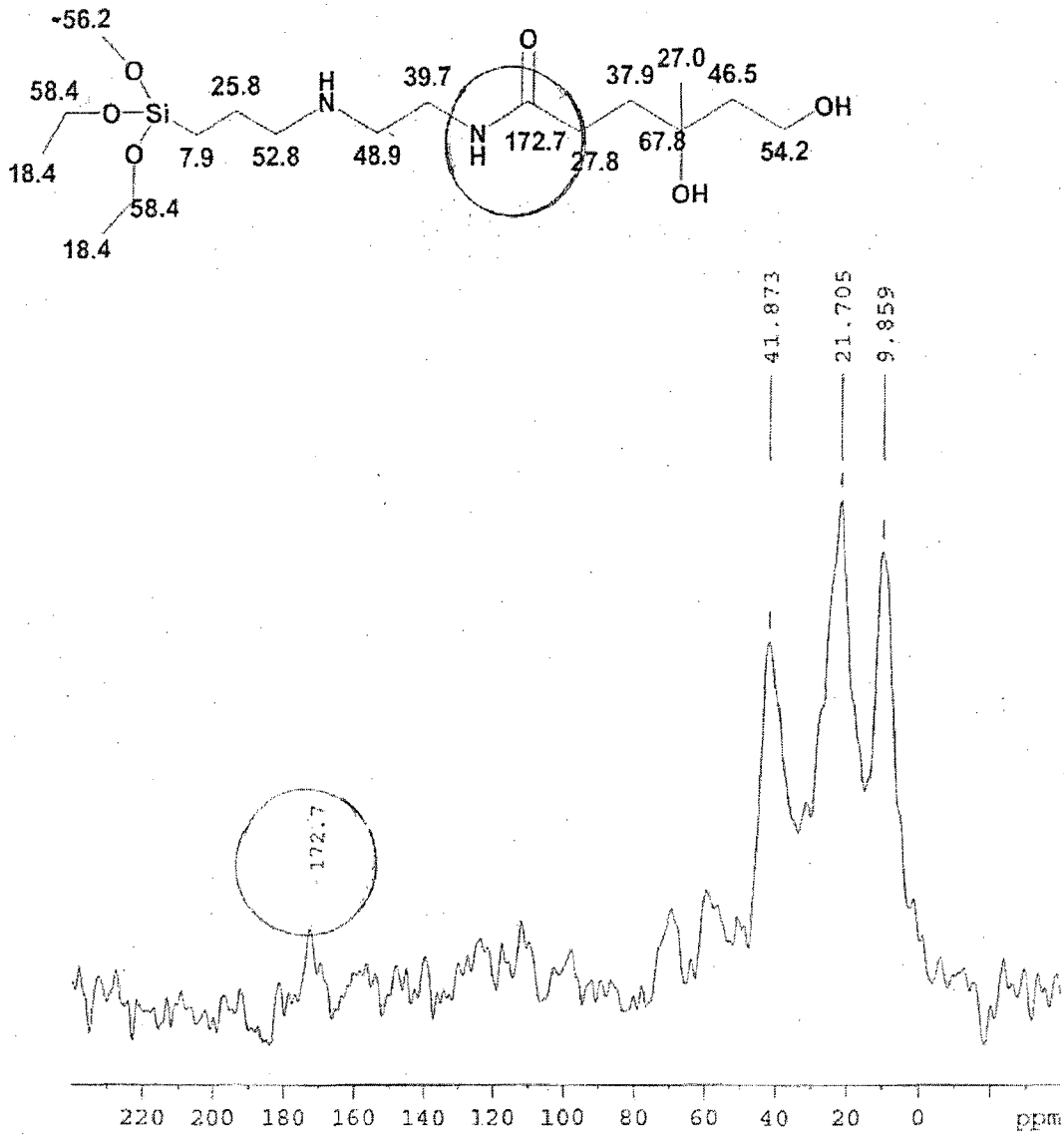
3-(2-hydroxyethyl)-1,3-dioxolanyl)propanoic acid (2-15)



S-4,6-dihydroxy-isopropylhexanamide (5-8)



Solid State ^{13}C NMR



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