ASYMMETRIC OXIDATIONS USING "DESIGNER YEAST"

by

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Abstract

A genetically engineered baker's yeast strain that overexpresses cyclohexanone monooxygenase from the soil bacterium *Acinetobacter* sp. NCIB 9871 was used as a biological version of chiral peracids to perform asymmetric oxidations. Baeyer-Villiger oxidations of prochiral 4-substituted cyclohexanones, 2- and 3-alkyl substituted cyclopentanones, and 2-substituted cyclopentanones with functionalized side chains were performed. The results were interpreted by model studies and theoretical calculations. To examine the reactivity of this "designer yeast" in its electrophilic mode, various sulfides, dithiolanes and dithianes were tested. The complexity of whole-cell biotransformations was revealed in a series of control experiments. An alternative *E. coli* overexpression system was tested in sulfur oxidations, and in some cases it gave improved reactivities and selectivities.

A short route towards the synthesis of mevinolin analogues was designed using the yeast reagent to obtain the key chirality in the final product. This synthetic design was aimed to demonstrate the potential usefulness of the designer yeast in organic synthesis.

In order to create new yeast reagents, the cloning of diketocamphane monooxygenase from *Pseudomonas putida* ATCC17453 was initiated. The *CAM* plasmid was isolated and two different approaches to identify the desired gene were tested. A DNA library of over 600 fragments was constructed and the subsequent cloning is an on-going project at the University of Florida.

Preface

Asymmetric oxidations by genetically engineered baker's yeast were studied and the results are presented in this thesis in the following order:

Chapter One: *Introduction.* This section introduces the basic background of enzymatic reactions, whole-cell biotransformations, the concept of designer yeast, and the target of this research.

Chapter Two: *Yeast-Mediated Baeyer-Villiger Oxidation of Prochiral Cyclohexanones.* This chapter discusses the syntheses of prochiral 4-substituted cyclohexanones and their oxidations by bioengineered yeast.

Chapter Three: *Yeast-Mediated Baeyer-Villiger Oxidations of Cyclopentanones.* This chapter discusses the syntheses and oxidations of 2- and 3-substituted cyclopentanones.

Chapter Four: *Yeast-Mediated Oxidations at Sulfur.* This chapter reports the syntheses of dithiolanes, dithianes, acyclic sulfides and their oxidations. The complication of oxidations at sulfur was revealed in a series of control reactions, and a partial solution was developed using an engineered *E. coli* strain as a bioreagent.

Chapter Five: *Oxidations of Compounds with Functionalized Side Chains.* This chapter reports preliminary results from oxidations of functionalized cyclic ketones and sulfur compounds.

Chapter Six: *Attempted Synthesis of Mevinolin Analogues.* This chapter reports the preliminary efforts towards the synthesis of mevinolin analogues in order to illustrate the potentials of the yeast reagent in organic synthesis.

Chapter Seven: *Cloning of Diketocamphane Monooxygenase.* This chapter describes

the attempts to clone a new yeast expressing diketocamphane monooxygenase from a *Pseudomonas putida* strain as a further development of the designer yeast reagent.

Chapter Eight: *Conclusions*. This section summarizes the results and serves as a review for this research project.

Appendix I: *Protocols Used in Yeast- and* E.coli*-mediated Oxidations*. This section summarizes the development of protocols used in yeast- and *E. coli*-mediated biotransformations. It also discusses several difficulties encountered in the oxidations and their possible solutions.

APPENDIX II: *General Experimental*. This section provides the general description of the analytical instrumentation, the treatment of reagents and solvents, and the suppliers.

APPENDIX III: *Recipes*. This is a collection of recipes for TLC detection solutions, the media for yeast and *E. coli* propagation and reactions.

APPENDIX IV: *BASIC Program for Internal Standard Method Calculations*. This section provides the computer programs used in interpreting the results from GC analysis.

APPENDIX V: *Spectra of Selected Compounds*. This is a collection of spectroscopic evidence of representative compounds.

Each chapter is designed to be self-contained, with its own introduction, results and discussion, experimental, and reference sections. To preserve the structural integrity and clarity, the recipes for solutions used in the molecular cloning are kept within Chapter Seven rather than in the general recipe section.

Acknowledgments

This thesis work would not be possible without all the people that have given me so much generous help over the years. Words are simply insufficient in expressing my deepest gratitude. In particular I thank Dr. Margaret M. Kayser, my supervisor, not only for her guidance in my study and research, but also for the affection and care in treating me as a family member by her and her husband, Dr. Edmond Kayser. On the Fredericton campus of UNB, I need to thank Dr. Cathleen Crudden for the help and material support she gave me; and Dr. D. MaGee and Dr. G. Deslongchamps for their excellent teaching and helpful discussions; I thank Dr. L. Calhoun for his outstanding NMR support. Special thanks should be given to Dr. C. K. Tompkins, Dr. R. Humphries, Dr. J. Wagstaff, F. Fullerton, J. McCrory, and other members in the Department of Physical Sciences on the Saint John campus of UNB for their help and support. I also extend my thanks to Dr. J. D. Stewart (University of Florida), Dr. D. Hooper (Dalhousie University) and Dr. F. Rochon (UQAM) for their help in my research work.

Thanks are also due my friends and colleagues Dr. J. Zhu, Dr. M. Mihovilovic, Dr. A. Feicht, S. DeVarenne at UNBSJ; L. Ren, M. L. Lee, G. Q. Li at UNBF; and C. Martinez at UFL. Almost everybody in the Chemistry Departments of these universities has given me various degrees of help, it is simply not possible to list all of them. Here I would like to express my sincere thanks to all of them.

Financial support from NSERC, UNB Graduate School, Department of Physical Sciences, and the O'Brien Foundation Humanitarian Fund is greatly appreciated. Thanks to Cerestar USA Inc. for their generous gift of cyclodextrin samples. And finally I would like to say thank you to the members of my reading and examining committees.

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List of Abbreviations

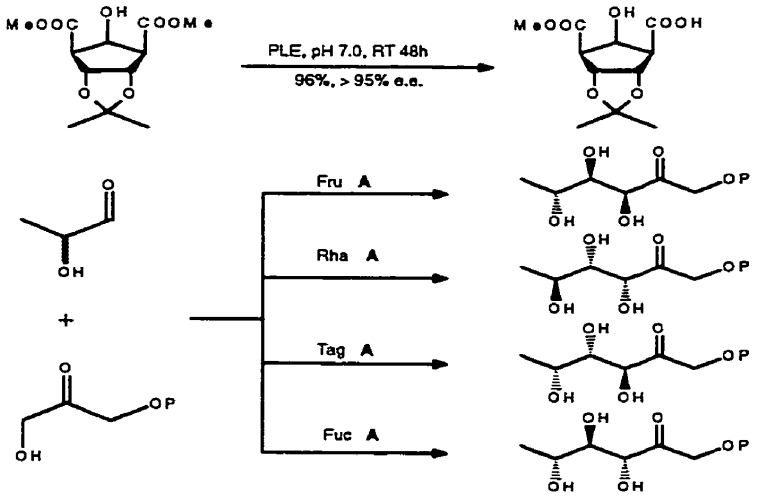
Ac	acetyl
Ar	aromatic
Bu	butyl
CHMO	cyclohexanone monooxygenase
DME	1,2-dimethoxyethane
DMS	dimethyl sulfide
DMSO	dimethyl sulfoxide
DPTBS	diphenyl-*t*-butylsilyl
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid, disodium salt
ELISA	enzyme linked immuno sorbent assay
Et	ethyl
FID	flame ionization detector
GC	gas chromatography
Hex	hexyl
HMDS	hexamethyldisilazane
HPLC	high performance liquid chromatography
Me	methyl
Oct	octyl
PMA	phosphomolybdic acid
Ph	phenyl

Pr	propyl
SM	starting material
TBDMS	*t*-butyldimethylsilyl
TE	Tris-EDTA buffer
TFA	trifluoroacetic acid
THAC	tetrahexylamonium chloride
THF	tetrahydrofuran
TLC	thin layer chromatography
TMS	trimethylsilyl
Und	undecyl
URA	uracil
UV	ultra violet
YPD	yeast extract/peptone/dextrose medium
YPG	yeast extract/peptone/galactose medium

Chapter One: Introduction

Enzymes are important chiral catalysts in organic synthesis. Frequently they have high catalytic activities, high regio- and enantioselectivities, and require only mild aqueous reaction conditions. With the increasing pressure for optical purities of chiral products and the environmental compatibility of their syntheses, enzymes become an attractive option for chemical reactions both in research laboratories and in industry.[1]

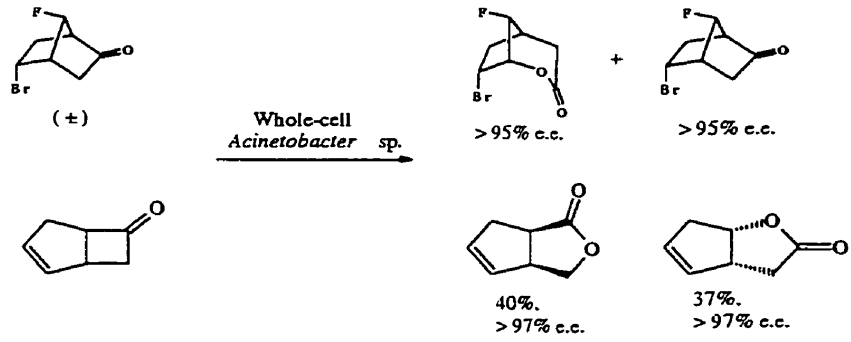
A reaction catalyzed by an enzyme can be straightforward if the enzyme is readily available, has sufficient stability, and does not require any cofactors. In such a case, the reaction simply involves incubating the substrate with an enzyme until the transformation is complete. Because there is only one enzyme catalyzing a single reaction, the product is generally very pure and its isolation is very simple. Examples of such cases include many of the hydrolytic enzymes[2] and aldolases[3] which have been widely used in organic synthesis. Several examples are shown in Scheme 1.[4]



Scheme 1

Unfortunately, many enzymes that catalyze useful reactions are not easily accessible, have low stabilities, and require expensive cofactors.* In addition, enzyme isolation is often tedious, costly, and requires specialized equipment and experience. These drawbacks can seriously offset any advantage of using such enzymes in chemical reactions and thereby limit their applications. Although there have been partial solutions to these problems, such as using immobilized enzymes and regenerating the cofactors,[5] those methods inevitably add complexity and cost to enzymatic reactions.

Sometimes these problems can be overcome by using a live microorganism that produces the desired enzyme in the biotransformations. Since growing cells constantly supply the enzyme and the cofactor, the problems associated with enzyme stability and cofactor regeneration are avoided. Experimentally, this strategy simply involves incubating the microorganism with the substrate in a suitable culture medium and isolating the product from the mixture when the conversion is complete.[5b, 6] (Scheme 2)



Scheme 2

* For example (Ref. 10a), only 0.7mg of cyclohexanone monooxygenase could be isolated from a 1L culture of soil bacterium *Acinetobacter* sp. (NCIB 9871) grown on cyclohexanol. This enzyme has a $t_{1/2}$ of 24 hours at 0°C, and requires NADPH as its cofactor.

However whole-cell biotransformations are known to have their own limitations (Figure 1).[5b] First of all, the same microorganism may further metabolize the product and lower the chemical yield. Secondly, there can be other enzymes in the microorganism that accept the same compound as their substrate, but transform it to different products. Thirdly, some useful enzymes are produced only in the presence of certain inducers.* The use of such compounds can create difficulties in the subsequent product isolation. Furthermore, some microorganisms that perform useful biotransformations are pathogenic, which makes their usage unattractive to organic chemists.

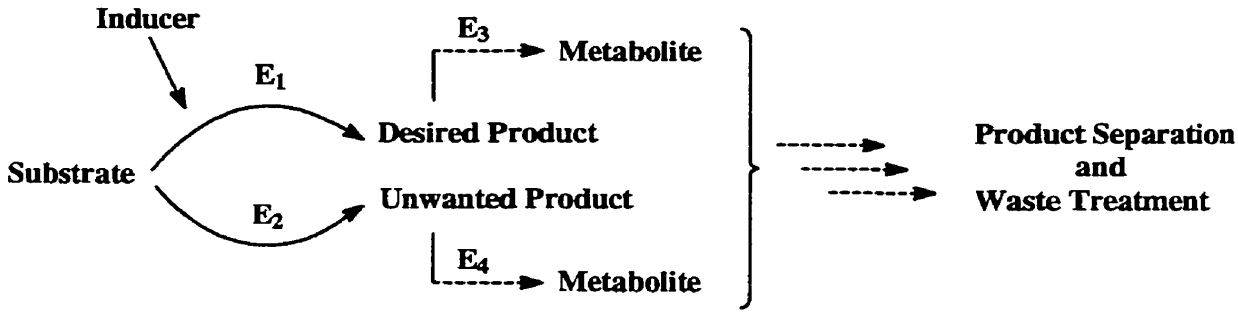


Figure 1: Complications in whole-cell biotransformations

A relatively new and general solution to the problems associated with whole-cell biotransformation is under development in this laboratory in collaboration with Dr. Stewart's group at the University of Florida. In this methodology, the gene for an enzyme that catalyzes useful reactions is isolated from the parent microorganism and

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*For example (Ref. 10a), in *Acinetobacter* sp., cyclohexanone monooxygenase is only produced when the bacterium is grown on cyclohexanol as the sole carbon source.

cloned into baker's yeast (*Saccharomyces cervisiae*), and the new "designer yeast" is used as a whole-cell bioreagent to catalyze the desired reactions.[7]

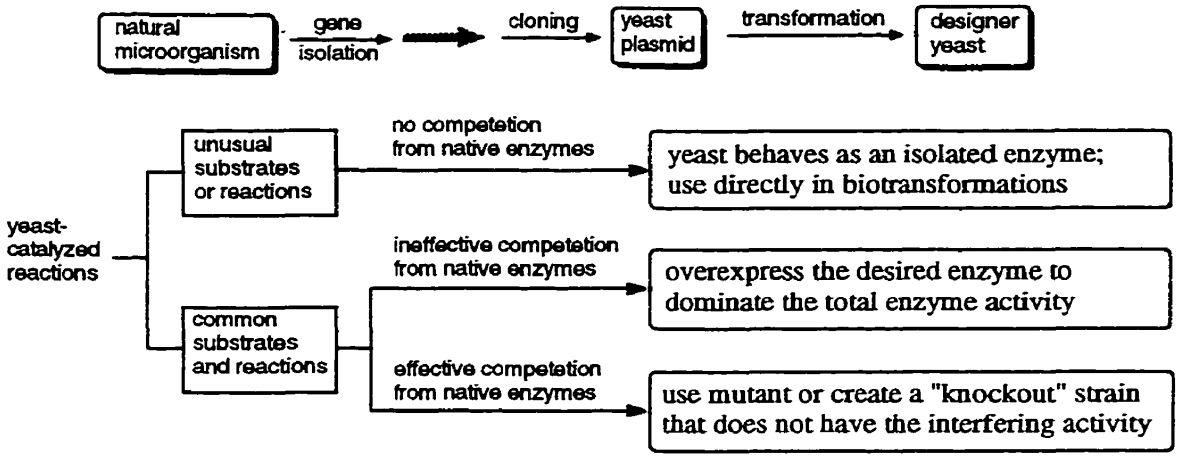

Figure 2: Strategies for designer yeast reagent

Several strategies are available to address the overmetabolism and competing enzyme problems (Figure 2). First of all, if the alien enzyme accepts unusual substrates or catalyzes reactions that are unnatural to the carrier strain, there would be no significant competition or overmetabolism from the native enzymes, and the whole-cell bioreagent will behave like an isolated enzyme and give the desired product in high yield. Secondly, if the carrier yeast can accept the same substrate or further degrade the product, but the native metabolism for that compound is not very efficient, a suitable cloning vector can be chosen that has multiple copies in each cell, so that the desired enzyme is overexpressed* to a much higher level than it would be in the natural microorganism. Therefore, the reaction catalyzed by the desired enzyme dominates the total enzyme

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* With the use of a relaxed control plasmid and an efficient promoter, the production of a protein of interest may reach 30% of the host's total cellular protein. Such genetically engineered organisms are called overproducers. (Voet, D., Voet, J., *Biochemistry*, 2nd. ed., John Willey and Sons, Inc., NY, 1995. page 906.)

activity in the whole-cell biotransformation, and the desired product can still be obtained in a good yield. Thirdly, if there are interfering enzymes in the host yeast that efficiently compete for the substrate or further metabolize the product, it is possible to choose a suitable mutant or bioengineer a "knockout" yeast that is deficient in the conflicting activity. Unless the latter interferes with the host cell's vital functions, the bioengineered yeast should still be able to produce the desired product in good yields. In general, overexpressing the desired gene would lead to increased activity unless a high concentration of the foreign enzyme interferes with the healthy growth of the host cells.

Instead of the more commonly used *E. coli*, baker's yeast was chosen as the host organism since it offers several advantages. First of all, baker's yeast is non-pathogenic; therefore it is simple to handle without any requirements for specialized equipment or expertise in microbiology. Also, organic chemists have been using baker's yeast for a long time and readily accept this type of biotransformation.[4,8] Furthermore, baker's yeast can be dried and stored for extended periods of time without losing activity.* In addition, unlike *E. coli* that can only express prokaryotic genes, baker's yeast can express both prokaryotic genes and eukaryotic genes, which makes it a more versatile and more attractive host in designing new generations of bioreagents.

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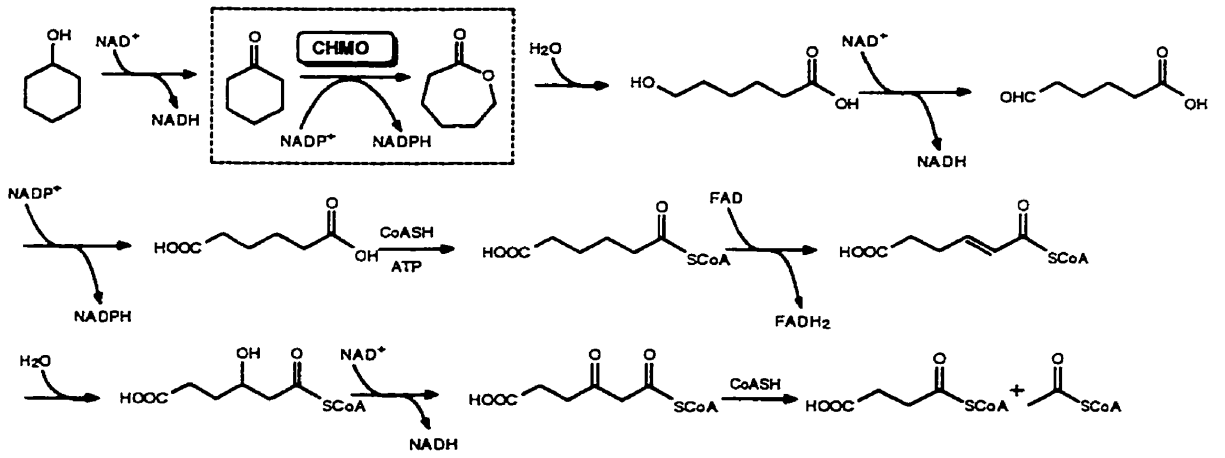
* Active dried baker's yeast is commercially available from local grocery stores; 31 (28%) *Saccharomyces cervisiae* laboratory strains from American Type Culture Collection are supplied in dried form; development of dried yeast strains overexpressing cyclohexanone monooxygenase is a collaborative project with Lallemand Inc.

Several criteria were followed when choosing the enzyme to be expressed in yeast. It should catalyze a synthetically valuable reaction, and have demonstrated good enantio- and diastereoselectivities over a broad spectrum of substrates, so that the designer yeast can be used as a general reagent in organic synthesis. Also, such an enzyme should be difficult to obtain, or be unstable *in vitro*, and preferably require cofactors, so that the use of purified enzyme on a large scale is not practical.

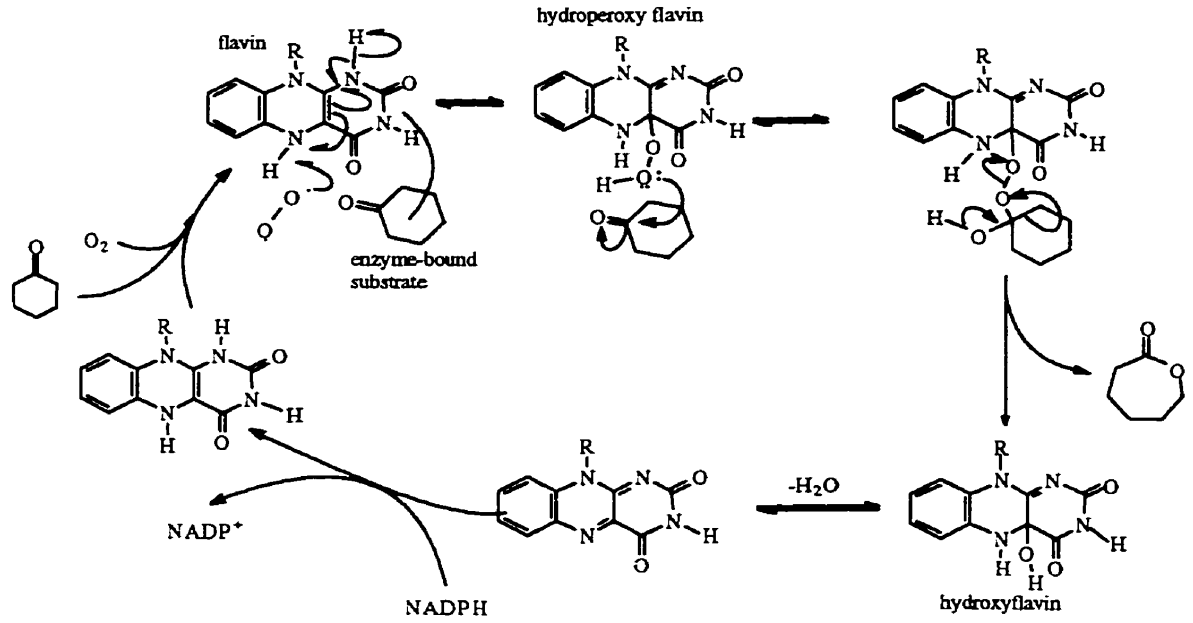
Based on these criteria, cyclohexanone monooxygenase (CHMO, E.C. 1.14.13.22) was chosen in the construction of the first designer yeast. This enzyme was first isolated from the soil bacterium *Acinetobacter* sp. (NCIB 9871).[9] It enables the bacterium to degrade cyclohexanol to acetyl CoA and succinyl CoA and thereby utilize cyclohexanol as its sole carbon and energy source (Scheme 3).[10]



Scheme 3

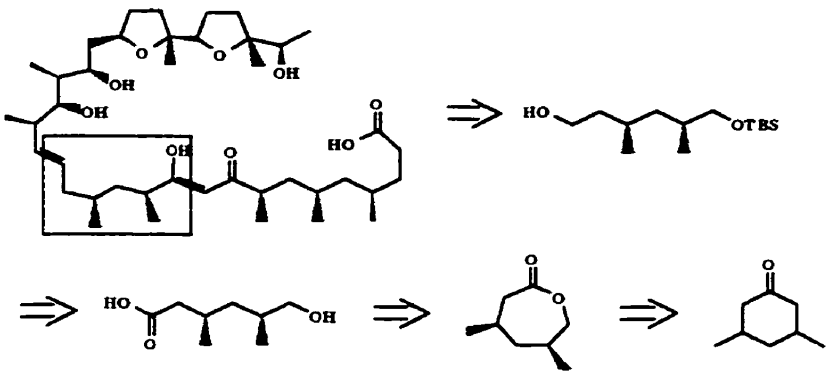
CHMO is a 58kd single unit protein,[11] a flavin subunit is tightly bound to the enzyme, and the hydroperoxy flavin derived from the reaction with molecular oxygen is believed

to be the active species.[12] It has been demonstrated that in CHMO-catalyzed Baeyer-Villiger oxidations, substrate binding occurs before oxygen enters the enzyme's active site. Thus the formation of hydroperoxy flavin intermediate is controlled by the fitting of a substrate into the active site, which in turn affects the overall reaction rate. This gives the enzyme an additional chance to control the stereoselectivity.[10a] The proposed mechanism for CHMO-catalyzed Baeyer-Villiger oxidations is illustrated in Scheme 4.[12,13]



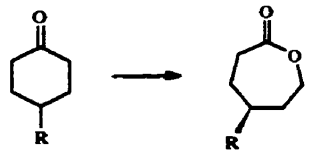
Scheme 4

This enzyme has demonstrated a broad substrate acceptability. Various cyclic ketones were successfully oxidized by CHMO to the corresponding lactones with good to excellent enantioselectivities (Table 1, Table 2).[5b,10b,14] It has been successfully used in the synthesis of natural products, such as the antibiotic *Ionomycin* (Scheme 5).[15]

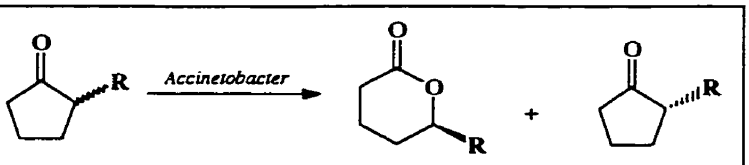


Scheme 5

Table 1: CHMO-catalyzed oxidation of monosubstituted cyclohexanones[5b,7,]

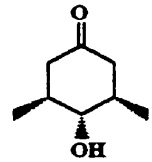
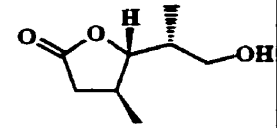
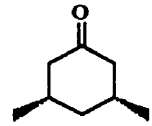
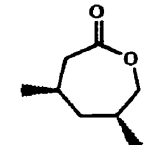
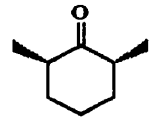
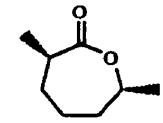
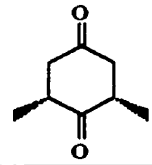
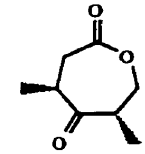


R	e.e.%
Me	>98
MeO-	75
Et	>98
n-Pr	>98
i-Pr	>98
t-Bu	>98
n-Bu	52
-CH_2OH	>98
-OH	9.6



R	e.e.%	e.e.%
n-C_5H_{11}	97	Not Reported
n-C_7H_{15}	95	
n-C_9H_{19}	85	
n-$C_{11}H_{23}$	73	

Table 2: CHMO-catalyzed oxidation of multisubstituted cyclohexanones[16]

substrate	product	yield (%)	e.e. (%)
		88	>98
		73	>98
		27	>98
		25	>98

Despite the fact that CHMO has broad substrate acceptability and exhibits very good enantioselectivity, its application in organic synthesis, either as an isolated enzyme or in whole-cell transformations, has been relatively limited. This is largely because of the low abundance and low stability of the enzyme, the necessity to grow the bacterium on cyclohexanol to induce the enzyme production, the use of NADPH as a cofactor, and the pathogenic nature of the parent bacterium.* [17] These inconveniences and the great practical potential of this enzyme in organic synthesis made CHMO an ideal candidate for expression in yeast.

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* See also the footnote on page 2.

To construct the designer yeast, the CHMO gene was amplified from *Acinetobacter* sp. genomic DNA (Figure 3) and inserted into an expression vector. The final yeast plasmid pKR001[7, 18] (Figure 4) was used to transform baker's yeast strain 15C to give the designer yeast reagent 15C(pKR001).

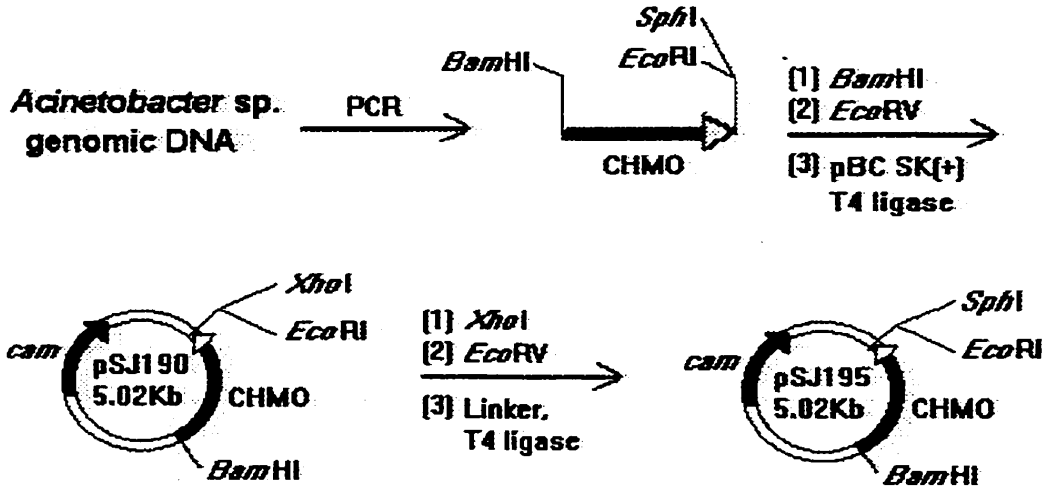


Figure 3: Amplification of CHMO gene

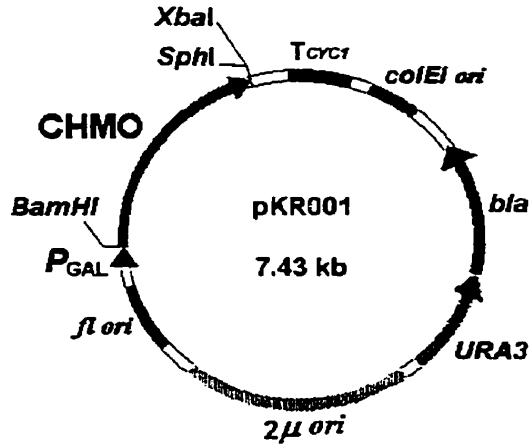
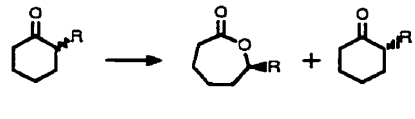
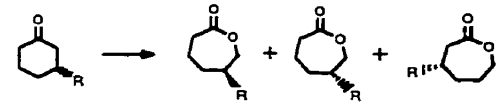
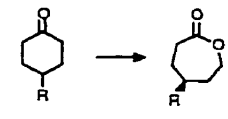


Figure 4: Yeast plasmid expressing CHMO

In the preliminary tests, this yeast reagent effectively oxidized cyclohexanone and cyclopentanone to the corresponding lactones in high yields.[7] The competing yeast

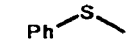
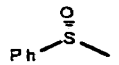
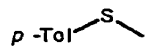
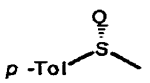
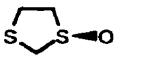
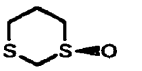
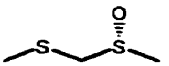
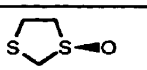
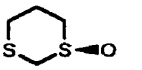
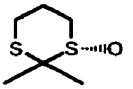
reductions were very minor and the products were not further metabolized. In subsequent experiments, 2-, 3-, and 4-alkyl substituted cyclohexanones were oxidized and in many cases the products were isolated with high optical purities and good yields (Table 3).[18] The introduction of designer yeast that overexpresses CHMO greatly simplified the use of this enzyme, and made it available for transformations in standard organic laboratories.

Table 3: Oxidation of substituted cyclohexanones by 15C(pKR001)[18,19]

							
R	yield % (e.e. %)				yield % (e.e. %)		yield % (e.e.%)
Me	50(49)	--			71(98)	60(98)	83(98)
Et	79(95)	69(98)			18(70)	20(70)	74(98)
n-Pr	54(97)	66(92)		8(83)	11(98)		63(92)
i-Pr	41(98)	46(96)		no	reaction		60(98)
allyl	59(98)	58(98)		9(98)	15(97)		62(95)
n-Bu	59(98)	64(98)		11(84)	37(56)		no reaction

Besides Baeyer-Villiger oxidations, various sulfides, dithianes, and dithiolanes have also been oxidized to the corresponding sulfoxides with different degrees of enantioselectivities, either by isolated CHMO or the parent bacterium *Acinetobacter* sp. (Table 4).[20] This reactivity towards sulfur parallels that of organic peracids.[21]

Table 4: Oxidation at sulfur center by *Acinetobacter* sp. or isolated CHMO

Substrate	Product	Biocatalyst	Yield (%)	e.e. (%)
		CHMO	88	99
		CHMO	91	92
		CHMO	94	98
		CHMO	81	98
		whole-cell	79	94
		whole-cell	71	95
		whole-cell	74	93
		whole-cell	66	60

The broad substrate acceptability combined with high enantioselectivities in CHMO-catalyzed asymmetric oxidations promoted speculations on the nature of the active site of this enzyme.[22] A cubic model was proposed by Furstoss (Figure 5)[14,22a] and a box model was proposed by Ottolina (Figure 6).[22b] In addition, Taschner also proposed a model based on the sequence homology between CHMO and human and *E. coli* glutathione reductase, for which the X-ray structures have been reported.[23]

The Furstoss cubic model was deduced from Baeyer-Villiger oxidations. The substrate is oriented in such a way as to minimize the steric hindrance, and the oxygen is delivered from the bottom. The migrating bond is determined by stereoelectronic requirements in the Criegee intermediate, i.e., the migrating bond needs to be antiperiplanar to the peroxy bond, and antiperiplanar to one of the lone pairs in the hydroxyl group.[24] These factors determine the final stereochemistry of the product (Figure 5).

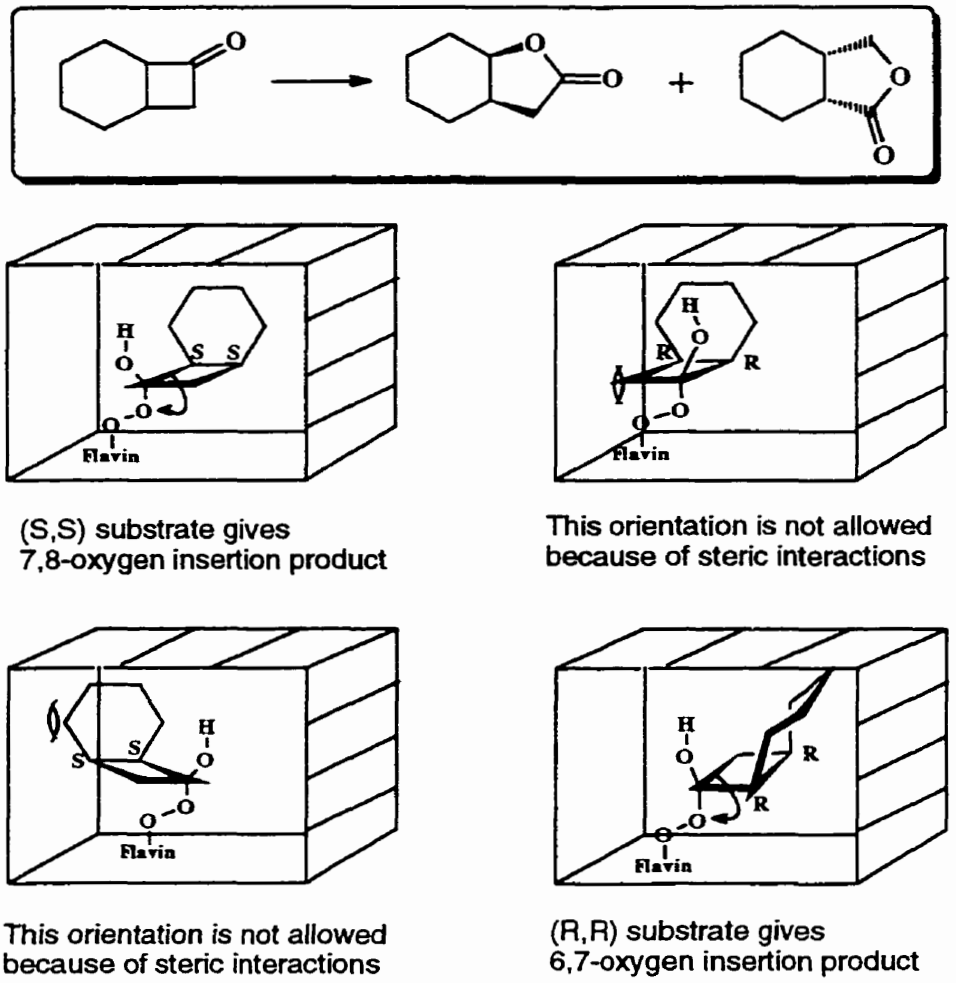


Figure 5: Furstoss cubic model

The Ottolina box model was originally deduced from the oxidation of sulfur compounds (Figure 6). It was constructed by overlaying the sketches of successfully oxidized substrates and enclosing their contours in "cubic space".[25] This model has also been used to predict the correct stereochemistry in several Baeyer-Villiger oxidations. In this model, the substrate is oriented to minimize the steric interactions, and the attacking oxygen appears to be delivered from the top of the active site.

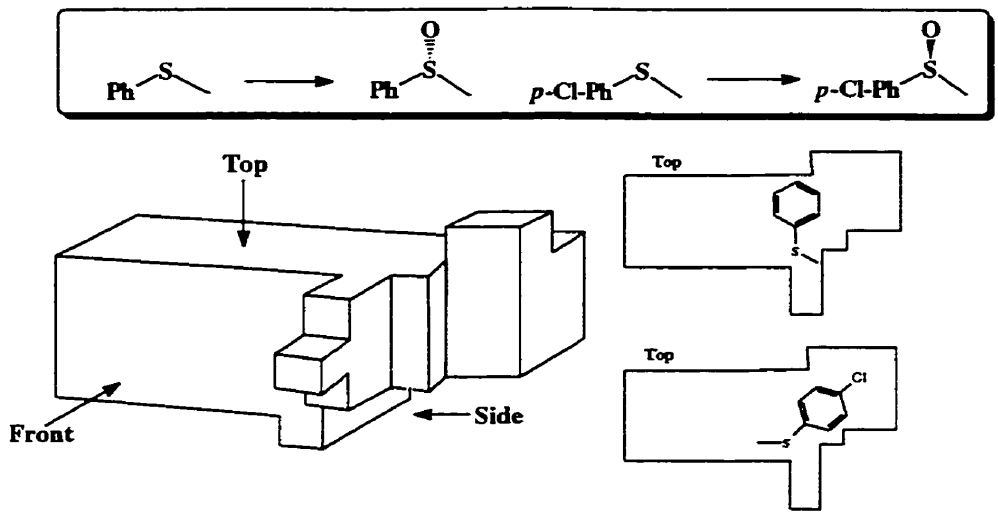


Figure 6: Ottolina box model for the active site in CHMO

To further develop the application of the oxidizing yeast, the following projects were conducted: (1) As a continuation in the search for suitable substrates in asymmetric Baeyer-Villiger oxidations, more prochiral 4-substituted cyclohexanones were tested, and a comprehensive survey of 2- and 3-substituted cyclopentanones were conducted; (2) To extend the application of the new yeast reagent in other oxidation reactions, it was used in asymmetric oxidation of sulfur-containing compounds; (3) The protocols for yeast propagation, yeast oxidation, reaction monitoring and product isolation were adapted to the new reactions: (4) Some preliminary work was conducted to apply the designer yeast in natural product synthesis; (5) The cloning of a new designer yeast was initiated.

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[17] *Acinetobacter* sp. NCIB9871 is a *Class II* pathogen. The classification is based on the degree of risk to an individual and a community:

Class I: low individual and community risk;
Class II: moderate individual risk, limited community risk;
Class III: high individual risk, low community risk;
Class IV: high individual risk, high community risk.

A *Class II* pathogen can cause human or animal disease, but under normal circumstances it is unlikely to be a serious hazard to laboratory workers, the community, livestock, or the environment. Laboratory exposures rarely cause infection leading to serious disease; effective treatment and preventive measures are available and the risk of spread is limited. (in reference to on-line lecture note http://duke.usask.ca/whiterv/bioman47.html)

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[19] Yields for the oxidation of 2-alkylcyclohexanones were based on ideal kinetic resolution yield of 50%. Yields for *(R)*- and *(S)*-6-methyloxepan-2-one were obtained from two separate reactions using optically pure starting materials. For other 3-substituted cyclohexanones, *(R)*-lactones were obtained from the beginning of reactions when the fractional conversions were low; near the end of each reaction, the *(S)*-enriched ketones were isolated and re-submitted to yeast oxidation conditions to be regeospecifically converted to the *(S)*-lactones.

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Chapter Two: Yeast-Mediated Baeyer-Villiger Oxidations of Prochiral Cyclohexanones

Introduction

Optically active ε-caprolactones and their linearized derivatives are important building blocks for pharmaceutically important compounds (Figure 7).[1] There have been numerous approaches to their synthesis, including chromatographic separation of diastereomeric mixtures[2], enzymatic resolution of racemic lactones[3], chemical Baeyer-Villiger oxidation of enantiomerically enriched ketones[4], and catalytic asymmetric Baeyer-Villiger oxidation of racemic ketones (Scheme 6).[5]

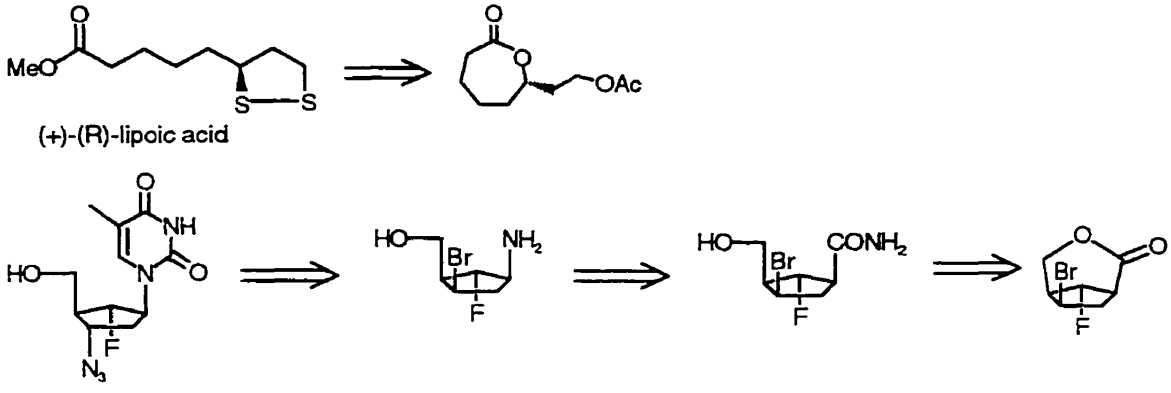
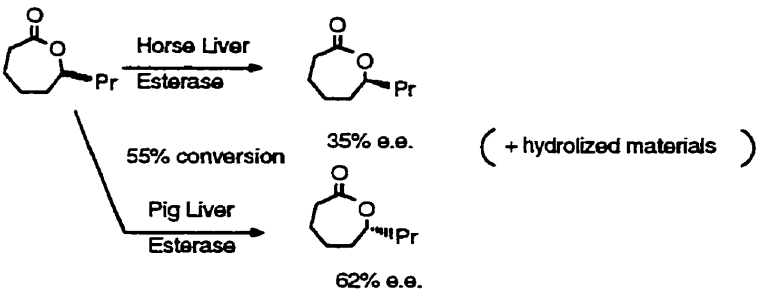
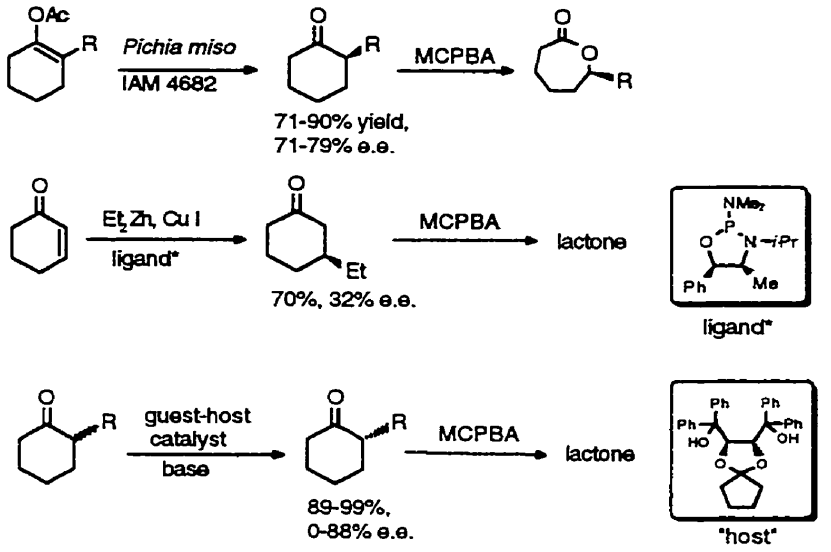


Figure 7: Biologically active compounds derived from chiral ε-caprolactones

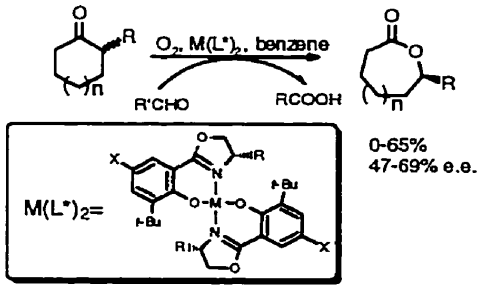
Enzymatic resolution of racemic lactones



Chemical Baeyer-Villiger oxidation of enantiomerically enriched ketone

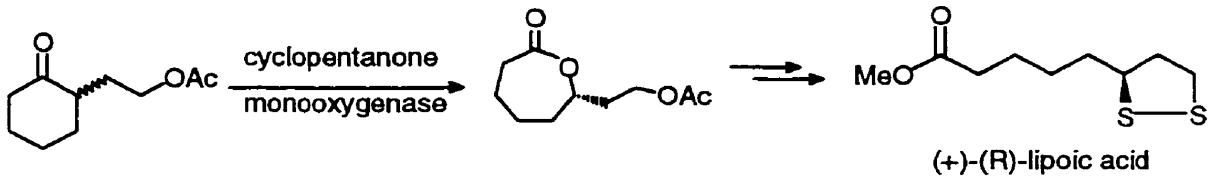


Catalytic asymmetric oxidations



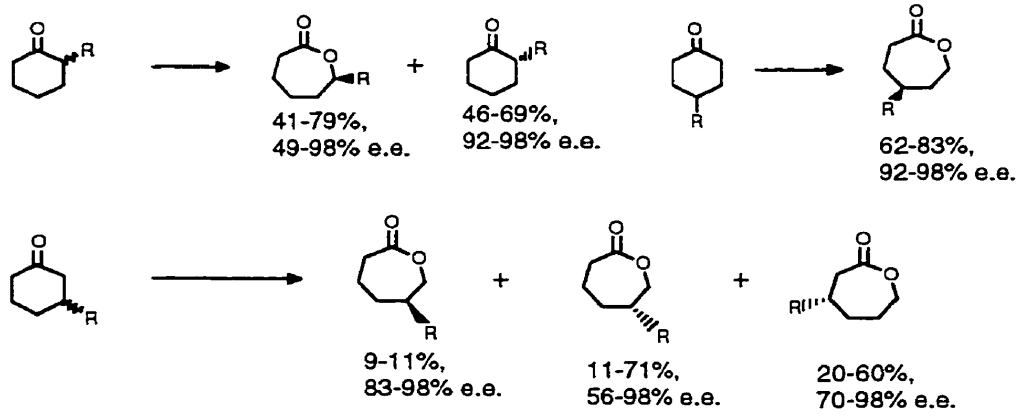
Scheme 6

Besides these methods, monooxygenases from various microorganisms,* [6] either in the form of isolated enzymes or whole-cell transformations,[7] have been used to catalyze asymmetric Baeyer-Villiger oxidations of cyclic ketones as a key step in natural product synthesis (Scheme 7).[1b]



Scheme 7

In a recent report,[8] the recombinant baker's yeast strain overexpressing cyclohexanone monooxygenase from soil bacterium *Acinetobacter* sp. NCIB 9871 was used in the kinetic resolution of 2- and 3-substituted cyclohexanones and the desymmetrization[9] of prochiral 4-substituted cyclohexanones, and showed excellent enantioselectivities (Scheme 8).



Scheme 8

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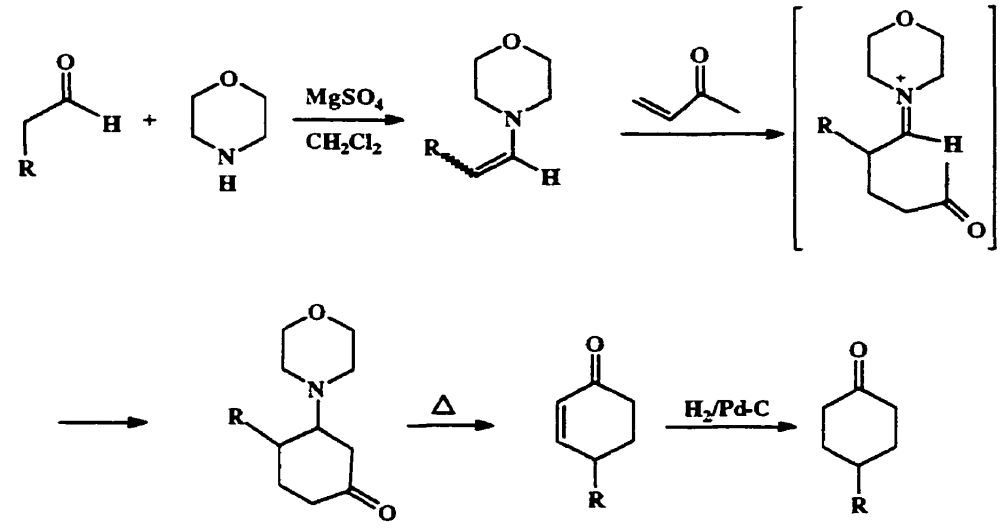
* Examples of natural microorganisms carrying Baeyer-Villiger enzymes are bacteria of the genera *Acinetobacter, Pseudomonas, Xanthobacter, Rhodococcus,* and *Norcardia*; fungi of the genera *Curvularia, Dreschlera, Exphilia, Cunninghamella,* and *Cylindrocarpon.*

In view of the experimental advantages in using the bioengineered baker's yeast as an asymmetric reagent in organic synthesis, it was important to identify other substrates suitable for yeast-catalyzed oxidations. Therefore, a further investigation was conducted on yeast-mediated asymmetric Baeyer-Villiger oxidations of prochiral 4-substituted cyclohexanones bearing various functional groups suitable for further transformations.

Results and Discussion

Preparation of substrates

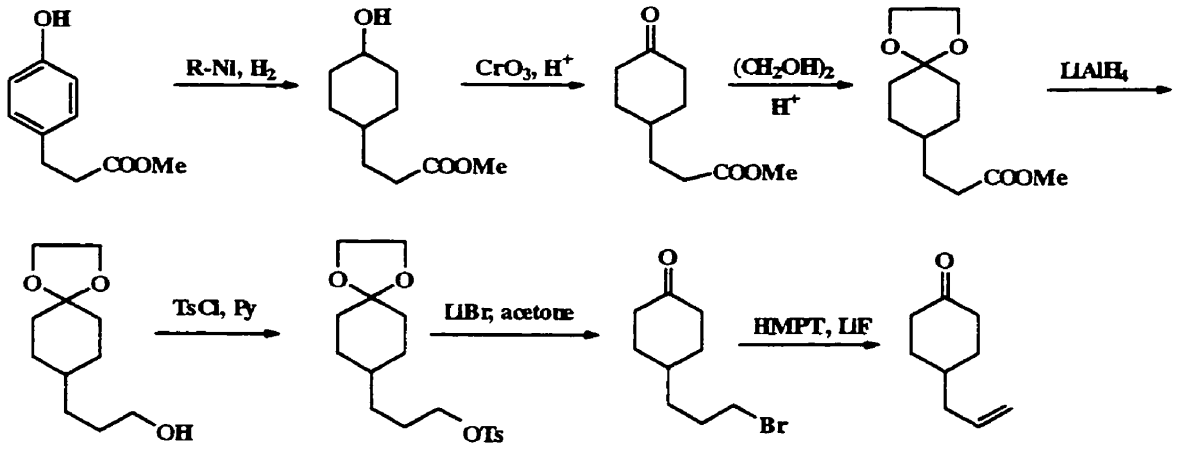
Many different approaches for the synthesis of 4-substituted cyclohexanones have been reported in the literature, such as hydrogenation of *para*-substituted phenol derivatives[10], Robinson annulation,[11] and ring construction via Diels-Alder reactions.[12] A variant of the Robinson annulation method illustrated in Scheme 9 was adapted for the synthesis of 4-alkyl substituted cyclohexanones.[13]



Scheme 9

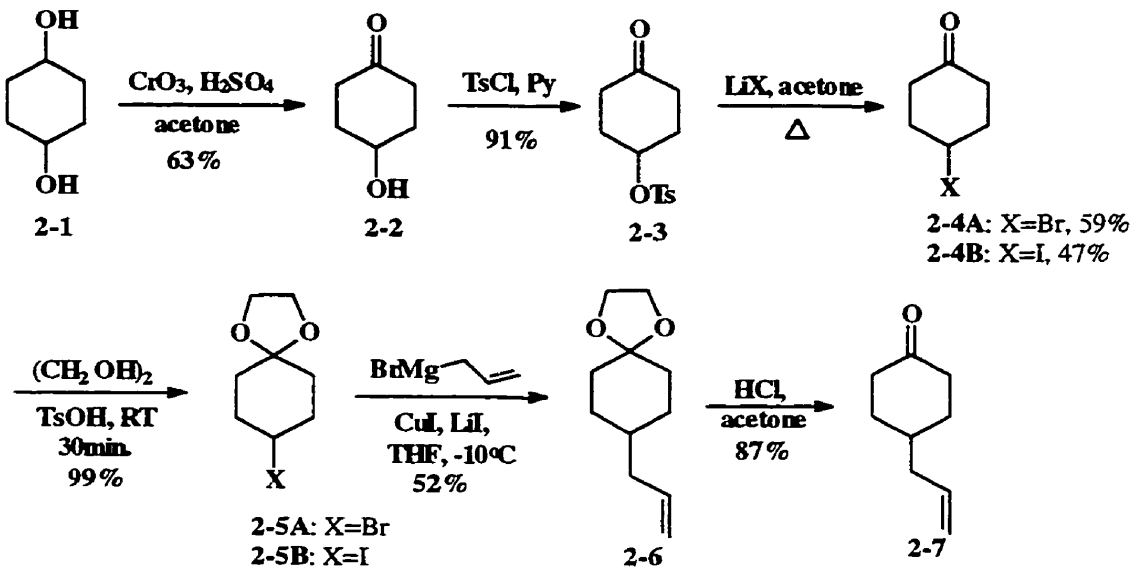
This method, however, could not be used in the synthesis of 4-allylcyclohexanone because of the hydrogenation step. Although there are methods to selectively reduce a double bond that is in conjugation with a carbonyl group, such as nickel-catalyzed silane reduction,[14] $Zn\text{-}NiCl_2$ under sonification,[15] or Wilkinson's catalyst in absolute ethanol,[16] no such attempt was made at this stage because the presence of transition metals could pose a threat for double bond migration, polymerization, and other side reactions.

A synthesis based on catalytic hydrogenation of phenol derivatives (Scheme 10) had been reported for the synthesis of 4-alkenyl substituted cyclohexanones.[17] However, in addition to the rather tedious synthesis, low overall yield, and the requirement for a high pressure and high temperature hydrogenator, this method lacked generality for the synthesis of other 4-substituted cyclohexanones.



Scheme 10

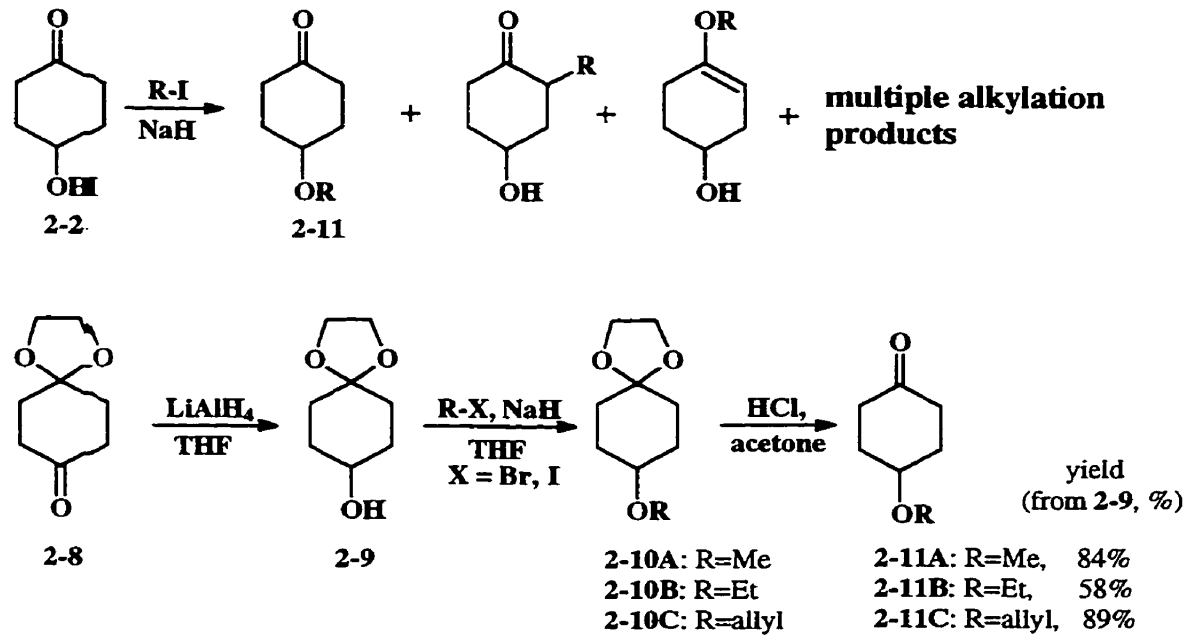
A different approach starting from 1,4-cyclohexanediol (**2-1**) was successfully developed for the synthesis of 4-allyl cyclohexanone (Scheme 11). This inexpensive method could be easily adapted for the synthesis of other 4-substituents simply by choosing appropriate nucleophiles, and most steps afforded reasonably good yields without extensive optimizations. In addition, the intermediate hydroxyketone **2-2** and 4-haloketone **2-4** could themselves be used as substrates. The 4-tosylated ketone **2-3** was not tested, because compounds with longer than *n*-butyl chains at the 4-position were known not to be substrates for CHMO.[18]



Scheme 11

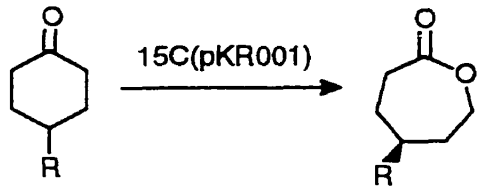
Although it should be possible to directly alkylate the intermediate hydroxyketone **2-2** to synthesize 4-alkoxy cyclohexanones (**2-11**), in several trials under different conditions[19] **2-2** either did not react, or gave a complex mixture of products according to GC analysis. This probably resulted from a combination of alkylation at the hydroxyl group

and at the α-positions of the ketone group (Scheme 12). The products in these reactions were not isolated or positively identified, and no further attempts were made to directly alkylate **2-2**. Instead, a different approach starting from the commercially available 1,4-cyclohexanedione monoethylene ketal was used for their synthesis (Scheme 12).



Scheme 12

Yeast-mediated oxidations



2-12

2-12A:* R=OH
2-12B: R=OMe
2-12C: R=OEt
2-12D: R=O-allyl
2-12E: R=Br
2-12F: R=I

Scheme 13

The yeast-mediated oxidations are illustrated in Scheme 13; all substrates were subjected to standard yeast oxidation conditions, and the results are listed in Table 5. For comparison, literature values for the oxidation of related compounds are shown in Table 6.[8,20] The stereochemistry of the lactone products was determined by X-ray crystallographic method[21] or by analogy to literature reports.[8,20] In general, prochiral 4-substituted cyclohexanones with *short, non-oxygenated* substituents are good substrates for the recombinant yeast, both moderately polar (Table 5, entry 5,6) and non-polar (Table 5, entry 7,8; Table 6, entry 1,2,3,5) groups are acceptable and give very high enantioselectivities. On the other hand, *oxygenated* substituents are less favourable (Table 5, entry 1,2). Although the oxidation of 4-methoxycyclohexanone (**2-11A**) was reasonably fast, the enantioselectivity was significantly lower than that of a substrate with a hydrophobic alkyl substituent (Table 6, entry 1). Substrates with longer

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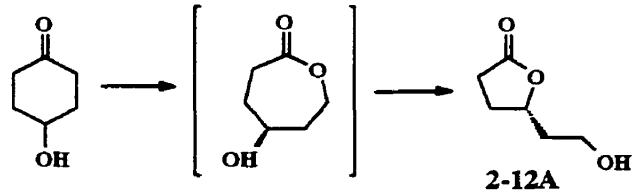
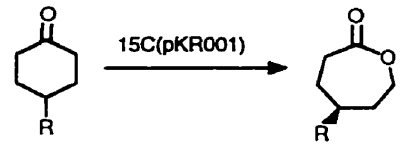
* The original ε-lactone will rearrange to give a 5-membered γ-lactone. **2-12A** refers to the final rearranged product.



2-12A

oxygenated substituents, such as **2-11B** and **2-11C**, do not seem to fit into the active site of the enzyme[22] (Table 5, entry 3,4). Thus when **2-11B** was incubated with the oxidizing yeast, no reaction was detected even after prolonged periods. This is in agreement with the general trend that longer 4-substituents are unsuitable in CHMO-catalyzed oxidations (Table 6, entry 2 *vs* 3, and entry 4 *vs* 5). A similar relationship between selectivity and chain length has been observed earlier.[20] Interestingly, substrates with longer alkoxy side chains (Table 5, entry 4) seem to fit into the active site(s) of the native reductase(s) in yeast. When **2-11C** was subjected to yeast-oxidation conditions, a significant amount of this compound was *reduced* to 4-allylcyclohexanol after an extended incubation period. It is not clear at this stage if the double bond in the side chain plays any role in the reduction of this compound. The oxidation of 4-hydroxycyclohexanone **2-2** (Table 5, entry 1), a substrate with a short but more hydrophilic substituent, was much slower than that of its methyl protected counterpart (Table 5, entry 2), and the enantioselectivity was negligible.

Table 5: Oxidation of prochiral 4-substituted cyclohexanones



Entry	R	Biocatalyst	Time(h)	SM (%)[1]	Lactone(%)[2]	% e.e.	$[\alpha]_D$
1	OH	15C(pKR001)	96	25	61[3]	12	--
2	OMe	15C(pKR001)	30	0	84	72(S)	+10.0, c1.1
3	OEt	15C(pKR001)	120	100	0	--	--
4	O-allyl	15C(pKR001)	120	0	0[4]	--	--
5	Br	15C(pKR001) BL21(pMM04)	36 7	0 0	63 79	97(S)	-10.2, c6.4
6	I	15C(pKR001) BL21(pMM04)	20 14	0 0	60 51	97(S)	-26.6, c1.0
7	Me	15C(pKR001)	48	3	83	>99(S)	-44.9[5], c1.4
8	4-allyl[5]	15C(pKR001)	--	--	62	95(R)[6]	-34.1[5], c4.1

(1) GC yield. (2) Isolated yield after chromatography. (3) See footnote on p.p. 2-8. (4) 4-Allyloxy-cyclohexanol recovered (41%) as a single isomer, tentatively assigned to be *trans*. (5) Cited from Ref. 19 in Chapter One. (6) A change in priority numbering is responsible for the (R)-configuration.

Table 6: CHMO-catalyzed oxidations of 4-alkylcyclohexanones[8, 20]

entry	R	Biooxidant	lactone(%)	e.e. (%)	$[\alpha]_D$
1	Et	15C(pKR001)	74	98	-28.7, c3.2
2	*n*-Pr	15C(pKR001)	63	92	-22.1, c0.88
3	*i*-Pr	15C(pKR001)	60	98	-26.5, c2.0
4	*n*-Bu	CHMO	70	52	+18.5, c2.74
5	*t*-Bu	CHMO	17	98	-34.9, c0.78

Model study for CHMO

In order to predict the stereochemistry of yeast-mediated Baeyer-Villiger oxidations, a corner model (Figure 8) has been proposed which resembles a modified Furstoss cubic model, but is generally easier to use and still gives correct predictions. In this model, a side wall, a back wall, and a floor define the pocket in the active site. A substrate arranges itself to minimize steric interactions within the active site. Thus all the side chains should point away from the side wall and occupy the equatorial position

whenever possible. As in the Furstoss cubic model, the attacking hydroperoxy flavin occupies a fixed position at the corner floor and is delivered from the bottom. There could be an amino acid residue on the back wall that is capable of stabilizing an axial hydroxy group in the Criegee intermediate, presumably by hydrogen bonding. The bond that is antiperiplanar to the peroxy bond will migrate to give the lactone product.

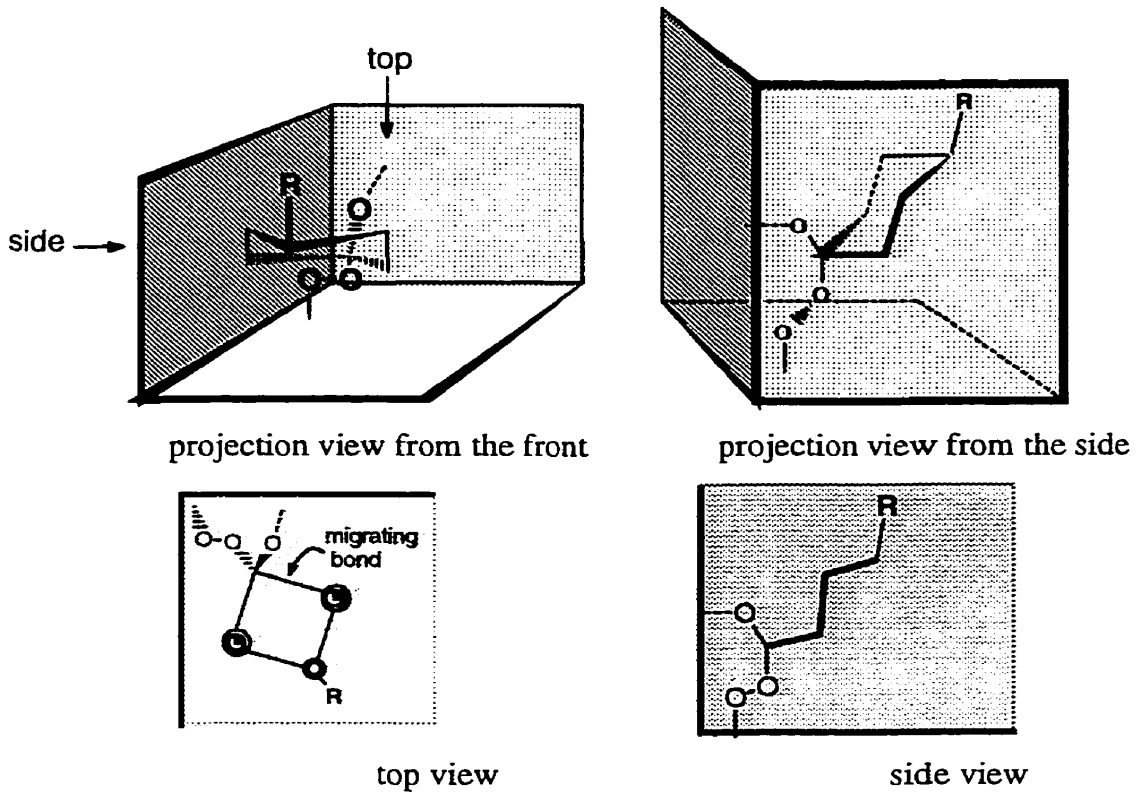


Figure 8: Corner model for CHMO

When this model is applied to the oxidation of prochiral 4-substituted cyclohexanones, one can predict that the product should have D-configuration,* providing that a

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* When the product is drawn with the inserted oxygen on the right side. the substituent would be facing *UP*. In this general description it can be misleading to use *(R)/(S)* notation because the change in priority numbering can alter the apparent absolute configuration of the product.

4-substituent occupies the equatorial position in the starting material. The requirement for a substituent to assume equatorial position in this reaction can also have significant influence in the stereoselectivity because of pre-reaction conformational equilibrium,[23] which is illustrated in Figure 9.

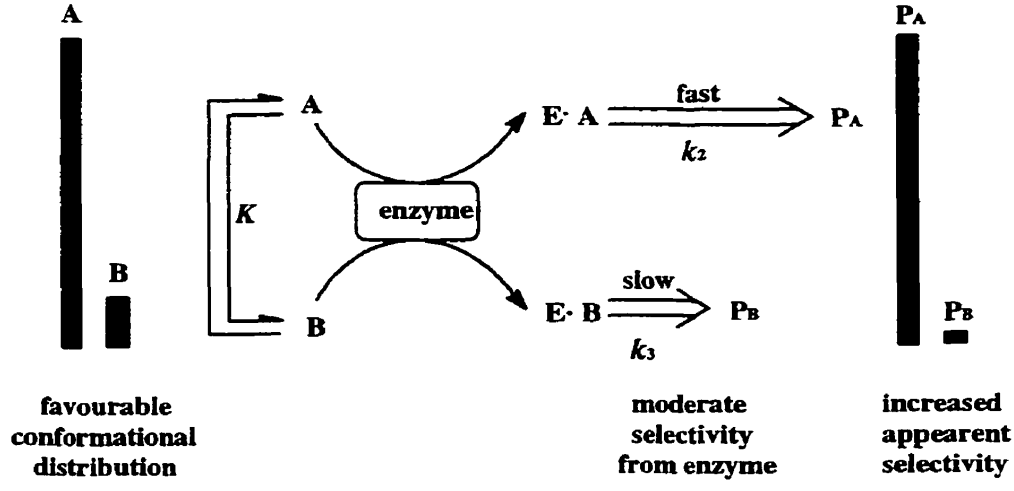
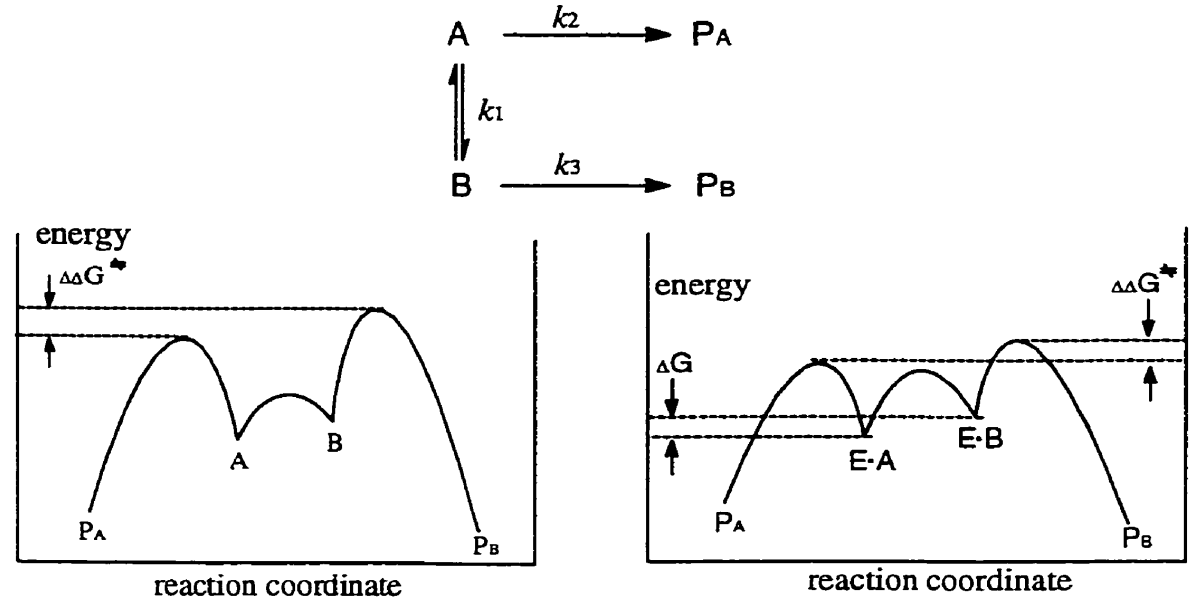


Figure 9: Enhancement of stereoselectivity by pre-reaction conformational equilibrium

Assume the starting material has two conformers **A** and **B** which are in equilibrium, **A** is the major conformer; if the enzyme has a preference for one conformer, ie. $k_2 \neq k_3$, the final product distribution will be the combined effect of the population distribution between **A** and **B** and the intrinsic enzymatic selectivity k_2/k_3.

According to the Curtin-Hammett principle,[24] the above pre-equilibrium model will only work when the conversion of the enzyme-substrate complex **E·A** and **E·B** to product $\mathbf{P_A}$ and $\mathbf{P_B}$ are faster than the conversion between the two conformers **A** and **B**. In ordinary chemical reactions, steps involving bond cleavage and bond formation

usually require higher activation energy than the conformational equilibration step, and the final product distribution may not reflect the conformer distribution of the starting material (Figure 10, left). In enzymatic reactions, however, the activation energy for the transformation of enzyme-substrate complex to the product can be greatly reduced, the activation energy of the conformational equilibration may fall in the same range as that of the actual reaction step, and the conformational distribution of starting material will affect the ratio of the final product (Figure 10, right).



When the activation energy for A⇌B is much lower than those for A→P_A and B→P_B, the product distribution is only determined by $\Delta\Delta G^{\neq}$ and is irrelevant to the equilibrium between A and B.

effective energy barrier = $\Delta\Delta G^{\neq}$

When the activation energy for A⇌B is comparable to those for A→P_A and B→P_B, relative concentration of A and B will affect the final product distribution.

effective energy barrier = $\Delta\Delta G^{\neq} + \Delta G$

Figure 10: Curtin-Hammett principle

The conformer distribution of selected starting materials are listed in Table 7. For a 4-alkylcyclohexanone, the predominant form in solution is the required equatorial conformer. If CHMO have low to moderate intrinsic selectivity towards the equatorial isomer, high enantioselectivity can be easily achieved with the assistance of a favourable conformational equilibrium.*

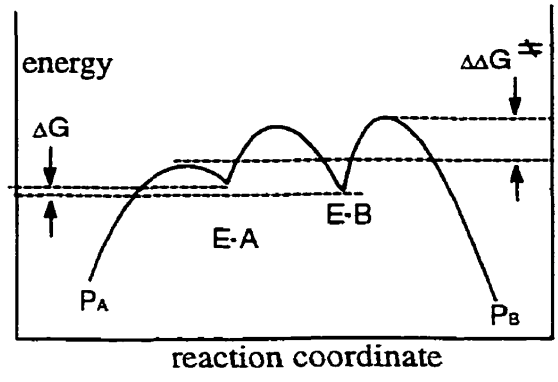
Table 7: Conformational equilibrium of 4-substituted cyclohexanones[25]

X	ΔG* (kcal/mol)	eq. %	ax. %
Et	+1.8	95	5
Me	+1.7	94	6
OMe	+0.35	64	36
OH	-0.09	46	54
Br	-0.24	40	60
I	-0.32	37	63

* Benzene solutions at 30°C

* If one takes 20:1 as an average eq./ax. ratio for 4-alkylcyclohexanones, and the enzyme has an intrinsic selectivity towards the equatorial conformer by a factor of 2, the total selectivity would have been 40:1, which can easily give >95% e.e. in the oxidations.

In case of 4-halocyclohexanones, the 4-axial conformers are slightly preferred due to transannular dipole-dipole interactions with the carbonyl group.[25a-c] According to the pre-reaction conformational equilibrium analysis, one would expect these compounds to be oxidized with low selectivities. The fact that they were all oxidized with very high optical purities suggests other factors contribute to the selectivity. A possible hypothesis is that a positively charged aminoacid residue in the active site stabilizes the 4-equatorial isomer by electrostatic interactions. If this stabilizing effect significantly lowers the activation energy in the breakdown of the Criegee intermediate of the equatorial isomer, the intrinsic enzymatic selectivity may outperform the unfavourable conformer distributions (Figure 11).



Small energy difference in the two conformers can be outperformed by the significant lowering of $\Delta\Delta G^{\ddagger}$ by dipole-dipole interactions.

Figure 11: Hypothesized energy diagram for the oxidation of 4-halocyclohexanones

In case of 4-methoxycyclohexanone, the relative short C-O bond (1.5Å, as opposed to 1.91Å for C-Br and 2.10Å for C-I)* may position the oxygen atom too far away from the positively charged aminoacid to have significant effect on the activation energy in the oxidation step. The observed moderate selectivity is a combined result of low equatorial

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* Calculated from the corresponding covalent radii.

conformer ratio (eq./ax.=1.8) and the low intrinsic selectivity from the enzyme.* Similarly, the low selectivity in the oxidation of 4-hydroxycyclohexanone would result from the unfavourable conformational distribution, weak dipole-dipole interaction, and low selectivity from the enzyme itself. In addition, since a hydroxy group is both a hydrogen bond donor and a hydrogen bond acceptor, the axial-hydroxy group could be stabilized by hydrogen bonding, which leads to lowered selectivity towards equatorial conformers.

Experimental

Synthesis of 4-substituted cyclohexanones

4-Hydroxylcyclohexanone **2-2** 1,4-cyclohexanediol **2-1** (4.8g, 40mmol) was suspended in 150mL acetone in a 250mL Erlenmeyer flask and stirred vigorously at room temperature. Jones reagent[26](10mL, 80mmol) 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 3 times with ethyl acetate. 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 1:1 petroleum ether and ethyl acetate as the eluent. A vanillin dip (Appendix III)

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* From the e.e. of the product (72%), the enzyme should contribute an extra 3.4:1 selectivity towards the equatorial conformer.

was used as the visualization method. 4-Hydroxycyclohexanone **2-2** was obtained as a colourless oil, 2.88g (63%). IR ν_{max} (neat): 3421(br, vs), 2940(vs), 2865(vs), 1709(vs), 1422(s), 1236(m), 1067(s), 951(s) cm^{-1}. ^1H NMR δ: 4.02(1H, m, br), 3.52(1H, s, br), 2.44(2H, qd, J_1=6.3, J_2=2.4), 2.16(2H, m), 1.89(2H, m), 1.82(2H, m) ppm. ^{13}C NMR δ: 212.0, 65.5, 36.9, 33.3 ppm.

p-Toluene sulfonic acid 4-oxocyclohexyl ester **2-3**[27] *p*-Toluene sulfonyl chloride (10.5g, 55mmol) was dissolved in 20mL pyridine and stirred at room temperature for 30 minutes. The solution was cooled in an ice-water bath and **2-2** (5.7g, 50mmol) was added. The mixture was stirred at room temperature for 2 hours until TLC showed complete consumption of the starting material. The mixture was poured onto ice cubes and extracted with 250mL ethyl acetate in 4 portions. The combined extracts were washed with a minimum amount of 2M HCl to bring the pH to slightly acidic conditions. After washing with saturated $NaHCO_3$ and brine, the organic phase was dried over anhydrous $MgSO_4$, and the solvent was removed on a rotary evaporator to give **4-3** as light brown crystals, 12.1g (91%), mp. 93-94°C. IR ν_{max} ($CHCl_3$): 3100(w), 3050(m), 2960(s), 2870(m), 1720(vs), 1600(s), 1440(s), 1350(vs), 1180(vs), 1100(s), 910(vs), 850(s), 810(s), 750(s) cm^{-1}. ^1H NMR δ: 7.84(2H, dt, J_1=8.4, J_2=1.6), 7.38(2H, d, J=8.0), 4.90(1H, quintet, J=2.8), 2.55(2H, qd, J_1=10.8, J_2=5.6), 2.45(3H, s), 2.29(2H, dt, J_1=15.2, J_2=5.2), 2.15(2H, m), 1.99(2H, m) ppm. ^{13}C NMR δ: 208.0, 144.6, 133.5, 129.6, 127.2, 76.4, 36.0, 30.6, 21.2 ppm.

General procedures for synthesizing 4-halocyclohexanones

LiI or NaBr (2-4 fold excess) was dissolved in 50mL acetone in a 100mL round bottom flask equipped with a refluxing condenser and a nitrogen balloon. *p*-Toluene sulfonic acid 4-oxocyclohexyl ester (**2-3**) was added and the system was flushed with nitrogen. The solution was gently refluxed until TLC showed complete consumption of the starting material (usually within 1-2 hours). The solvent was removed by rotary evaporation, the residue was dissolved in a minimum amount of water and extracted with 150mL of ethyl acetate in 3 portions. The combined extracts were washed once with 0.1M KOH and once with brine. After drying with anhydrous $MgSO_4$, the solvent was removed on a rotary evaporator. The product was purified by flash chromatography using 3:1 petroleum ether and ether as the eluent, and subsequent chromatography using CH_2Cl_2 as eluent to give the haloketone **2-4**.

4-Bromocyclohexanone **2-4A**[28] LiBr (10g, 112mmol) and **2-3** (4.04g, 15mmol) were reacted according to general procedures to give 4-bromocyclohexanone **2-4A** as a colourless oil, 1.58g(59%). IR ν_{max} (neat): 2960(m), 2920(w), 2875(w), 2850(w), 1720(vs), 1440(m), 1235(s) cm^{-1}. ^1H NMR δ: 4.56(1H, m), 2.67(2H, m), 2.35-2.20(6H, m) ppm. ^{13}C NMR δ: 208.5, 49.1, 38.4, 35.6 ppm.

4-Iodocyclohexanone **2-4B**[29] LiI (7g, 50mmol) and **2-3** (2.7g, 10mmol) were reacted according to general procedures to give 4-iodocyclohexanone **2-4B** as white crystals. 1.05g(47%), mp. 43-43.5°C. IR ν_{max} ($CDCl_3$): 3005(w), 2960(w), 2910(w), 2850(w), 1615(vs), 1435(m), 1120(m), 1210(vs), 1140(m), 930(w) cm^{-1}. ^1H NMR δ: 4.65(1H,

heptet, br, J=2.8), 2.55(2H, m), 2.33-2.20(4H, m), 2.16-2.08(2H, m) ppm. ^{13}C NMR δ: 208.2, 40.6, 37.8, 27.6 ppm.

General procedures for synthesizing 4-halocyclohexanone ethylene ketals

p-Toluenesulfonic acid (1 equivalent) was dissolved in ethylene glycol (3-10mL, excess) and 4-halocyclohexanone **2-4** was added. The mixture was stirred at room temperature for 30 minutes and poured into ice-cold 0.2M KOH. The aqueous phase was adjusted to basic conditions and extracted with 80mL petroleum ether in 3 portions. The combined extracts were dried with anhydrous Na_2SO_4, and the solvent was removed on a rotary evaporator to give the ketal **2-5**.

8-Bromo-1,4-dioxa-spiro[4.5]decane **2-5A** *p*-Toluene sulfonic acid (0.82g, 4.3mmol), ethylene glycol (3mL, excess) and 4-bromocyclohexanone **2-4A** (0.7g, 4mmol) were reacted according to general procedures to give the product as a light yellow oil, 0.78g (90%). IR ν_{max} (neat): 2960(vs), 2940(s), 2890(s), 1490(m), 1340(m), 1300(s), 1240(m), 1190(m), 1110(vs), 1070(s) cm^{-1}. ^1H NMR δ: 4.31(1H, heptet), 3.93(4H, m), 2.19-2.02(4H, m), 1.90(2H, dq, J_1=13.2, J_2=4.4), 1.60(2H, dq, J_1=12.8, J_2=4.8) ppm. The crude product was used in the next step without further purification and characterizations.

8-Iodo-1,4-dioxa-spiro[4.5]decane **2-5B** *p*-Toluenesulfonic acid (5g, 23mmol), ethylene glycol (20mL, excess), and 4-iodocyclohexanone **2-4A** (5.0g, 22mmol) were reacted according to general procedures to give **2-5B** as a colourless oil, 5.85g (98%). IR ν_{max} (neat): 2954(s), 2888(s), 1453(s), 1374(s), 1242(s), 1183(s), 1104(vs), 1032(s),

913(s) cm^{-1}. ^1H NMR δ: 4.39(1H, heptet, J=3.76), 3.95-3.87(4H, multiplet of 14 peaks), 2.17-2.01(4H, m), 1.83-1.74(2H, m), 1.63-1.54(2H, multiplet of 8 peaks) ppm. ^{13}C NMR δ: 107.4, 64.32, 64.26, 36.2, 34.7 ppm.

4-Hydroxycyclohexanone ethylene ketal **2-9** $LiAlH_4$ (0.65g, 17mmol) was suspended in 20mL THF in a 50mL round bottom flask under nitrogen. After stirring in an ice bath for 10 minutes, **2-8** (3.12g, 20mmol in 10mL THF) was added via a syringe over 5 minutes. After stirring at 0°C for 1 hour, 10mL saturated tartaric acid solution was added carefully to quench the reaction. The mixture was filtered through celite and the filtration cake was washed with ether. The aqueous layer was extracted with 100mL ether in 3 portions. The combined extracts were washed with saturated $NaHCO_3$ and the aqueous layer was back-extracted once with ether. The combined extracts were dried with anhydrous Na_2SO_4, and the solvent was removed by rotary evaporation to give **2-9** as a sticky, colourless oil, 2.85g (91%). IR ν_{max} (neat): 3400(s, br), 2950(s), 2870(s), 2700(w), 1450(m), 1370(m), 1100(s, br), 940(s, br), 660(m) cm^{-1}. ^1H NMR δ: 3.90(4H, m), 3.74(1H, heptet, J=4.6), 1.87-1.73(5H, m), 1.65-1.49(4H, m) ppm. ^{13}C NMR δ: 108.2, 68.0, 64.22, 64.20, 31.9, 31.5 ppm.

General procedures for synthesizing 4-alkoxycyclohexanone ethylene ketals

Method A: NaH (50% dispersion in mineral oil, 2-4 fold excess) was placed in a 100mL round bottom flask under nitrogen and washed twice with 20mL THF. Alkyl halide (50-100% excess) in 40mL THF was added and the mixture was cooled in an ice bath. 4-hydroxycyclohexanone ethylene ketal (**2-9**, neat) was added via a syringe. After 20 minutes, the ice bath was removed and the reaction was allowed to proceed

until GC showed complete consumption of the starting material. The mixture was poured onto ice cubes, and extracted with 100mL of petroleum ether in 3 portions. The combined extracts were dried with anhydrous $MgSO_4$ and the solvent was removed by rotary evaporation. The residue was purified by flash chromatography when necessary using 20:1 petroleum ether and ethyl acetate as the eluent, or it was used directly in the next step.

Method B: NaH (50% dispersion in mineral oil, 2-4 fold excess) was placed in a 100mL round bottom flask equipped with a refluxing condenser under nitrogen and washed twice with 20mL THF. A solution of **2-9** in 40mL THF was added via a syringe and the mixture was heated to reflux for 1 hour. The mixture was maintained at 40-50°C and alkyl halide (4-10 fold excess, neat) was added in small portions over a 12-24 hour period until GC or TLC showed complete consumption of the starting material. The same workup procedures as in **method A** were used to give the product.

General procedures for deketalization

4-Alkoxycyclohexanone ethylene ketal **2-10** was dissolved in a mixture of 20mL acetone and 5mL 2M HCl and stirred at room temperature until GC showed complete conversion. The acetone was removed by rotary evaporation and the residue was extracted with 75mL CH_2Cl_2 in 4 portions. The combined extracts were washed once with saturated $NaHCO_3$ and dried with anhydrous $MgSO_4$. The solvent was removed by rotary evaporation and the product was purified by flash chromatography on silica gel using 10:1 petroleum ether and acetone as the eluent to give 4-alkoxycyclohexanone **2-11** as a colourless oil.

4-Methoxycyclohexanone **2-11A**[30] NaH (0.8g), MeI (2.5mL, neat, excess) and **2-9** (1.58g, 10mmol in 40mL THF) were reacted according to **method A** to give 4-methoxycyclohexanone ethylene ketal **2-10A**. IR ν_{max} (neat): 2950(s), 2980(s), 2830(m), 1450(m), 1380(s), 1100(vs), 1040(s), 940(s) cm^{-1}. ^1H NMR δ: 3.90(4H, m), 3.29(3H, s), 3.26(1H, m), 1.83-1.71(4H, m), 1.71-1.61(2H, m), 1.54-1.46(2H, m) ppm. ^{13}C NMR δ: 108.4, 76.1, 64.2, 55.7, 31.2, 28.1 ppm. Deketalization of the crude product according to general procedures gave 4-methoxycyclohexanone **2-11A** as a colourless oil. IR ν_{max} (neat): 2950(m), 2900(m), 2875(m), 2830(m), 1720(vs), 1120(m), 1100(s), 1080(s) cm^{-1}. ^1H NMR δ: 3.56(1H, heptet, J=2.9), 3.36(3H, s), 2.50(2H, ddd, J_1=15.6, J_2=10.6, J_3=5.8), 2.24-2.17(2H, m), 2.08-2.00(2H, m), 1.93-1.83(2H, m) ppm. ^{13}C NMR δ: 211.1, 74.1, 56.0, 37.0, 30.0 ppm.

4-Ethoxycyclohexanone **2-11B**[31] NaH (2g), ethyl bromide (10mL in 4 portions, excess), and **2-9** (0.8g, 5mmol) were reacted according to **method B** to give 4-ethoxycyclohexanone ethylene ketal **2-10B**, 0.9g. Spectroscopic data were recorded from the crude mixture. IR ν_{max} (neat): 2954(m), 2881(m), 1381(m), 1117(s), 1045(m), 920(m) cm^{-1}. ^1H NMR δ: 3.74-3.68(4H, m), 3.45(2H, q, J=7.0), 3.39-3.32(1H, m), 1.85-1.74(2H, m), 1.72-1.62(2H, m), 1.55-1.46(2H, m), 1.32-1.22(2H, m), 1.16(3H, t, J=7.0) ppm. ^{13}C NMR δ: 74.5, 67.9, 64.2, 63.1, 31.5, 28.7, 15.6 ppm. Deketalization according to general procedures and flash chromatography using 20:1 petroleum ether and ethyl acetate as the eluent gave **2-11B** as a colourless oil, 0.42g (two steps, 58%). IR ν_{max} (neat): 2967(s), 2940(s), 2868(s), 1723(vs), 1341(w), 1111(s), 1085(m), 966(w) cm^{-1}. ^1H NMR δ: 3.66(1H, heptet, J=2.9), 3.51(2H, q, J=7.0), 2.57-2.49(2H, multiplet

of 7 peaks), 2.25-2.17(2H, m), 2.06-1.97(2H, m), 1.94-1.85(2H, m), 1.19(3H, t, J=7.0) ppm. ^{13}C NMR δ: 211.3, 72.5, 63.6, 37.2, 30.6, 15.6 ppm.

4-Allyloxycyclohexanone **2-11C** NaH (1g), allyl bromide (1mL, 12mmol), and **2-9** (1.0g, 6.3mmol) were reacted according to **method A** to give 4-allyloxycyclohexanone ethylene ketal **2-10C** as a light yellow oil, 1.12g (89%). ^{1}H NMR δ: 5.94-5.83(1H, m), 5.24(1H, dq, J_1=17.3, J_2=1.7), 5.12(1H, m), 3.95(2H, dt, J_1=5.47, J_2=1.7), 3.90(4H, dd, J_1=3.9, J_2=2.9), 3.42(1H, heptet, J=3.2), 1.84-1.75(4H, m), 1.70(2H, q, J=7.7), 1.55-1.48(2H, m) ppm. ^{13}C NMR δ: 135.4, 116.3, 108.5, 74.1, 68.9, 64.3, 31.4, 28.5 ppm. Deketalization according to general procedures gave **2-11C** as a colourless oil. 0.87g (>99%). IR ν_{max} (neat): 3090(w), 3010(w), 2950(s), 2870(s), 1720(vs), 1650(w), 1420(m), 1340(m), 1100(s), 1060(s), 930(m) cm^{-1}. ^{1}H NMR δ: 5.92-5.81(1H, m), 5.23(1H, dq, J_1=17.3, J_2=1.7), 5.10(1H, dq, J_1=10.4, J_2=1.4), 3.96(2H, dt, J_1=5.5, J_2=1.7), 3.68(1H, heptet, J=2.9), 2.55-2.46(2H, m), 2.21-2.14(2H, m), 2.05-1.96(2H, m), 1.91-1.82(2H, m) ppm. ^{13}C NMR δ: 210.9, 134.9, 116.4, 72.0, 69.1, 37.0, 30.4 ppm.

8-Allyl-1,4-dioxa-spiro[4.5]decane **2-6** 8-Bromo-1,4-dioxa-spiro[4.5]decane **2-5A** (0.8g, 3.6mmol) was dissolved in THF (10mL), and CuI (0.5g, 2.6mmol) was added. The system was flushed with nitrogen, cooled in an ice bath and allyl magnesium bromide (1M in ether, 10mL, 10mmol) was added slowly. The black mixture was stirred at room temperature for 2 hours and the reaction was quenched with water. The mixture was extracted with 100mL ether in 3 portions. The combined extracts were washed once with water, once with brine and dried with anhydrous Na_2SO_4. The

solvent was removed by rotary evaporation to give the crude product, 0.73g (53% purity by GC, 60% yield). Purification by flash chromatography using 4:1 hexane and ethyl acetate as the eluent gave **2-6** as a colourless oil. IR ν_{max} (neat): 3075(w), 2928(s), 2840(m), 1640(m), 1446(m), 1374(m), 1106(s), 1035(m), 925(m) cm^{-1}. ^1H NMR δ: 5.83-5.68(1H, m), 4.97(1H, d, J=6.3), 4.93(1H, s), 3.92(4H, s), 2.02-1.93(2H, m), 1.76-1.64(4H, m), 1.58-1.42(2H, m), 1.40-1.22(2H, m) ppm. ^{13}C NMR δ: 137.3, 115.5, 109.1, 64.2, 40.7, 36.2, 34.5, 29.9 ppm.

4-Allyl cyclohexanone **2-7** **2-6** was dissolved in a mixture of acetone (10mL) and HCl (1M, 2mL) and stirred at room temperature for 1 hour. The mixture was diluted with water (50mL) and extracted with 60mL ethyl acetate in 3 portions. The combined extracts were washed once with saturated $NaHCO_3$ and once with brine. The solvent was removed by rotary evaporation and the residue was purified by flash chromatography using CH_2Cl_2 as the eluent to give **2-7** as a colourless oil. IR ν_{max} (neat): 3076(w), 2955(s), 2925(s), 2858(m), 1720(vs), 1449(w), 1162(w), 912(w) cm^{-1}. ^1H NMR δ: 5.87-5.72(1H, m), 5.05(1H, d, J=4.2), 5.00(1H, s), 2.43-2.23(4H, m), 2.11-1.98(4H, m), 1.80(1H, multiplet of 13 peaks), 1.40(2H, AB quartet of doublet, J_1=12, J_2=5.7) ppm. ^{13}C NMR δ: 212.0, 136.5, 116.3, 40.7, 39.9, 35.9, 32.3 ppm.

5-(2-Hydroxyethyl)oxolane-2-one **2-12A** Yeast-mediated oxidation of 4-hydroxy-cyclohexanone (**2-2**, 50μL) was performed in the presence of β-cyclodextrin according to general procedures except that half of the substrate concentration was used. When GC indicated that substrate consumption had stopped, the reaction medium was continuously extracted for 18 hours with CH_2Cl_2. The solvent was removed by rotary

evaporation and the residue was purified by flash chromatography using 2:1 petroleum ether and acetone as the eluent to give **2-12A** as a colourless oil, 35mg (61%). IR ν_{max} (neat): 3434(br, m), 2960(m), 2894(w), 1775(vs), 1190(s), 1052(s), 959(m), 670(w) cm^{-1}. ^1H NMR δ: 4.71-4.63(1H, multiplet of 12 peaks), 3.78(2H, t, J=6.3), 2.54-2.49(2H, m), 2.39-2.30(2H, including an overlapping hydroxy peak, multiplet of 9 peaks), 1.95-1.81(3H, m) ppm. ^{13}C NMR δ: 177.2, 78.5, 59.0, 38.1, 28.7, 28.1 ppm.

5-Bromooxepan-2-one **2-12E** Yeast-mediated oxidation was performed with 100mg of 4-bromocyclohexanone (**2-4A**, 0.56mmol) in the presence of 0.5g β-cyclodextrin according to general procedures. Chromatography on silica gel using 4:4:1 petroleum ether : CH_2Cl_2 : ether followed by repeated chromatography using 1:1 petroleum ether and ether as eluent gave **2-12E** as a colourless oil, 72mg (66%). *E. coli*-mediated oxidation was performed with 140mg of **2-4A** (0.79mmol) in presence of 0.5g β-cyclodextrin according to general procedures. Chromatography on silica gel using 4:1 petroleum ether and ethyl acetate as eluent gave the product as a colourless oil, 120mg (79%). IR ν_{max} (neat): 2954(w), 1736(vs), 1438(w), 1337(w), 1201(m), 1065(m), 963(w) cm^{-1}. ^1H NMR δ: 4.57(1H, m), 4.49(1H, ddd, J_1=13.3, J_2=8.04, J_3=2.1), 4.13(1H, dddd, J_1=13.2, J_2=5.8, J_3=2.7, J_4=0.5), 3.02(1H, m), 2.54(1H, m), 2.31(2H,m), 2.20(2H,m) ppm. ^{13}C NMR δ: 174.6, 64.6, 51.5, 38.6, 32.1, 30.5 ppm.

5-Iodooxepan-2-one **2-12F** Yeast-mediated oxidation was performed with 100mg of 4-iodocyclohexanone (**2-4B**, 0.45mmol) in the presence of 0.5g β-cyclodextrin according to general procedures. Chromatography on silica gel using 4:1 petroleum ether and ethyl acetate as the eluent gave **2-12F** as a colourless oil, 120mg (79%),

which solidified upon standing to give white crystals. IR ν_{max} (neat): 2954(m), 2888(w), 1736(vs), 1190(m), 933(m) cm^{-1}. ^1H NMR δ: 4.67(1H, s, br), 4.35(1H, dd, J_1=13.3, J_2=8.7), 4.16(1H, ddd, J_1=13.5, J_2=7.2, J_3=1.4), 2.90(1H, t, br, J=13.7), 2.58(1H, ddd, J_1=14.5, J_2=8.9, J_3=1.5), 2.34-2.10(4H, m) ppm. ^{13}C NMR δ: 174.6, 66.9, 40.5, 34.1(br), 33.4 ppm.

5-Methoxyoxepan-2-one **2-12B** Yeast-mediated oxidation was performed with 100μL of 4-methoxycyclohexanone (**2-11A**, 0.78mmol) according to general procedures. Chromatography on silica gel using 5:1 petroleum ether and acetone as the eluent gave **2-12B** as a colourless oil, 95mg(84%). $[\alpha]_D^{20}$= +10°, IR ν_{max} (neat): 2947(s), 2835(m), 1743(vs), 1446(s), 1302(s), 1150(s), 1098(s) cm^{-1}. ^1H NMR δ: 4.37(1H, ddd, J_1=13.2, J_2=9.58, J_3=1.4), 3.96(1H, ddd, J_1=13.0, J_2=6.2, J_3=1.5), 3.47(1H, heptet, J=2.7), 3.24(3H, s), 2.84(1H, ddd, J_1=14.0, J_2=12.0, J_3=1.9), 2.31(1H, ddd, J_1=14.2, J_2=8.4, J_3=1.2), 1.99-1.81(3H, m), 1.78-1.69(1H, m) ppm. ^{13}C NMR δ 175.9, 75.3, 63.1, 55.7, 33.2, 27.5, 26.6 ppm.

trans-4-Allyloxycyclohexanol 4-Allyloxycyclohexanone (**2-11C**, 100mg) was subjected to standard oxidation conditions. After 5 days of incubation, GC showed consumption of the starting material had stopped. The culture was extracted with ethyl acetate, the solvent was removed by rotary evaporation and the residue was purified by flash chromatography with 10:1 petroleum ether and acetone according to standard protocols to give a colourless oil, (42mg, 41%), which was shown to be 4-allylcyclohexanol, tentatively assigned to be *trans*. IR ν_{max} (neat): 3376(s, br), 3084(w), 2936(s), 2874(s), 1648(w), 1450(m), 1362(m), 1128(m), 1076(s), 966(m), 922(m) cm^{-1}. ^1H NMR δ: 5.97-

5.86(1H, m), 5.27(1H, dq, J_1=17.1, J_2=1.7), 5.15(1H, dq, J_1=10.5, J_2=1.4), 4.01-3.96(2H, m), 3.73(1H, heptet, J=3.8), 3.43(1H, heptet, J=3.2), 2.55(1H, br), 1.87-1.79(2H, m), 1.74-1.60(4H, m), 1.60-1.50(2H, m) ppm. ^{13}C NMR δ: 135.3, 116.1, 76.1, 73.5, 68.6, 30.3, 27.4 ppm.

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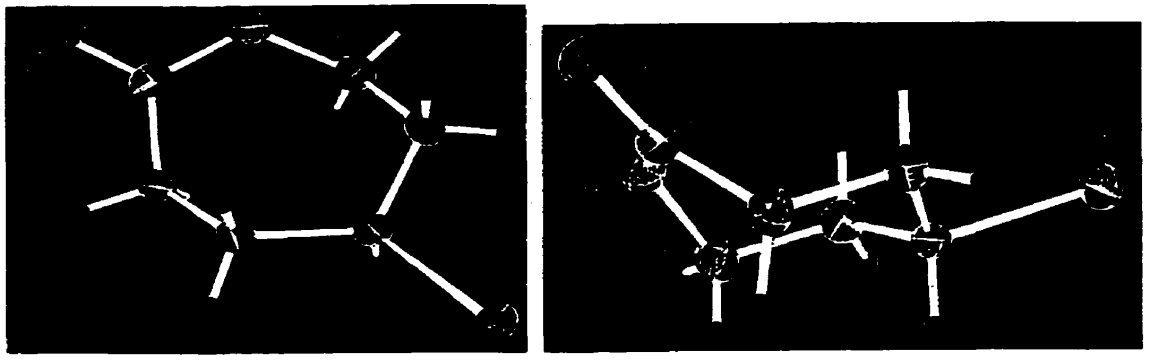
[21] Enantiomerically pure (>97% e.e.) 4-Iodooxepan-2-one **2-12f** was obtained from yeast-mediated oxidation of **2-4b**. It was crystallized and the absolute configuration was determined by X-ray crystallography to be (S)



[22] Substrate solubility or membrane transportation is unlikely to be responsible for the failure in the oxidation of **2-11B** and **2-11C**, because short-chain substrate **2-11A** was oxidized, and long-chain substrate **2-11C** was reduced, it means these compounds are soluble enough, and can be transported through the cell membrane. The chain length for **2-11B** is in between, and it can be expected to have similar behavior.

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Chapter Three: Yeast-Mediated Baeyer-Villiger Oxidations of Cyclopentanones

Introduction

Chiral δ-valerolactones and their derivatives are key asymmetric fragments in a variety of biologically active compounds[1] (Figure 12). Although several chemical and chemoenzymatic methods for the total or partial synthesis of these compounds have been reported,[2] many of them involved tedious steps, hazardous reagents, or starting materials that are not readily available.* Enantioselective Baeyer-Villiger oxidation of racemic substituted cyclopentanones is a possible route for the convenient access to chiral lactones. At present, chemical oxidations in the presence of chiral catalysts have achieved some success, although the yields and enantiomeric excesses of the products were only low to moderate[3] (Scheme 14, Table 8).

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* In reference 2c, a precursor for mevinolin was synthesized using chemoenzymatic methods in 11 steps from commercially unavailable starting materials giving 5% total yield with 89% e.e. and 50% d.e.

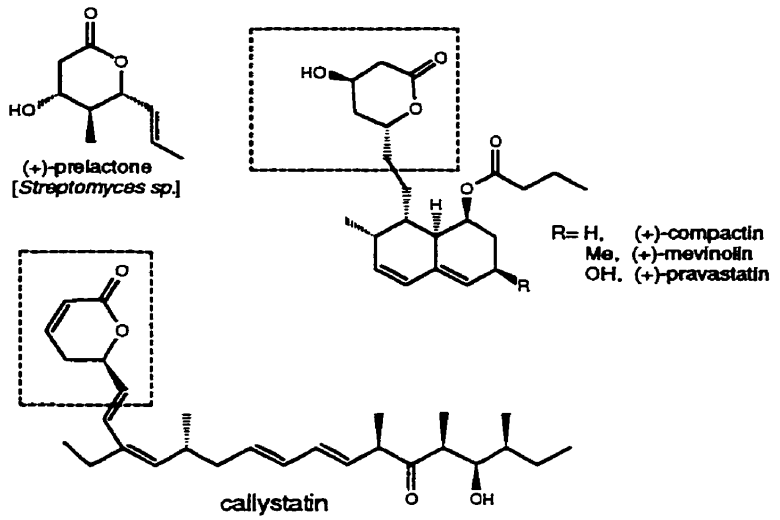
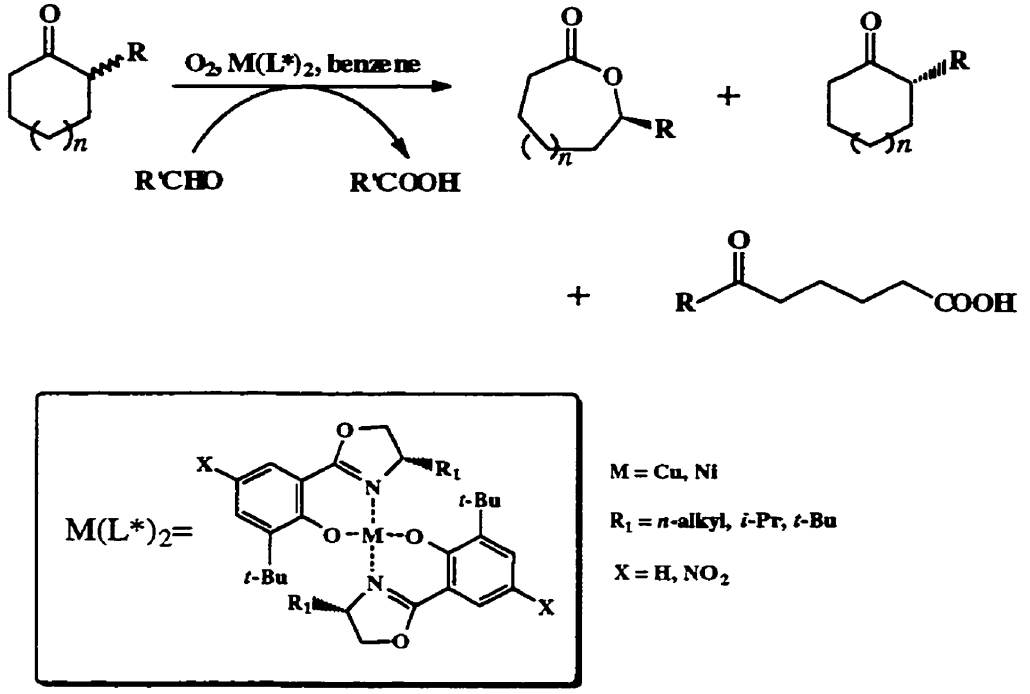


Figure 12: Examples of natural products having δ-valerolactone fragments



Scheme 14

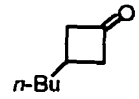
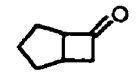
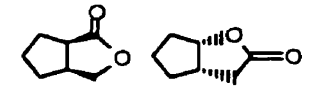
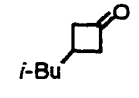
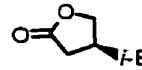
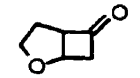
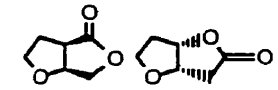
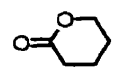
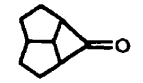
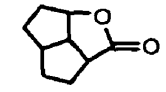
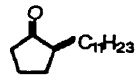
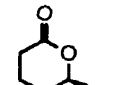
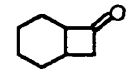
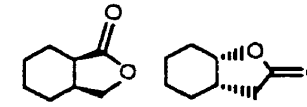
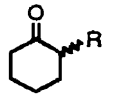
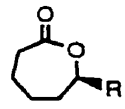
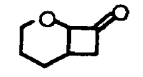
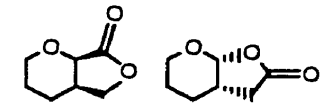
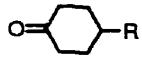
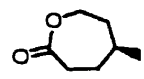
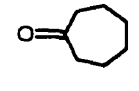
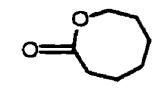
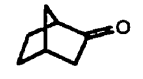
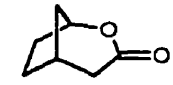
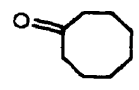
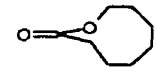
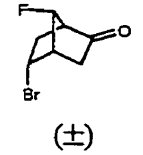
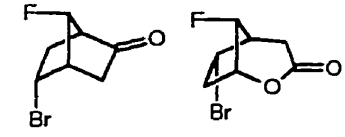
Table 8: Catalytic asymmetric Baeyer-Villiger oxidations[3]*

entry	*n*	R	R'	lactone (%)	e.e. (%)
1	1	Ph	*t*-Bu	41	65
2	1	Ph	*t*-Bu	47	69
3	1	Ph	*m*-Cl-Ph	32	49
4	1	Ph	*p*-MeO-Ph	no reaction	-
5	1	Ph	*i*-Bu	65	59
6	1	Ph	Ph	61	61
7	1	*p*-Cl-Ph	*t*-Bu	43	60
8	1	*p*-MeO-Ph	*t*-Bu	53	65
9	0	Ph	*t*-Bu	21	47
10	2	Ph	*t*-Bu	no reaction	-

* M=Cu (1% mol), X = NO_2, R_1 = *t*-Bu, room temperature 6h, O_2 1atm, GC/HPLC yield

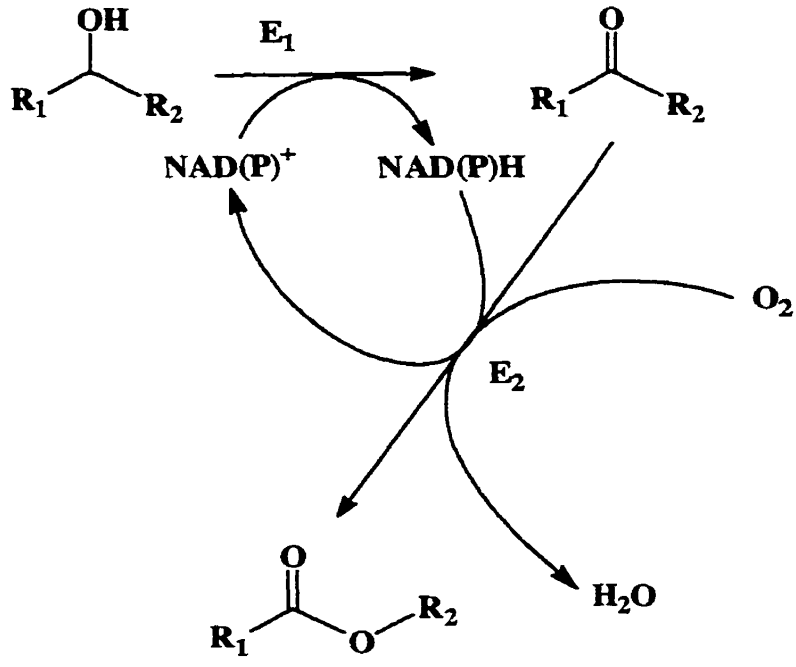
On the other hand, enzymatic asymmetric Baeyer-Villiger oxidations have shown significant success. Cyclohexanone monooxygenase (CHMO) isolated from soil bacterium *Acinetobacter* sp. (NCIB 9871) is the most extensively studied enzyme; and it has shown broad substrate spectrum and good stereoselectivity (Table 9).[4]

Table 9: CHMO-catalyzed Baeyer-Villiger oxidations [4]

substrate	product	yield (e.e.)%	substrate	product	yield (e.e.)%
		68(17)			31, 36 (95, 95)
		56(84)			35, 35 (97, 97)
		67			--[1] (98)
		25(73)			28, 52 (95, 60)
		21-38 (49-98)			33, 33 (70, 98)
		62-83 (92-98)			62(80)
		-- [1]			81(-- [1])
		-- [1]	 (±)		30, 36 (95, 95)

1. Not reported

The main factors that limited the wide use of CHMO are its low stability[5] and its dependence on the expensive cofactor NADPH. Although there have been successful precedents where the stability of CHMO was improved by using immobilized enzyme in a membrane reactor,[6] and NADPH was used in catalytic amount by a coupled recycling process[7] (Scheme 15), none of these technologies are suitable for large scale preparations.



E_1= alcohol dehydrogenase
E_2= monooxygenase

Scheme 15

An alternative method is to perform the biotransformations with live *Acinetobacter* cells, but this bacterium is a *Class II* pathogen,[8] and it needs to be grown on cyclohexanol to induce the production of CHMO. These facts and the relatively low enzyme production make *Acinetobacter* not a very convenient bioreagent to be used in ordinary organic laboratories.

Because chiral δ-valerolactones are very useful compounds, and the recombinant oxidizing yeast 15C(pKR001) had been successful in the asymmetric oxidation of cyclohexanones, it was desirable to extend the yeast-mediated oxidation to

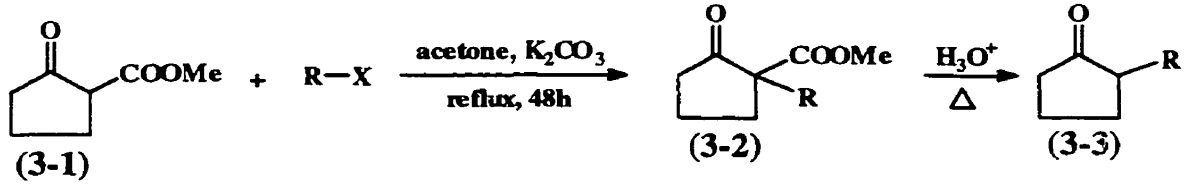
cyclopentanones. Therefore, a systematic investigation of 2- and 3-alkyl substituted cyclopentanones was conducted.

Results and discussion

Preparation of substrates

2-Alkylcyclopentanones

Most 2-alkyl substituted cyclopentanones were prepared by alkylation of methyl cyclopentanone-2-carboxylate (**3-1**) followed by decarboxylation (Scheme 16). The results are summarized in Table 10.



Scheme 16

Table 10: Synthesis of 2-alkyl substituted cyclopentanones

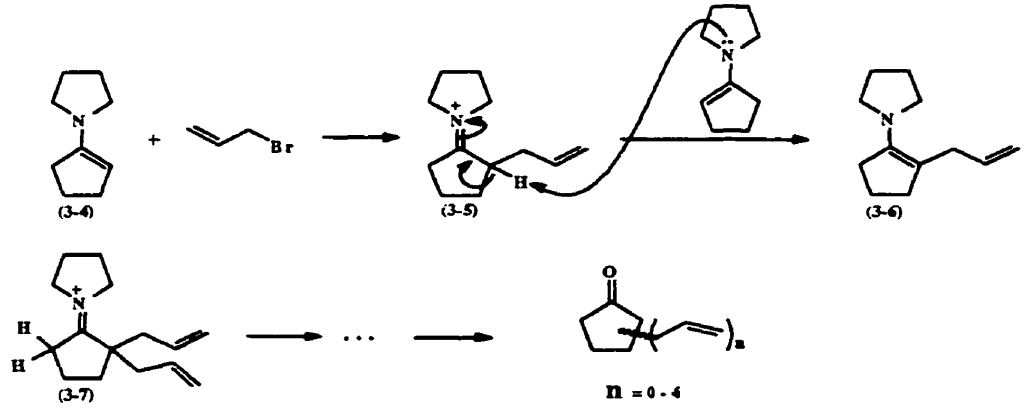
	R	(3-2) isolated yield (%)	(3-3) isolated yield (%)
B[1]	Ethyl	80	70
C	*n*-Propyl	77	92
E	*n*-Butyl	71	88
F	*i*-Butyl	70	44
H	*n*-Octyl	60	67
I	*n*-Undecyl	70	82

1. Letters **A** and **G** are reserved for commercially available 2-methyl and 2-*n*-hexyl cyclopentanones; **D** is reserved for 2-allylcyclopentanone, which was synthesized via a different method.

Some difficulties were encountered in the synthesis of 2-allylcyclopentanone (**3-3D**). Acidic hydrolysis of **3-2D** gave a complex mixture of products according to GC

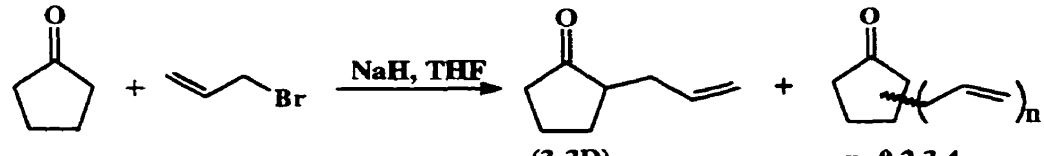
analysis, probably due to acid-catalyzed polymerization of the double bond moiety. Decarboxylation under neutral conditions by heating with wet NaCl in DMSO at 160°C[9] yielded dark coloured polymeric materials. Since hydrolysis under basic conditions also failed, probably due to ring cleavage by the hydroxide,[10] no further attempts were made to vary the reaction conditions.

Alkylation of cyclopentanone via its enamine derivative[11] afforded **3-3D**, but it was accompanied with large proportions of di-, tri-, and even tetra-substituted byproducts, and the final yield of **3-3D** was low (20-30% from the enamine). One possible mechanism for the formation of these multi-substituted products is illustrated in Scheme 17. In this mechanism, extraction of an activated α-proton from the positively charged imonium intermediate **(3-5)** by the basic nitrogen in the enamine starting material **(3-4)** regenerates the nucleophilic enamine **(3-6)** which is capable of a second round of alkylation. This process can repeat until all the α-positions have been alkylated. This type of multiple alkylation is particularly significant with active electrophiles like allyl bromide.[11]



Scheme 17

It was observed that direct alkylation of cyclopentanone with allyl bromide in the presence of NaH gave product distribution similar to that obtained from the enamine method (Scheme 18). Although the yield was still low, this method was faster and simpler, and the starting materials are readily available in bulk quantities. Therefore, this method was used to obtain sufficient starting material for this preliminary work without further optimizations.



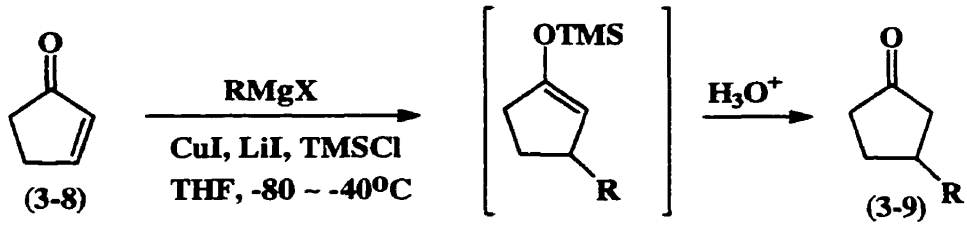
(3-3D)
~ 25% by GC

n=0,2,3,4

Scheme 18

3-Alkylcyclopentanones

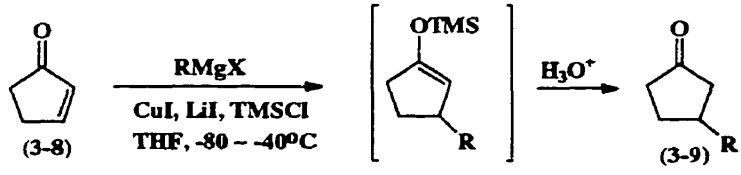
3-Substituted cyclopentanones were synthesized by Michael addition of the corresponding dialkyl cuprates to 2-cyclopentenone (Scheme 19). 3-*n*-Butylcyclopentanone was prepared using Gilman reagent generated *in situ* from commercially available *n*-butyl lithium and cuprous iodide. For other side chains, readily accessible Grignard reagents, prepared from alkyl halides and magnesium metal, were used in place of the alkyl lithium reagents.



Scheme 19

Some optimization was made to the standard Gilman addition conditions:[12] (1) Excess of LiI was added to solubilize the cuprous catalyst which facilitated the formation of dialkyl cuprates. (2) One equivalent of TMSCl was added to trap the intermediate enolate, which both accelerated the reaction rate and prevented possible side reactions.[13] The TMS ethers were readily decomposed during the acidic workup to give the corresponding 3-alkylcyclopentanones. Under these conditions, all reactions produced the 3-alkyl substituted ketones in reasonably good yields as shown in Table 11.

Table 11: Synthesis of 3-alkylcyclopentanones via Michael additions

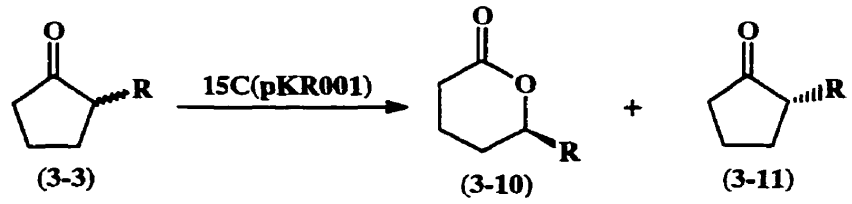


R	Product	yield (%)
Ethyl	**3-9B**[1]	78
n-Propyl	**3-9C**	62
Allyl	**3-9D**	93
n-Butyl	**3-9E**	36
n-Hexyl	**3-9F**	74
n-Octyl	**3-9G**	82
n-Undecyl	**3-9H**	28

1. Code **3-9A** is reserved for commercially available 3-methyl cyclopentanone

Yeast-Mediated Baeyer-Villiger oxidations

Oxidations of 2-substituted cyclopentanones



(3-3) (3-10) (3-11)

Scheme 20

2-Alkylcyclopentanones are kinetically resolved into the *(S)*-lactones and the *(R)*-ketones (Scheme 20). In order to describe the resolving capacity of the yeast-catalyzed Baeyer-Villiger oxidations, the E value (enantiomeric ratio) was used as a quantitative measurement.[15] The E value is defined as the ratio of specificity constants* of the fast- and slow-reacting enantiomers (Eq. 1), it reflects the relative rate at which the two enantiomers are converted to the products. It can be qualitatively estimated by plotting enantiomeric excess of the starting material [e.e.(S)] or the product [e.e.(P)] against fractional conversion (c) and visually comparing it with the theoretical plots. Alternatively, it can be calculated from Eq. 2. More precise values can be obtained by computer non-linear data fitting according to Sih's method.[14]

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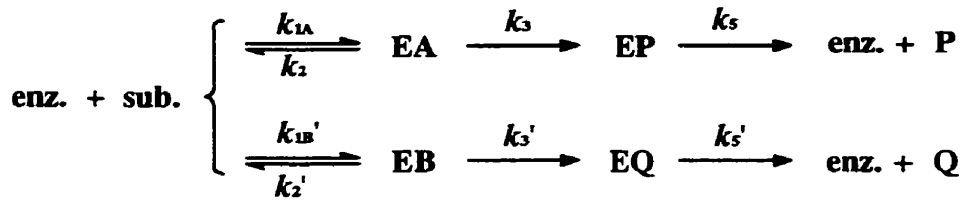
* Specificity constant is defined as V/K, where V is the maximum velocity of the reaction, and K is the Michaelis constant. For details, see Ref. 15(b, c).



EA, EB: enzyme-substrate complexes with the two enantiomers
EP, EQ: enzyme-product complexes with the two enantiomers
P, Q: two enantiomers of the product

The E value is defined as: $E = \frac{V_A / K_A}{V_B / K_B}$ **Eq. 1**

V_A, K_A and V_B, K_B stand for maximum velocity and Michaelis constants of the fast and slow reactions. In practice, the following equation can be used for its calculation:

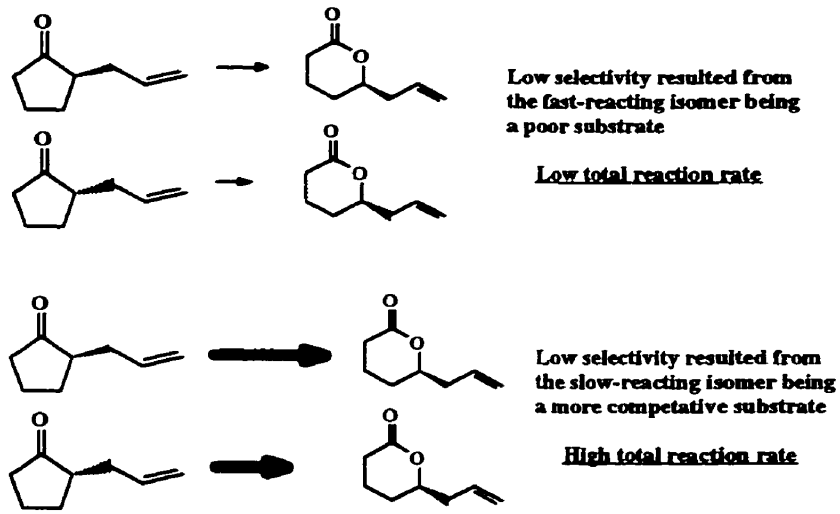
$$E = \frac{\ln(A / A_0)}{\ln(B / B_0)} = \frac{\ln[(1-c)(1-e.e.(S))]}{\ln[(1-c)(1+e.e.(S))]} = \frac{\ln[1-c(1+e.e.(P))]}{\ln[1-c(1-e.e.(P))]} \quad \textbf{Eq. 2}$$

A_0, B_0, A, B stand for the initial and current concentrations of the two enantiomers of the starting material; e.e.(S) and e.e.(P) are the enantiomeric excess of the starting material and the product, c is the fractional conversion.

A previous investigation of yeast-mediated oxidations of 2-alkylcyclohexanones[15] showed these oxidations were highly enantioselective for substituents longer than methyl. Both ketones and lactones were isolated from the same transformation in essentially optically pure form. A similar trend was observed for the oxidation of 2-alkylcyclopentanones, although high enantioselectivities were not achieved until the chain length was four carbons and longer.

The oxidation of 2-allylcyclopentanone **3-3D** occurred with only a marginal preference for the *(S)*-ketone (E value 2.3). The selectivity is even lower than that of 2-methylcyclopentanone although **3-3D** has a side chain of three carbons. The fact that

this reaction is relatively fast suggests the low selectivity is caused by the *(S)*-enantiomer being a more competitive substrate, rather than the *(R)*-isomer being less favoured (Scheme 21).



Scheme 21

The low enantioselectivity in the oxidation of **3-3D** may look unattractive in asymmetric synthesis, however this reaction is relatively fast, and the double bond is not affected. Potentially this reaction could be coupled with kinetic resolution by lipase-mediated hydrolysis of the lactone and produce enantiomerically pure products with a double bond in the side chain suitable for further derivatizations.

In an independent series of reactions designed for the preparation and isolation of optically active lactones and ketones, yeast-catalyzed oxidations were stopped at about 50% conversion for short chain (C_1-C_3) substrates, or allowed to proceed until the ratio of the remaining ketone and the lactone product remained constant over an extended

period of time (4-8 hours depending on the substituents) for longer chains. As shown in Table 12, the time required to reach 50% conversion is linked to the length of the alkyl chain. While the oxidation of 2-butylcyclopentanone was complete in 8 hours, 2-undecylcyclopentanone required 72 hours.

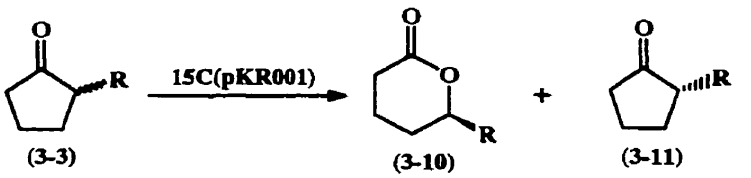
In general, rapid oxidations (24 hours or shorter) gave relatively pure products while during extended reaction periods significant amounts of yeast metabolites were always formed. Although these contaminants could be removed, extensive chromatographies did lower the isolated yields in some cases. The major metabolite was isolated and identified to be 2-phenyl-ethanol, but neither its biological function nor the mechanism for its generation in this strain are known.

Most of the substrates tested have sufficient solubility to allow the oxidations to proceed smoothly. It was only for 2-octyl and 2-undecylcyclopentanones that their low solubilities retarded the reactions. For these two compounds, a stoichiometric amount of γ-cyclodextrin was included in the culture medium to solubilize the substrates by formation of an inclusion complex.* [14] No significant toxicity effects were observed for any of the 2-substituted substrates.

---

* In a control experiment **3-3A** was stirred with β-cyclodextrin and an aliquote was extracted with hexane. GC analysis of the extract showed no significant change in the ratio of the two enantiomers.

Table 12: Baeyer-Villiger oxidations of 2-alkylcyclopentanones with 15C(pKR001)

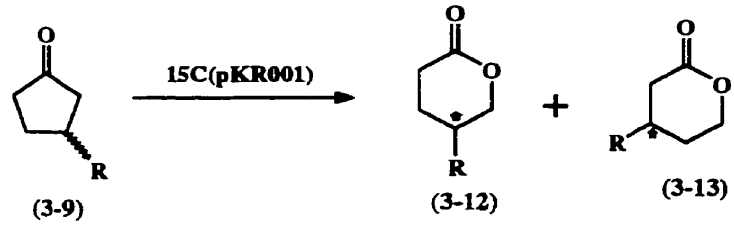


(3-3) (3-10) (3-11)

Substrate	R	Time (h)	E values[1]	Ketone **3-11** % yield[2] (e.e.)	$[\alpha]_D^{23}$ (in $CDCl_3$)	Lactone **3-10** % yield[2] (e.e.)	$[\alpha]_D^{23}$ (in $CDCl_3$)
3-3A	Me	11	3.6	34[3] (44)	-	36[2](32)	-
3-3B	Et	7	3.7	37[3] (46)	-	44[2] (39)	-
3-3C	*n*-Pr	7	30	21 (72)	-106 (c=0.68)[4]	51 (67)	-35.9 (c=0.97)[4]
3-3D	allyl	8	2.3	10 (51)	-105 (c=0.42)[4]	76 (32)	-25.1 (c=1.08)[4]
3-3E	*n*-Bu	9	>200	32 (>95)	-164.5 (c=0.78)	18 (>95)	-48.4 (c=0.83)
3-3G	*n*-hexyl	24	>200	42 (>95)	-112 (c0.70)	32 (>95)	-44.2 (c=0.98)
3-3H (γ-CD)	*n*-octyl	48	>200	14 (>95)	-113 (c=0.46)	25 (>95)	-35.0 (c=1.38)
3-3I (γ-CD)	*n*-undecyl	72	>200	37 (>95)	-70.5 (c=2.19)	39 (>95)	-12.0 (c=0.55)

1. E values were obtained from separate set of reactions run to completion.
2. Isolated yields after flash chromatography except where otherwise indicated.
3. Yields based on GC integrations.
4. The rotations have been corrected to 100% e.e.

Oxidations of 3-alkylcyclopentanones



A) R = Me
B) R = Et
C) R = *n*-Pr
D) R = allyl
E) R = *n*-Bu
F) R = *n*-Hex
G) R = *n*-Oct
H) R = *n*-Und

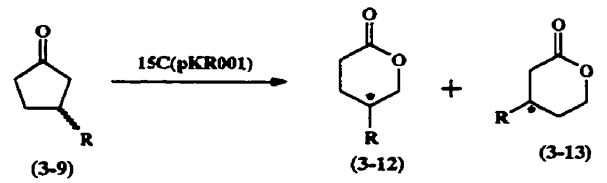
Scheme 22

Yeast-mediated oxidations of 2-substituted cyclopentanones gave strictly the proximal oxygen insertion products without any trace of the other regioisomer. This result is in agreement with the migratory aptitude rule established from chemical Baeyer-Villiger oxidations. Since the breakdown of the Criegee intermediate is a sigmatropic rearrangement to an electron-poor oxygen, the group that has higher electron density would have better nucleophilicity and therefore be the migrating group. The electron donating effect by hyperconjugation from a 2-alkyl substituent dictated that proximal** oxygen insertion product is the predominant product. For a 3-substituted substrate, however, there is no such electronic effect, therefore chemical oxidations with an organic peracid give mixtures of regioisomers in an almost 50:50 ratio regardless of the 3-substitutions. On the contrary, the regiochemistry of yeast-mediated oxidations depends on the length of the substituent (Scheme 22). For 3-methyl, ethyl, *n*-propyl and allyl substituted cyclopentanones (**3-9A** to **3-9D**), both regioisomers were observed.

** In the oxidation of cyclopentanones, *proximal* product means the oxygen is inserted between C_1 and C_2; *distal* product means the oxygen is inserted between C_1 and C_5

Interestingly, there is a distinct reversal in regio-preferences between 3-methyl and 3-propyl substituents. A distal oxygen insertion (**3-13**) was preferred for methyl and ethyl substituents, and a proximal oxygen insertion (**3-12**) for propyl. A similar trend was observed in the oxidation of 3-alkylcyclohexanones. For substrates with longer substituents, yeast-mediated oxidation gave regiospecifically the proximal oxygen insertion product (**3-12**). These results can provide useful insights into the shape and size of the active site in CHMO. Again, 3-allylcyclopentanone (**3-9D**) gave lower regioselectivity than what would be expected from its chain length.

Table 13: Baeyer-Villiger oxidations of 3-alkylcyclopentanone with 15C(pKR001)

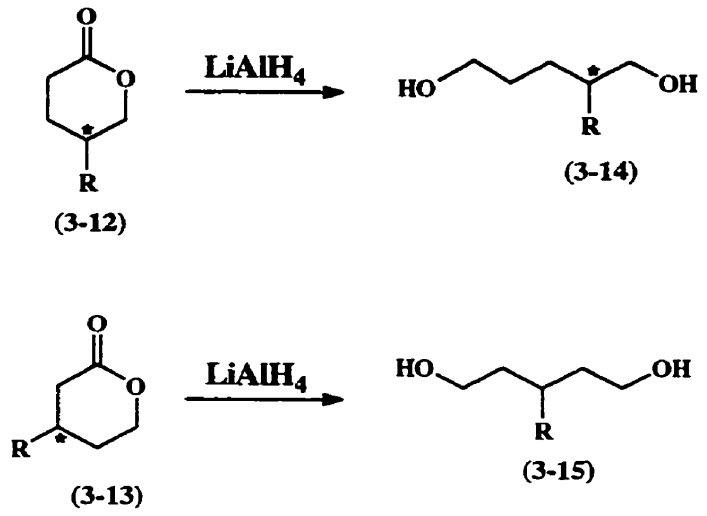


substrate 3-9		time (days)	conversion %[1]	3-9 %[2] (e.e.)	3-9 $[\alpha]_D^{23}$ (in $CDCl_3$)	lactones % (12+13)[2]	12:13 Ratio	3-12 e.e. %	3-13 e.e. %
A	Me	1	100	-	-	95	13 : 87	9	36
B	Et	1	86	10[1] 19)	-	80[1]	20 : 80	33	19
C	*n*-Pr	3	53	27 (13)	+18.2, c2.5	44	83 : 17	33	60
D	allyl	1.5	43	55[1](--)	-	42[1]	44 : 56	23	-
E	*n*-Bu	3	44	44 (25)	+15.8, c1.4	34	>99 : -[3]	38[4]	-
F	*n*-Hex	4.5	32	54 (29)	+18.0, c1.5	20	>99 : -[3]	60[4]	-
G	*n*-Oct	8	31	44 (8)	-	19	>99: -[3]	18[5]	-
H	*n*-Und	7	23	54 (16)	-	20	>99 : -[3]	54[5]	-
H[6]	*n*-Und	4	49	32 (24)	-	37	>99 : -[3]	25[5]	-

1. Based on GC analysis. 2. Isolated yields after flash chromatography except where otherwise indicated. 3. Not detected on a capillary GC column. 4. Estimated from the reduced products (**3-14**) which were resolved on the chiral GC column. 5. Calculated, based on the e.e. values for the remaining ketone. 6. The concentration of **3-9H** was reduced by half.

The regioselectivity of the yeast-mediated oxidations was determined from 2-D NMR spectra and were experimentally confirmed by reducing the lactones to diols as shown in

Scheme 23. The structures of asymmetric diols **3-14E** and **3-14F** unambiguously supported the regioselectivities assigned to the parent lactones. Because the enantioselectivity of these reactions was low for all substrates regardless of the substituents' chain length, no attempt was made to assign the absolute configurations to the isolated ketones and lactones. As a preparative method, a yeast-catalyzed oxidation of 3-alkylcyclopentanone would be of little interest but for the very high regioselectivities with substrates having chains of 4-carbons or longer. These ketones produce exclusively 5-alkyltetrahydropyran-2-ones, in a transformation that cannot be duplicated by organic peracids.



Scheme 23

Because 3-alkylcyclopentanones lack the electron donating effect as that in 2-substituted counterparts to assist the sigmatropic rearrangement during the breakdown of the Criegee intermediate,* they are poorer substrates for the Baeyer-Villiger

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* See the discussion on page 64.

oxidations. The time required for the reactions was much longer, and for substrates with long substituents the reactions were incomplete even after prolonged incubation periods. As in the 2-alkylcyclopentanone series, a solubility problem was encountered for 3-octyl and 3-undecylcyclopentanones. But unlike with the 2-substituted counterparts, the addition of cyclodextrins did not solve the problem. The substrates formed an insoluble complex with cyclodextrin and precipitated from the reaction medium; the conversion rate and isolated yield were even worse than in reactions without any additives. Both β- and γ-cyclodextrins were tested and were found unsuitable. Preliminary results indicated that yields may be improved by carrying out oxidations at lower substrate concentrations (**3-9H**, last entry in Table 13).

Model study for the oxidation of cyclopentanones

The corner model described in Chapter Two successfully predicted the stereochemistry of the Baeyer-Villiger products in the oxidation of 2-substituted cyclohexanones and cyclopentanones (Figure 13). The existence of a side wall also explains why 3-substituted cyclohexanones and cyclopentanones with long side chains can only produce the 1,2-oxygen insertion products.

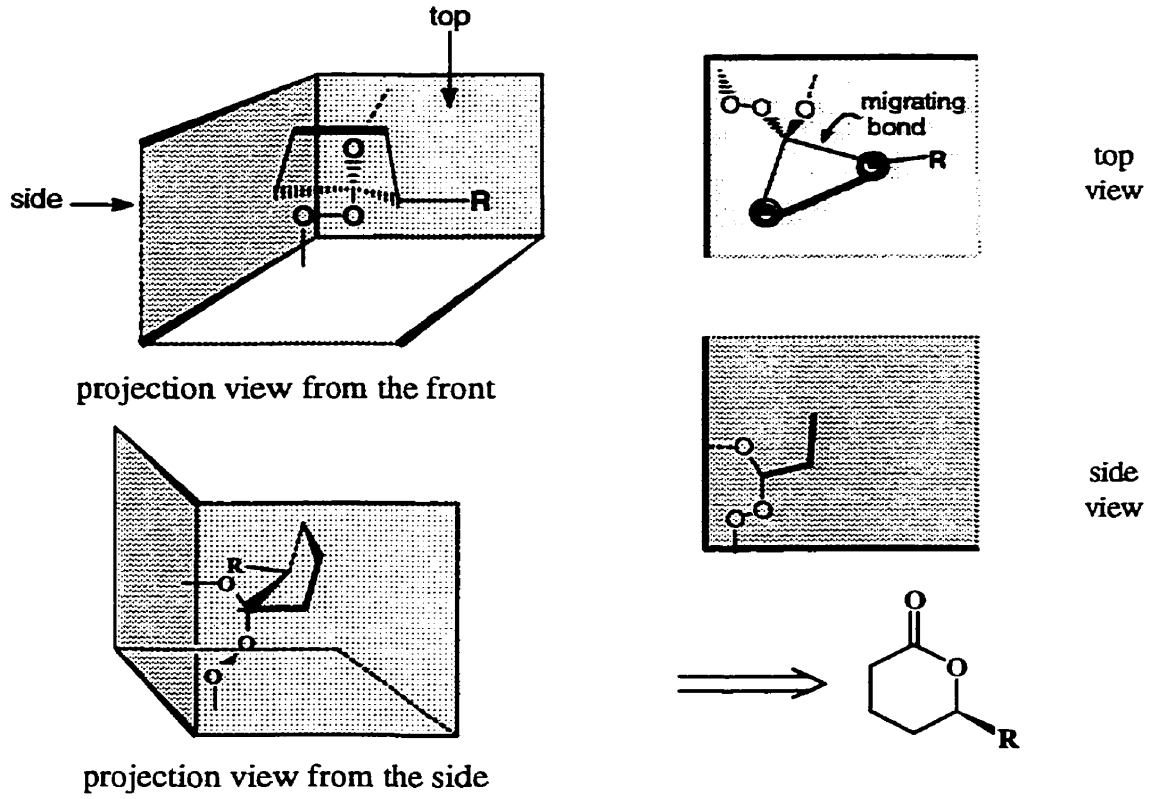

Figure 13: Prediction of stereochemistry in CHMO-catalyzed Baeyer-Villiger oxidations of cyclopentanones by a corner model

In order to rationalize the enantioselectivities in these reactions and the differences between 3-alkylcyclohexanones and 3-alkylcyclopentanones, it is necessary to look at the effect of pre-reaction conformational equilibrium,[16] which is illustrated in Figure 14.

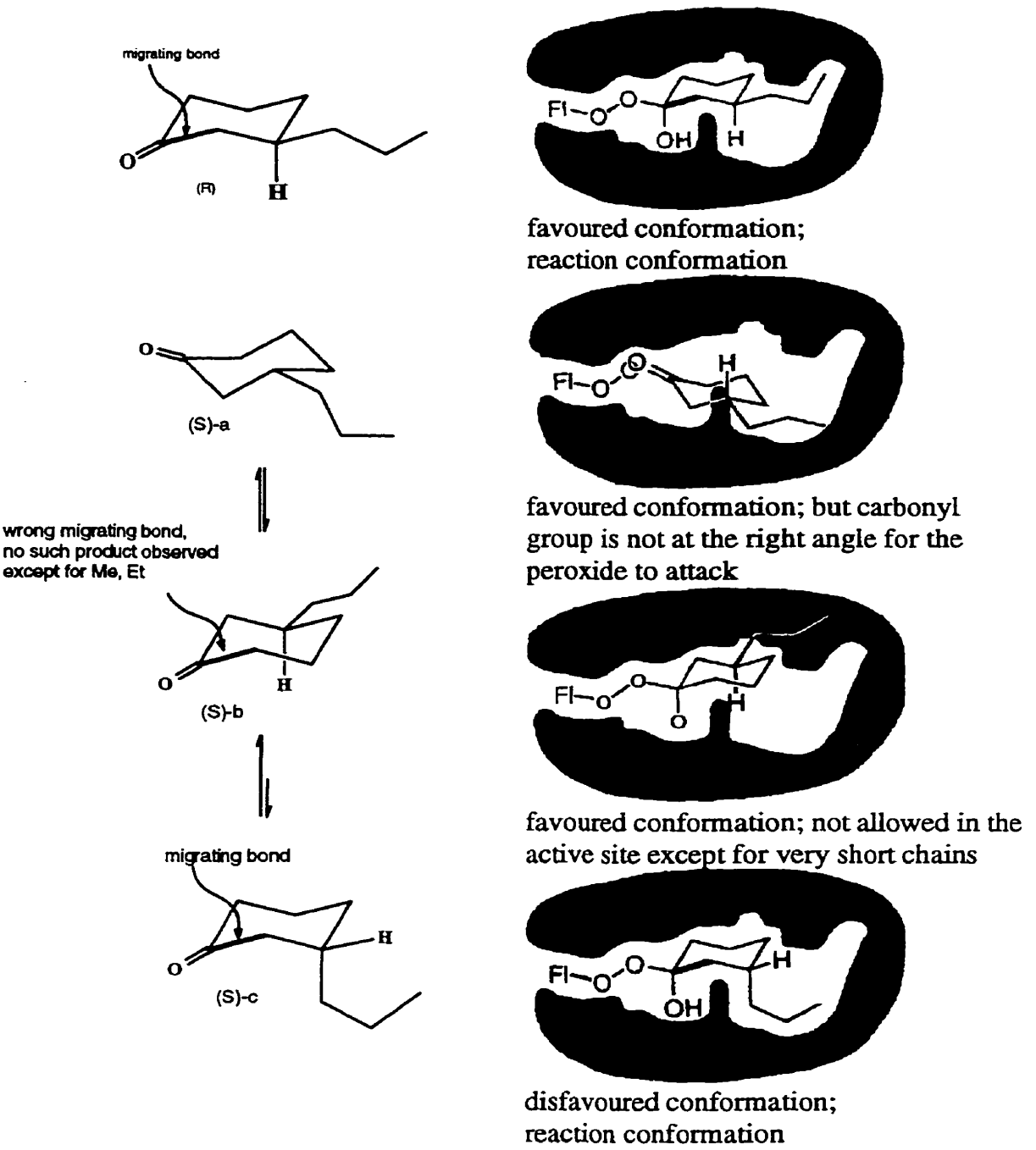

Figure 14: Conformational equilibria analysis

The shape of the active site of the enzyme was constructed by overlaying 2-, 3-, and 4-substituted cyclohexanones that had been successfully oxidized by the engineered yeast and orienting the molecules in such a way that their carbonyl groups and the migrating bonds overlapped; the aggregation of the side chains defined the contour of the active site. For a 3-*(R)* substrate, the thermodynamically favourable conformation with the side chain in an equatorial position fits well into the active site, therefore the only step that affects the reaction rate is the enzymatic oxidation. A 3-*(S)* substrate, however, has to assume the unfavourable 3-axial conformation in order to react. For its 3-equatorial conformer, either the carbonyl group is at a wrong angle for the peroxide attack, or the molecule does not fit into the active site.

Because the 3-*(S)*-substrate needs to adopt the unfavoured conformation before it can bind to the enzyme's active site, the total reaction rate difference between 3-*(R)* and 3-*(S)* substrates is a combined result of enantioselectivity of the enzyme and the conformational equilibrium of the substrate.* Similar analysis also applies to 3-substituted cyclopentanones.

The energy difference between an axial and equatorial 3-alkylcyclohexanone or cyclopentanone can be estimated using an MM2 optimization program.† The average energy difference for 3-alkylcyclohexanones was found to be about 1.8kcal/mol; while for a 3-alkylcyclopentanones it was only in the order of 0.1-0.2kcal/mol, because of the

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* For discussions justifying the suitability of pre-reaction equilibrium analysis in regard to Curtin-Hammett principle, see Chapter Two, page 32.

† ChemOffice® from Cambridge Softwares was used for the calculation.

flexibility of the 5-membered ring. From these values, the ratio of axial to equatorial conformers can be calculated to be around 20:1 for a 3-alkylcyclohexanone, and close to 1:1 for a 3-alkylcyclopentanone. Since the experimental results and all the models for CHMO suggest that the preferred substrate is oxidized with the side chain in an equatorial position, the higher population of the equatorial conformer serves to enhance the resolving power of CHMO.

Conclusion

Both 2- and 3-substituted cyclopentanones were successfully oxidized into the corresponding δ-valerolactones by recombinant yeast overexpressing cyclohexanone monooxygenase from *Acinetobacter* sp. NCIB 9871. 2-Alkyl substituted cyclopentanones were oxidized to give *(S)*-lactones and *(R)*-ketones in good yields and high enantioselectivities. The kinetic resolution was complete for substrates with side chains longer than *n*-butyl. Both the ketone and the lactone were isolated from a single reaction in optically pure forms. Proximal oxygen insertion products were the only regioisomers isolated in this reaction. Although the reaction and isolation procedures have not been fully optimized, these results demonstrated the potential utility of the oxidizing yeast as a general reagent for asymmetric synthesis of 6-substituted δ-valerolactones via enantioselective Baeyer-Villiger oxidations.

3-Alkyl cyclopentanones were oxidized to lactones with low to moderate enantioselectivities. Both proximal oxygen insertion and distal oxygen insertion products were observed for substrates with short substituents, and there is a reversal in

the regiopreference between methyl and propyl substrates. The regioselectivity for proximal oxygen insertion is complete for substituents longer than butyl. MM2 calculations revealed the importance of pre-reaction conformational equilibria in enzymatic reactions.

In the oxidation of 2-substituted cyclopentanones, addition of one equivalent of γ-cyclodextrin helped to solve the solubility problems and improve the yield; however this strategy was not suitable for 3-substituted substrates because of the precipitation of the substrate-cyclodextrin complex. Lower substrate concentrations appeared to improve the yields.

Both 2- and 3-allyl substituted cyclopentanones were oxidized with much lower selectivities than expected for their chain length. These results may provide useful information about the active site of CHMO. In addition, these reactions were relatively fast, and the double bond was not touched in the reaction, they can be still useful in organic synthesis.

Experimental

Synthesis of 2-alkylcyclopentanones

Anhydrous K_2CO_3 (5.5g, 40mmol), and acetone (20mL) were placed in a 100mL 3-neck round bottom flask equipped with a reflux condenser and a nitrogen balloon. After flushing the system 3 times with nitrogen, methyl cyclopentanone-2-carboxylate (**3-1**, 20mmol) and alkyl halide (20mmol) were added. The mixture was gently refluxed for

2 days with vigorous stirring until all starting material was consumed or a significant amount of by-products started to form as shown by GC. The mixture was cooled to room temperature and poured into ice water. The aqueous phase was extracted with 200mL ethyl acetate in 3 portions. The combined extracts were washed once with brine and dried over anhydrous Na_2SO_4. The solvent was removed on a rotary evaporator to gave 2-alkylketoester (**3-2**) as a pale yellow oil. The crude product was purified by flash chromatography on silica gel using a mixture of hexanes and ethyl acetate as the eluent. The chromatography was monitored by TLC using PMA[171] dipping solution for visualization.

The 2-alkylketoester (**3-2**, 10mmol) was dissolved in a mixture of glacial acetic acid (20mL) and HCl (6M, 10mL) and refluxed gently under nitrogen until all the starting material was consumed as shown by GC (usually between 4-6 hours). The reaction mixture was cooled to room temperature, poured into ice water and extracted with 150mL ethyl acetate or petroleum ether in 3 or 4 portions. Combined extracts were washed once with water and once with saturated $NaHCO_3$. After drying with anhydrous Na_2SO_4, the solvent was removed on a rotary evaporator to give the crude product, which was purified by vacuum distillation.

2-Ethylcyclopentanone **3-3B** **3-1** (2.5mL, 20mmol), ethyl bromide (2.0mL, 27mmol), and K_2CO_3 (5.5g, 40mmol) were reacted according to general procedures. Flash chromatography using 6:1 hexane and ethyl acetate as the eluent gave methyl 2-ethylcyclopentanone-2-carboxylate **3-2B** as a colourless oil, 2.15g (80%). IR ν_{max}(neat):

2960(s), 2870(w), 1750(vs), 1730(vs), 1260(s), 1160(s), 1060(s) cm^{-1}. ^1H NMR δ: 3.67(3H, s), 2.54-2.45(1H, m), 2.42-2.33(1H, m), 2.27-2.16(1H, m), 2.02-1.78(4H, m), 1.60(1H, dt, J_1=21.4, J_2=7.3), 0.85(3H, t, J=7.5) ppm. ^{13}C NMR δ: 214.9, 171.5, 60.9, 52.4, 38.1, 32.2, 26.9, 19.5, 9.2 ppm. **3-2B** was decarboxylated according to general procedures to give **3-3B** as a colourless oil. IR ν_{max}(neat): 2970(s), 2940(s), 2880(s), 1740(s), 1220(m), 1160(m) cm^{-1}. ^1H NMR: 2.27-2.10(2H, m), 2.08-2.00(1H, m), 1.98-1.80(3H, m), 1.76-1.64(1H, m), 1.52-1.42(1H, m), 1.31-1.18(1H, m), 0.87(3H, t, J=7.5) ppm. ^{13}C NMR: 214.0, 50.4, 38.2, 28.9, 22.5, 20.6, 11.8 ppm.

2-*n*-Propylcyclopentanone **3-3C** **3-1** (2.5mL, 20mmol), *n*-propyl iodide (2.2mL, 20mmol), and K_2CO_3 (5.5g, 40mmol) were reacted according to general procedures. Flash chromatography using 6:1 hexane and ethyl acetate as the eluent gave methyl 2-*n*-propylcyclopentanone-2-carboxylate **3-2C** as a colourless oil, 2.85g (77%). IR ν_{max}(neat): 2961(s), 2875(s), 1752(vs), 1727(vs), 1462(s), 1224(s) cm^{-1}. ^1H NMR δ: 3.64(3H, s), 2.47 (1H, m), 2.33 (1H, m), 2.19 (1H, m), 1.88 (4H, m), 1.48 (1H, m), 1.22 (2H, m), 0.85 (3H, t, J=7.2) ppm. ^{13}C NMR δ: 214.8, 171.4, 60.6, 52.4, 37.9, 36.1, 32.6, 19.5, 18.2, 14.3 ppm. **3-2C** (1.70g, 20 mmol) was decarboxylated according to general procedures. The crude product was vacuum distilled at 50°C/3mmHg to give **3-3C** as a colourless oil, 1.07g (92%). IR ν_{max} (neat): 2960(s), 2927(s), 2874(m), 1743(vs), 1157(s) cm^{-1}. ^1H NMR δ: 2.24(1H, m), 2.16(1H, m), 2.06(1H, m), 1.96(2H, m), 1.70(2H, m), 1.47(1H, m), 1.32(2H, m), 1.19(1H, m), 0.86(3H, t, J=7.2) ppm. ^{13}C NMR δ: 221.6, 48.8, 38.1, 31.8, 29.5, 20.71, 20.68, 13.9 ppm.

2-*n*-Butylcyclopentanone **3-3E** **3-1** (2.5mL, 20mmol), *n*-butyl iodide (3.0mL, 20mmol), and K_2CO_3 (5.5g, 40 mmol) were reacted according to general procedures. Flash chromatography using 6:1 hexane and ethyl acetate as the eluent gave methyl 2-butylcyclopentanone-2-carboxylate **3-2E** as a colourless oil, 2.80g (71%). IR ν_{max} (neat): 2957(vs), 2876(s), 1758(vs), 1723(vs), 1434(s), 1260(s), 1157(s) cm^{-1}. ^1H NMR δ: 3.64 (3H, s), 2.47 (1H, m), 2.34 (1H, m), 2.19(1H, m), 1.87 (4H, m), 1.50 (1H, m), 1.22 (3H, m), 1.13 (1H, m), 0.82 (3H, t, J=7.0) ppm. ^{13}C NMR δ: 214.8, 171.4, 60.5, 52.4, 37.9, 33.7, 32.6, 26.9, 22.9, 19.5, 13.7 ppm. **3-2E** (1.57g, 8mmol) was decarboxylated according to general procedures. The crude product was vacuum distilled at 80°C/4mmHg to give **3-3E** as a colourless oil, (0.98g, 88%). IR ν_{max} (neat): 2859(vs), 2930(vs), 2860(s), 1742(vs), 1465(s), 1456(s), 1154(m), 827(w) cm^{-1}. ^1H NMR δ: 2.10(2H, m), 2.08(1H, quintet, J=5.6), 1.96(2H, m), 1.72(2H, m), 1.50(1H, m), 1.24(5H, m), 0.90(3H, t, J=6.7) ppm. ^{13}C NMR δ: 221.6, 49.1, 38.1, 29.7, 29.5, 29.3, 22.6, 20.7, 13.9 ppm.

2-*n*-Octylcyclopentanone **3-3H** **3-1** (2.5mL, 20mmol), octyl bromide (2.6mL, 20mmol), and K_2CO_3 (5.5g, 40mmol) were reacted according to general procedures. Flash chromatography using 6:1 hexane and ethyl acetate as the eluent gave methyl 2-octylcyclopentanone-2-carboxylate **3-2H** as a colourless oil, 3.06g (60%). IR ν_{max} (neat): 2961(s), 2933(vs), 2856(s), 1753(vs), 1733(vs), 1460(m), 1235(m), 1151(m) cm^{-1}. ^1H NMR δ: 3.66(3H, s), 2.50(1H, m), 2.36(1H, m), 2.22(1H, m), 1.90(4H, m), 1.52(1H, m), 1.25(12H, m), 0.84(3H, t, J=7.0) ppm. ^{13}C NMR δ: 214.9, 171.5, 60.6, 52.4, 38.0, 34.0, 32.6, 31.8, 29.8, 29.3, 29.1, 24.8, 22.6, 19.6, 14.0 ppm. **3-2H** (2.75g,

11mmol) was decarboxylated according to general procedures. The crude product was vacuum distilled at 100°C/0.15mmHg to give **3-3H** as a colourless oil, (1.53g, 67%). IR ν_{max} (neat): 2957(vs), 2925(vs), 2856(vs), 1740(vs), 1466(m), 1153(m) cm^{-1}. ^1H NMR δ: 2.36-1.94(5H, m), 1.76(2H, m), 1.53(1H, m), 1.28(13H, m), 0.88(3H, t, J=6.7) ppm. ^{13}C NMR δ: 221.8, 49.2, 38.2, 31.9, 29.7, 29.6, 29.5, 29.3, 27.6, 22.7, 20.8, 14.1 ppm.

2-*n*-Undecylcyclopentanone **3-3I** **3-1** (2.5mL, 20mmol), undecyliodide (4.6mL, 20mmol), and K_2CO_3 (5.5g, 40mmol) were reacted according to general procedures. Flash chromatography using 6:1 hexane and ethyl acetate as the eluent gave methyl 2-undecylcyclopentanone-2-carboxylate **3-2I** as a colourless oil, 4.02g (70%). IR ν_{max} (neat): 2957(s), 2932(vs), 2851(s), 1758(vs), 1732(vs), 1461(s), 1227(s), 1161(s) cm^{-1}. ^1H NMR δ: 3.70(3H, s), 2.55(1H, m), 2.30(2H, m), 1.93(4H,m), 1.55(1H, m), 1.93(18H, m), 0.89(3H, t, J=6.8) ppm. ^{13}C NMR δ: 215.0, 171.6, 60.7, 52.5, 38.0, 34.1, 32.7, 31.9, 29.9, 29.62, 29.55, 29.4, 24.9, 22.7, 19.6, 14.1 ppm. **3-2I** (2.96g, 10mmol) was decarboxylated according to general procedures. The crude product was vacuum distilled at 96-100°C/0.02mmHg to give **3-3I** as a colourless oil, 1.96g (82%). IR ν_{max} (neat): 2961(vs), 2926(vs), 2854(vs), 1742(vs), 1467(s), 1154(s), 723(w) cm^{-1}. ^1H NMR δ: 2.37-1.93(5H, m), 1.75(2H, m), 1.52(1H, m), 1.26(19H, m), 0.88(3H, t, J=6.1) ppm. ^{13}C NMR δ: 221.9, 49.2, 38.2, 31.9, 29.6, 29.5, 29.4, 27.6, 22.7, 20.8, 14.1 ppm.

2-Allylcyclopentanone **3-3D** NaH (4.8g, 50% dispersion in mineral oil, 100mmol) was placed in a 150mL round bottom flask under nitrogen. After washing the NaH with

THF (30mL x 3), THF (100mL) was added, followed by cyclopentanone (8.4g, 100mmol, neat) and allyl bromide(12.1g, 100mmol, neat). After stirring overnight, the mixture was poured into ice water, and the aqueous layer was extracted with 200mL ethyl acetate in 3 portions. The combined extracts were washed once with HCl(0.5M) and dried over anhydrous Na_2SO_4. The solvent was removed on a rotary evaporator and the crude product was fractionally vacuum distilled at 68-70°C/13mmHg to give **3-3D** as a colourless oil (0.91g, 7%). IR ν_{max} (neat): 3075(m), 2967(s), 2881(s), 1743(vs), 1641(s), 1407(s), 1154(vs), 1006(m), 913(s), 815(m) cm^{-1}. ^1H NMR δ: 5.72 (1H, m), 5.06-4.95 (2H, m), 2.46 (1H, m), 2.26 (1H, m), 2.20-1.90 (5H, m), 1.75 (1H, m), 1.55 (1H, m) ppm. ^{13}C NMR δ: 220.5, 135.9, 116.3, 48.6, 38.1, 33.8, 28.9, 20.6 ppm.

Synthesis of 3-alkylcyclopentanones

General procedures for the preparation of Grignard reagents

Magnesium turnings (1.2g, 50mmol) and a small crystal of iodine were placed in a 100mL 3-neck round bottom flask equipped with a reflux condenser, a thermometer and a pressure equalizing dropping funnel. The system was flame dried and flushed 3 times with nitrogen. Alkyl bromide or iodide (50mmol) was dissolved in 30mL of ether and placed in the dropping funnel. A small portion of *ca.* 3-5 mL was added to the flask. The flask was gently warmed with a heat gun until the reaction started. At that point, the remaining alkyl halide was added at a rate necessary to maintain vigorous but controllable reflux. After the addition, the reaction mixture was stirred for

1 hour at room temperature, then the Grignard reagent was transferred into a sealed, nitrogen flushed bottle.

General procedures for Michael additions

LiI (1.34g, 10mmol, vacuum dried at 100°C/0.01mmHg for 24h.) and CuI (1.9g, 10mmol, recrystalized from KI[18] and vacuum dried at 100°C/0.01mmHg for 12h.) were placed in a 100mL 3-neck round bottom flask equipped with a thermometer and a pressure equalizing dropping funnel. The system was flame dried and was flushed 3 times with nitrogen. THF (40mL) was added and the mixture was stirred at room temperature until a clear solution was obtained. The reaction flask was then cooled to -78°C and the previously prepared Grignard reagent (20mL, 20mmol) was added via a syringe at such a rate that the reaction temperature remained below -70°C. The mixture was stirred for 30 minutes at -80°C, then TMSCl (1.3mL neat, 10mmol) followed by 2-cyclopentene-1-one (0.9mL in 10mL THF, 10mmol) were added over a period of 10 minutes. The mixture was vigorously stirred for 40 minutes, during which time the temperature was raised to between -40 and -30°C. The reaction was quenched with HCl (20mL, 2M) and extracted with 150mL ethyl acetate in 3 portions. The combined extracts were washed once with 0.5M HCl, twice with NH_4Cl/NH_3, once with saturated NH_4Cl, and dried over anhydrous Na_2SO_4. The solvent was removed on a rotary evaporator, and the residue was purified by vacuum distillation or flash chromatography on silica gel using mixtures of hexane and ethyl acetate as the eluents.

3-Ethylcyclopentanone **3-9B** was prepared according to general procedures. The crude product was vacuum distilled at 60-80°C/40mmHg, to give **3-9B** as a colourless oil

(0.87g, 78%). IR ν_{max} (neat): 2964(m), 2930(m), 2877(m), 1747(vs), 1158(m) cm^{-1}. ^1H NMR δ: 2.33(1H, m), 2.24(1H, m), 2.16-1.98 (3H, m), 1.75(1H, m), 1.41(3H, sextet, J=7.3), 0.90(3H, t, J=7.3) ppm. ^{13}C NMR δ: 219.9, 44.9, 38.9, 38.5, 29.1, 28.4, 12.2 ppm.

3-*n*-Propylcyclopentanone **3-9C** was prepared according to general procedures. The crude product was vacuum distilled at 80°C/12mmHg, to give **3-9C** as a colourless oil (0.78g, 62%). IR ν_{max} (neat): 2962(vs), 2928(s), 2876(m), 1744(vs), 1467(m), 1404(m), 1157(s) cm^{-1}. ^1H NMR δ: 2.34(1H, m), 2.24(1H, m), 2.10(3H, m), 1.75(1H, m), 1.45(1H, m), 1.35 (4H, m), 0.88(3H, t, J=6.8) ppm. ^{13}C NMR δ: 220.0, 45.2, 38.5, 37.9, 36.9, 29.5, 20.9, 14.1 ppm.

3-Allylcyclopentanone **3-9D** was prepared according to general procedures. The crude product was purified by flash chromatography on silica gel using 6:1 hexane and ethyl acetate as the eluent to give **3-9D** as a yellow oil (1.15g, 93%). IR ν_{max} (neat): 3079(w), 2961(w), 2902(w), 1743(vs), 1405(w), 1156(m), 916(m) cm^{-1}. ^1H NMR δ: 5.74(1H, m), 5.04-4.95 (2H, m), 2.36-2.16(3H, m), 2.16-2.04(4H, m), 1.80 (1H, qd, J_1=8.8, J_2=1.4), 1.52(1H, m) ppm. ^{13}C NMR δ: 219.3, 136.1, 116.2, 44.5, 39.4, 38.2, 36.5, 28.8 ppm.

3-*n*-Butylcyclopentanone **3-9E** was prepared according to general procedures, but using BuLi (9mL of 2.5M in pentane, 22mmol) instead of the Grignard reagent. The crude product was vacuum distilled at 110-120°C/6mmHg, to give **3-9E** as a colourless oil (0.51g, 36%). IR ν_{max} (neat): 2958(s), 2924(s), 2859(m), 1743(vs), 1156(m) cm^{-1}. ^1H NMR δ: 2.32(1H, m), 2.23(1H, m), 2.10(3H, m), 1.75(1H, m), 1.51-1.34(3H, m),

1.27(4H, m), 0.86(3H, t, J=6.8) ppm. ^{13}C NMR δ: 220.0, 45.3, 38.5, 37.1, 35.3, 34.9, 30.0, 29.5, 22.7, 14.0 ppm.

3-*n*-Hexylcyclopentanone **3-9F** was prepared according to general procedures. The crude product was vacuum distilled at 80°C/3mmHg to give **3-9F** as a colourless oil (1.24g, 74%). IR ν_{max} (neat): 2956(s), 2927(vs), 1744(vs), 1159(s) cm^{-1}. ^{1}H NMR δ: 2.34(1H, m), 2.25(1H, m), 2.11(3H, m), 1.76(1H, qd, J_1=9.6, J_2=1.2), 1.52-1.36 (3H, m), 1.26(8H, m), 0.86(3H, t, J=6.8) ppm. ^{13}C NMR δ: 220.0, 45.3, 38.5, 37.2, 35.7, 31.8, 29.5, 29.3, 27.8, 22.6, 14.0 ppm.

3-*n*-Octylcyclopentanone **3-9G** was prepared according to general procedures. The crude product was vacuum distilled at 80-90°C/0.05mmHg to give **3-9G** as a colourless oil (1.49g, 82%). IR ν_{max} (neat): 2959(vs), 2928(vs), 2855(vs), 1746(vs), 1461(s), 1161(s), 722(w) cm^{-1}. ^{1}H NMR δ: 2.34(1H, m), 2.25(1H, m), 2.12(3H, m), 1.76(1H, qd, J_1=9.6, J_2=1.2), 1.52-1.36 (3H, m), 1.25(12H, m), 0.85(3H, t, J=6.8) ppm. ^{13}C NMR δ: 220.0, 45.3, 38.5, 37.2, 35.7, 31.8, 29.7, 29.5, 29.3, 27.9, 22.6, 14.1 ppm.

3-*n*-Undecylcyclopentanone **3-9H** was prepared according to general procedures. The crude product was purified by flash chromatography on silica gel using 20:1 hexane and ethyl acetate as the eluent to give **3-9H** as a colourless oil (0.67g, 28%). IR ν_{max} (neat): 2957(s), 2923(vs), 2854(vs), 1745(vs), 1465(m), 1157(m), 723(w) cm^{-1}. ^{1}H NMR δ: 2.34(1H, m), 2.25(1H, m), 2.12(3H, m), 1.76(1H, qd, J_1=9.6, J_2=1.2), 1.52-1.35 (3H, m), 1.25(18H, m), 0.84(3H, t, J=6.8) ppm. ^{13}C NMR δ: 220.0, 45.3, 38.5, 37.2, 35.7, 31.9, 29.7, 29.63, 29.60, 29.57, 29.5, 29.3, 27.9, 22.7, 14.1 ppm.

1.27(4H, m), 0.86(3H, t, J=6.8) ppm. ^{13}C NMR δ: 220.0, 45.3, 38.5, 37.1, 35.3, 34.9, 30.0, 29.5, 22.7, 14.0 ppm.

3-*n*-Hexylcyclopentanone **3-9F** was prepared according to general procedures. The crude product was vacuum distilled at 80°C/3mmHg to give **3-9F** as a colourless oil (1.24g, 74%). IR ν_{max} (neat): 2956(s), 2927(vs), 1744(vs), 1159(s) cm^{-1}. ^{1}H NMR δ: 2.34(1H, m), 2.25(1H, m), 2.11(3H, m), 1.76(1H, qd, J_1=9.6, J_2=1.2), 1.52-1.36 (3H, m), 1.26(8H, m), 0.86(3H, t, J=6.8) ppm. ^{13}C NMR δ: 220.0, 45.3, 38.5, 37.2, 35.7, 31.8, 29.5, 29.3, 27.8, 22.6, 14.0 ppm.

3-*n*-Octylcyclopentanone **3-9G** was prepared according to general procedures. The crude product was vacuum distilled at 80-90°C/0.05mmHg to give **3-9G** as a colourless oil (1.49g, 82%). IR ν_{max} (neat): 2959(vs), 2928(vs), 2855(vs), 1746(vs), 1461(s), 1161(s), 722(w) cm^{-1}. ^{1}H NMR δ: 2.34(1H, m), 2.25(1H, m), 2.12(3H, m), 1.76(1H, qd, J_1=9.6, J_2=1.2), 1.52-1.36 (3H, m), 1.25(12H, m), 0.85(3H, t, J=6.8) ppm. ^{13}C NMR δ: 220.0, 45.3, 38.5, 37.2, 35.7, 31.8, 29.7, 29.5, 29.3, 27.9, 22.6, 14.1 ppm.

3-*n*-Undecylcyclopentanone **3-9H** was prepared according to general procedures. The crude product was purified by flash chromatography on silica gel using 20:1 hexane and ethyl acetate as the eluent to give **3-9H** as a colourless oil (0.67g, 28%). IR ν_{max} (neat): 2957(s), 2923(vs), 2854(vs), 1745(vs), 1465(m), 1157(m), 723(w) cm^{-1}. ^{1}H NMR δ: 2.34(1H, m), 2.25(1H, m), 2.12(3H, m), 1.76(1H, qd, J_1=9.6, J_2=1.2), 1.52-1.35 (3H, m), 1.25(18H, m), 0.84(3H, t, J=6.8) ppm. ^{13}C NMR δ: 220.0, 45.3, 38.5, 37.2, 35.7, 31.9, 29.7, 29.63, 29.60, 29.57, 29.5, 29.3, 27.9, 22.7, 14.1 ppm.

General procedures for yeast-mediated Baeyer-Villiger oxidations

Ketone substrate (100μL) suspended in YPG (100mL) in a 250mL baffled Erlenmeyer flask was shaken at 30°C at 250rpm for 10-30 minutes to obtain a uniform dispersion. One vial of frozen yeast stock (contains *ca.* 0.2g wet yeast) was thawed and added to the reaction flask. The flask was shaken at 30°C, 250rpm and the reaction was monitored by GC. At approximately 50% conversion (or when the ratio of products remained constant over a 4-hour period), the reaction mixture was centrifuged and the yeast pellet was suspended in 50mL distilled water and extracted twice with ethyl acetate (100mL). The aqueous layer was saturated with NaCl and extracted with 200mL ethyl acetate in 3 or 4 portions. The combined extracts were washed once with brine and dried over anhydrous Na_2SO_4 or $MgSO_4$. The solvent was removed on a rotary evaporator and the residue was purified by flash chromatography on silica gel.

6-Methyltetrahydropyran-2-one **3-10A** [19] prepared as described in the general procedures was purified by flash chromatography on silica gel using 3:1 hexane and acetone as the eluent. IR ν_{max} (neat): 2973(m), 2934(m), 1736(vs), 1249(vs), 1071(s) cm^{-1}. ^1H NMR δ: 4.45(1H, m), 2.58(1H, m), 2.45(1H, m), 1.98-1.80(3H, m), 1.54(1H, m), 1.38(3H, d, J=6.3) ppm. ^{13}C NMR δ: 171.7, 76.8, 29.4, 29.0, 21.5, 18.4 ppm. MS m/e M^+ 114 (14%), 99(12), 71(25), 70(100), 55(39).

6-Ethyltetrahydropyran-2-one **3-10B** [20] prepared as described in the general procedures was purified by flash chromatography on silica gel using 1:1 hexane and diethyl ether as the eluent. IR ν_{max} (neat): 2947(s), 1717(s), 1468(m), 1387(m), 1343(m), 1241(s),

1080(m), 1036(m) cm^{-1}. ^1H NMR δ: 4.28(1H, m), 2.58(1H, m), 2.45(1H, m), 1.97-1.47 (6H, m), 1.00 (3H, t, J=7.7) ppm. ^{13}C NMR δ: 172.0, 81.8, 76.3, 29.5,28.7, 27.3, 18.5, 9.3 ppm.

6-*n*-Propyltetrahydropyran-2-one **3-10C** [19] prepared as described in the general procedures was purified by flash chromatography on silica gel using 1:1 hexane and diethyl ether as the eluent. IR ν_{max} (neat): 2959(s), 2935(s), 2874(m), 1732(vs), 1243(s), 1166(m) cm^{-1}. ^1H NMR δ: 4.27(1H, m), 2.34(1H, m), 2.40(1H, m), 1.92-1.74(3H, m), 1.66(1H, m), 1.50(3H, m), 1.39(1H, m), 0.90(3H, t, J=7.2) ppm. ^{13}C NMR δ: 171.9, 80.3, 53.4, 37.8, 29.4, 27.7, 18.4, 18.1, 13.8 ppm. MS m/e M^+ 142 (1.4 %), 99(100), 71(50), 70(35), 55(26).

6-Allyltetrahydropyran-2-one **3-10D** [21] prepared as described in the general procedures was purified by flash chromatography on silica gel using 3:1 hexane and diethyl ether, followed by 1:1 hexane and diethyl ether as the eluent. IR ν_{max} (neat): 3078(w), 2954(m), 2917(m), 1734(vs), 1242(s), 1048(s), 925(m) cm^{-1}. ^1H NMR δ: 5.78(1H, m), 5.14(2H, m), 4.32(1H, m), 2.55(1H, m), 2.50-2.40(2H, m), 2.36(1H, m), 1.89(2H, m), 1.80(1H, m), 1.51(1H, m) ppm. ^{13}C NMR δ: 171.6, 132.6, 118.5, 79.7, 40.0, 29.4, 27.1, 18.4 ppm. MS m/e (M^+-45) 99(100%), 71(66), 55(29).

6-*n*-Butyltetrahydropyran-2-one **3-10E** [19] prepared as described in the general procedures was purified by flash chromatography on silica gel using 3:1 hexane and diethyl ether, followed by 1:1 hexane and diethyl ether as the eluent. IR ν_{max} (neat): 2957(s), 2933(s), 2863(m), 1731(vs), 1244(m), 1166(m) cm^{-1}. ^1H NMR δ: 4.26(1H, m), 2.55(1H, m), 2.42(1H, m), 1.94-1.76(3H, m), 1.67(1H, m), 1.60-1.40(3H, m), 1.32(3H,

m), 0.88(3H, t, J=7.2) ppm. ^{13}C NMR δ: 172.0, 80.6, 35.5, 29.4, 27.8, 27.0, 22.5, 18.5, 13.9 ppm. MS m/e M^{+} 156 (1%), 99(100), 71(46), 70(27), 55(27).

6-*n*-Hexyltetrahydropyran-2-one **3-10F** prepared as described in the general procedures was purified by flash chromatography on silica gel using 3:1 hexane and diethyl ether, followed by 1:1 hexane and diethyl ether as the eluent. IR ν_{max} (neat): 2960(s), 2934(s), 2855(m), 1743(vs), 1242(s), 1052(m) cm^{-1}. ^{1}H NMR δ: 4.25(1H, m), 2.55(1H, m), 2.42(1H, m), 1.92-1.74(3H, m), 1.67(1H, m), 1.50(3H, m), 1.25(7H, m), 0.84(3H, t, J=7.0) ppm. ^{13}C NMR δ: 171.9, 80.6, 35.8, 31.6, 29.4, 29.0, 27.7, 24.8, 22.5, 18.5, 14.0 ppm. MS m/e M^{+} 184 (1 %), 99(100), 71(40), 70(29), 55(27).

6-*n*-Octyltetrahydropyran-2-one **3-10G** [19] prepared as described in the general procedures was purified by flash chromatography on silica gel using 3:1 hexane and diethyl ether, followed by 1:1 hexane and diethyl ether as the eluent. IR ν_{max} (neat): 2927(s), 2861(m), 1743(vs), 1236(s), 1045(m) cm^{-1}. ^{1}H NMR δ: 4.26(1H, m), 2.55(1H, m), 2.42(1H, m), 1.92-1.75(3H, m), 1.68(1H, m), 1.50(3H, m), 1.26(11H, m), 0.86(3H, t, J=7.2) ppm. ^{13}C NMR δ: 172.0, 80.6, 35.8, 31.8, 29.5, 29.44, 29.43, 29.41, 29.2, 27.8, 24.9, 22.6, 18.5, 14.1 ppm. MS m/e (M^{+} - 18) 194(1 %), 99(100), 71(36), 70(29), 55(31).

6-*n*-Undecyltetrahydropyran-2-one **3-10H** [22] prepared as described in the general procedures was purified by flash chromatography on silica gel using 10:1 hexane and acetone as the eluent. IR ν_{max} (neat): 2928(vs), 2855(s), 1741(s), 1463(m), 1246(s), 1047(s) cm^{-1}. ^{1}H NMR δ: 4.24 (1H, m), 2.55 (1H, m), 2.42 (1H, m), 1.94-1.75 (3H, m), 1.66 (1H, m), 1.60-1.40 (3H, m), 1.33-1.18 (17H, m), 0.86 (3H, t, J=6.4) ppm. ^{13}C

NMR δ: 171.9, 80.6, 35.8, 31.9, 29.61, 29.59, 29.54, 29.45, 29.40, 29.3, 27.8, 24.9, 22.7, 18.5, 14.1 ppm. MS m/e (M^+-18)236(2 %), 99(100), 71(41), 70(36), 55(45).

4- and 5-Methyltetrahydropyran-2-one **3-13A** and **3-12A** Oxidation of **3-9A** according to general procedures gave an unseparatable mixture of two lactone regioisomers in the ratio of 87:13. Characteristic spectral data were recorded from the mixture. IR ν_{max} (neat): 2956(m), 2929(m), 2908(w), 2873(w), 1745(vs), 1731(vs), 1400(m), 1228(s), 1089(s) cm^{-1}. **3-13A**: ^1H NMR δ: 4.43(1H, m), 4.27(1H, m), 1.07(3H, d, J=6.2) ppm. ^{13}C NMR δ: 171.2, 68.5, 38.1, 30.5, 26.4, 21.3 ppm. MS m/e M^+ 114(31%), 70(24), 56(56), 55(100). **3-12A**: ^1H NMR δ: 4.32(1H, dd, J_1=4.6, J_2=2.2), 3.91(1H, t, J=10.1), 3.86(1H, t, J=6.7), 1.01(3H, d, J=6.7) ppm. ^{13}C NMR δ: 171.2, 74.8, 39.1, 29.0, 27.4, 16.4 ppm. MS m/e M^+ 114(15%), 84(25), 70(31), 56(100), 55(45).

4- and 5-Ethyltetrahydropyran-2-one **3-13B** and **3-12B** Oxidation of **3-9B** according to general procedures gave an unseparatable mixture of two lactone regioisomers in the ratio of 80:20. Characteristic spectral data were assigned from the mixture. IR ν_{max} (neat): 2967(m), 2927(m), 1743(vs), 1262(m), 1223(m), 1071(m) cm^{-1}. **3-13B**: ^1H NMR δ: 4.39(1H, m), 4.22(1H, td, J_1=10.4 , J_2=3.6), 0.91(3H, t, J=7.3) ppm. ^{13}C NMR δ: 171.5, 68.5, 36.3, 33.1, 28.9, 28.5, 10.9 ppm. **3-12B**: ^1H NMR δ: 4.33(1H, ddd, J_1=11.2, J_2=8.8, J_3=2.0), 3.93(1H, dd, J_1=11.1, J_2=9.7), 0.87(3H, t, J=7.3) ppm. ^{13}C NMR δ: 171.5, 73.4, 34.4, 25.1, 24.5, 22.4, 11.3 ppm. GC MS did not resolve the two lactones. MS m/e M^+ 128(4%), 99(100), 71(), 70(84), 56(30), 55(33).

4- and 5-*n*-Propyltetrahydropyran-2-one **3-13C** and **3-12C** Oxidation of **3-9C** according to general procedures gave an unseparatable mixture of two lactone

regioisomers in the ratio of 17:83. Characteristic spectral data were recorded from the mixture. IR ν_{max} (neat): 2960(s), 2927(s), 2875(m), 1743(vs), 1460(w), 1407(w), 1190(m), 1052(m) cm^{-1}. **3-13C**: ^{1}H NMR δ: 4.37(1H, m), 4.21(1H, m) ppm. ^{13}C NMR δ: 171.57, 68.5, 38.3, 36.5, 31.1, 19.5, 18.7, 13.9 ppm. MS m/e (M^+ - 43) 99(82%), 84(34), 70(57), 69(68), 56(100), 55(95). **3-12C**: ^{1}H NMR δ: 4.30(1H, m), 3.91(1H, m) ppm. ^{13}C NMR δ: 171.62, 73.6, 33.6, 32.5, 29.0, 25.4, 19.9, 14.0 ppm. MS m/e M^+ 142(5%), 84(100), 70(33), 69(32), 56(51), 55(74).

4- and 5-Allyltetrahydropyran-2-one **3-13D** and **3-12D** Oxidation of **3-9D** according to general procedures gave an unseparatable mixture of two lactone regioisomers in the ratio of 56:44. Characteristic spectral data were recorded from the mixture. IR ν_{max} (neat): 3030(w), 2924(s), 2864(m), 1726(vs), 1643(w), 1453(m), 1252(s), 1169(s), 1051(m) cm^{-1}. **3-13D**: ^{1}H NMR δ: 4.39(1H, m), 4.24(1H, m), 2.64(1H, m), 2.50(1H, dd, J_1= 9.6, J_2= 7.0) ppm. ^{13}C NMR δ: 171.2, 134.5, 117.8, 68.4, 40.2, 36.1, 31.2, 28.4 ppm. **3-12D**: ^{1}H NMR δ: 4.32(1H, m), 3.96(1H, dd, J_1=11.3, J_2=9.1), 2.68(1H, dd, J_1=5.5, J_2=1.4), 2.59(1H, dd, J_1=6.8, J_2=4.4) ppm. All other ^{1}H NMR signals overlapped. ^{13}C NMR δ: 171.4, 134.6, 117.6, 73.1, 35.9, 32.4, 29.0, 25.1 ppm.

5-*n*-Butyltetrahydropyran-2-one **3-13E** prepared as described in general procedures was purified by flash chromatography on silica gel using 10:1 petroleum ether and acetone, followed by 3:1 petroleum ether and acetone as the eluent. IR ν_{max} (neat): 2960(m), 2858(w), 1740(s), 1457(w), 1182(m), 1052(w) cm^{-1}. ^{1}H NMR δ: 4.30(1H, ddd, J_1=11.1, J_2=4.6, J_3=2.1), 3.93(1H, dd, J_1=10.9, J_2=9.7), 2.59(1H, m), 2.46(1H, m), 1.98(1H, m), 1.87(1H, m), 1.50 (1H, m), 1.28(4H, m), 0.87(3H, t, J=6.8) ppm. ^{13}C

NMR δ: 171.6, 73.7, 32.8, 31.2, 29.0, 28.9, 25.5, 22.7, 13.9 ppm. MS m/e M^+ 156(2%), 98(100), 84(33), 70(65), 69(56), 56(72), 55(79).

5-*n*-Hexyltetrahydropyran-2-one **3-13F** prepared as described in general procedures was purified by flash chromatography on silica gel using 10:1 petroleum ether and acetone, followed by 3:1 petroleum ether and acetone as the eluent. IR ν_{max} (neat): 2960(s), 2927(vs), 2861(s), 1743(vs), 1460(m), 1242(m), 1183(s), 1058(m) cm^{-1}. ^1H NMR δ: 4.31(1H, ddd, J_1=11.1, J_2=4.4, J_3=1.9), 3.92(1H, dd, J_1=11.1, J_2=9.7), 2.59(1H, m), 2.47(1H, m), 1.98(1H, m), 1.86(1H, m), 1.50(1H, m), 1.20-1.36 (10H, m), 0.86(3H, t, J=7.2) ppm. ^{13}C NMR δ: 171.5, 73.7, 32.8, 31.6, 31.5, 29.3, 29.1, 26.7, 25.5, 22.6, 14.0 ppm. MS m/e (M^+-30) 154(1%), 128(23), 98(100), 84(30), 70(50), 69(50), 56(38), 55(71).

5-*n*-Octyltetrahydropyran-2-one **3-13G** prepared as described in general procedures was purified by flash chromatography on silica gel using 10:1 petroleum ether and acetone, followed by 3:1 petroleum ether and acetone as the eluent. IR ν_{max} (neat): 2937(s), 2853(s), 1742(vs), 1464(w), 1186(m), 725(w) cm^{-1}. ^1H NMR δ: 4.32(1H, ddd, J_1=11.1, J_2=4.6, J_3=2.0), 3.93(1H, dd, J_1=11.1, J_2=9.7), 2.60(1H, m), 2.48(1H, m) 0.86(3H, t, J=6.7) ppm. ^{13}C NMR δ: 171.6, 73.7, 32.8, 31.8, 31.5, 29.6, 29.4, 29.2, 26.8, 25.5, 22.7, 22.4, 14.1 ppm. MS m/e M^+ 212(1%), 150(33), 128(22), 98(100), 97(48), 83(47), 70(43), 69(48), 55(72).

5-*n*-Undecyltetrahydropyran-2-one **3-13H** prepared as described in the general procedures was purified by flash chromatography on silica gel using 10:1 petroleum ether and acetone, followed by 3:1 petroleum ether and acetone as the eluent. IR ν_{max}

(neat): 2937(vs), 2853(s), 1742(s), 1464(m), 1171(s), 725(w) cm^{-1}. ^1H NMR δ: 4.32(1H, ddd, J_1=11.1, J_2=4.6, J_3=1.9), 3.93(1H, dd, J_1=11.1, J_2=9.7), 2.60(1H, m), 2.48(1H, m), 0.86(3H, t, J=6.7) ppm. ^{13}C NMR δ: 171.7, 73.7, 40.2, 36.9, 32.8, 31.9, 31.7, 31.5, 31.0, 29.9, 29.7, 29.6, 29.4, 26.7, 22.7, 14.1 ppm. MS m/e M^+ 254(3%), 192(100), 141(38), 111(57), 98(89), 83(73), 69(66), 55(95).

2-*n*-Butyl-1,5-pentanediol **3-14E** A 5mL round bottom flask containing $LiAlH_4$ (20mg) was flushed with nitrogen. THF (1mL) was added via a syringe and the mixture was stirred at 0°C for 10min. 5-Butyltetrahydropyran-2-one **3-13E** (5mg in 100μL $CDCl_3$) was injected and the mixture was stirred for another 30 minutes at this temperature. The reaction was quenched by injecting 1mL saturated tartaric acid solution and was extracted with 15mL $CHCl_3$. The organic extract was dried over anhydrous Na_2SO_4 and the solvent was removed on a rotary evaporator. GC analysis of the sample gave the ratio of the two enantiomers present in the original lactonic mixture. IR: ν_{max} (neat): 3630(m), 3370(br, vs), 2930(vs), 2860(vs), 1710(m), 1465(s), 1450(s), 1030(br, s) cm^{-1}. ^1H NMR δ: 3.65(2H, t, J=6.4), 3.56(2H, qd, J_1=13.2, J_2=5.1), 1.7-1.2 (15H, m), 0.87(3H, t, J=6.3) ppm. ^{13}C NMR δ: 65.5, 63.2, 40.2, 30.7, 29.8, 29.1, 26.9, 23.1, 14.1 ppm.

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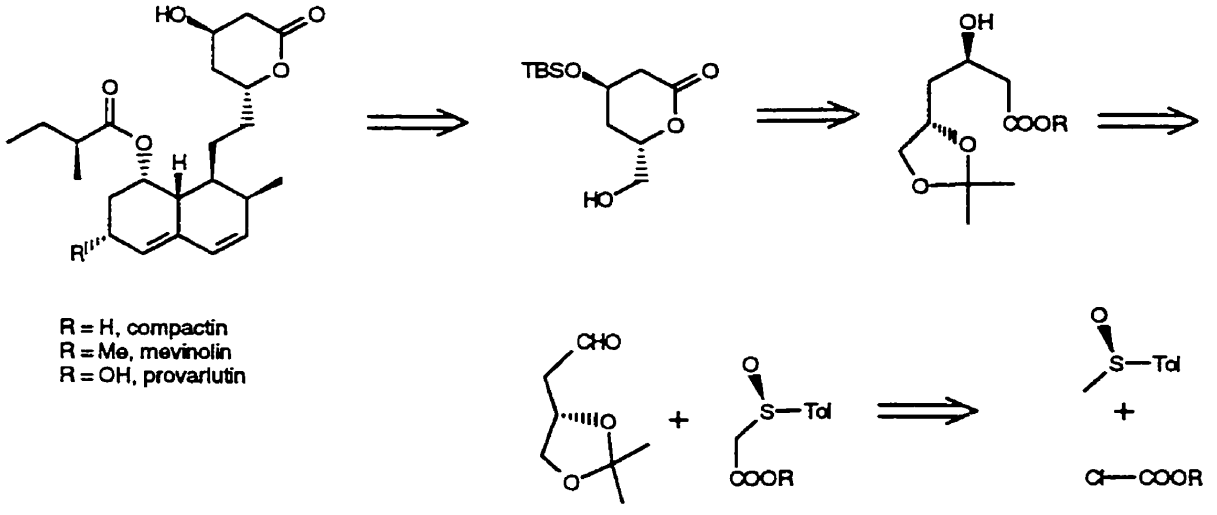
Chapter Four: Yeast- and *E. coli*-Mediated Oxidations at Sulfur Centres

Introduction

Optically pure sulfoxides are powerful stereodirecting groups, particularly as chiral synthons for asymmetric C-C bond formations,[1] and they have been widely used in natural product synthesis (Scheme 24).[2] Numerous approaches to their preparation have been investigated.[3] Although certain useful sulfoxides are available in high optical purities and good yields by chemical oxidations followed by diastereomeric separations,[4] it is more desirable to have direct access to chiral sulfoxides by asymmetric oxidations. Some catalytic asymmetric oxidation methods have been reported that give both high optical purity and high chemical yield,[5] while others give products with a variable degree of enantiomeric enrichment and yield. The results are highly dependent on the nature of the substrates.[6]



Scheme 24

The limitations encountered in chiral chemical oxidations and the ever growing interest in developing ecologically more benign processes inspired a search for bioorganic methods, which include reactions with purified enzymes or with live microorganisms.[7] Cyclohexanone monooxygenase (CHMO) from the soil bacterium *Acinetobacter* sp. (NCIB 9871) has been shown to perform enantioselective oxidations on a variety of sulfur substrates.[8] The success of the designer oxidizing yeast in the asymmetric Baeyer-Villiger reactions and the simplicity of the procedures encouraged the investigation of this bioreagent in the oxidations of sulfur. Several dithiolanes, dithianes and sulfides were tested, preliminary results are summarized in Table 14 to Table 16.*

Results and Discussion

Preparation of substrates

Since dithianes and dithiolanes are frequently used as protecting groups for carbonyl compounds, numerous methods have been reported for their synthesis.[9] Compounds **4-1a** to **4-1d** and **4-1f** were synthesized by condensation between the corresponding

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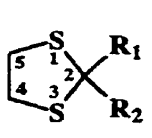
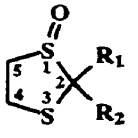
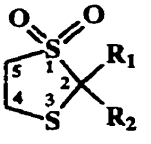
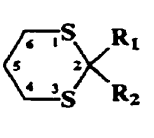
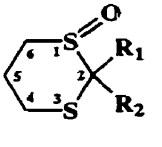
* In this report, the following nomenclature conventions were followed:

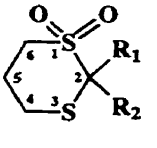

dithiolane


dithiolane-1-oxide


dithiolane-1-sulfone

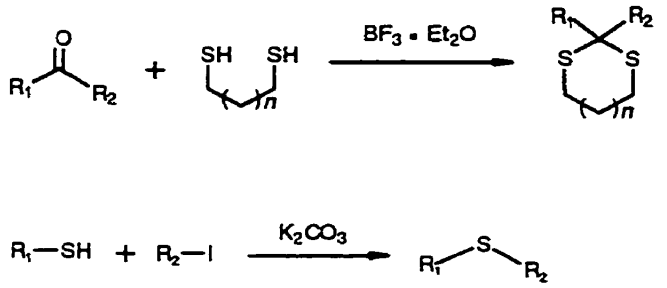

dithiane


dithiane-1-oxide


dithiane-1-sulfone

Since dithianes, dithiolanes and sulfides have sulfur atoms in the same oxidative state, they are all referred to as sulfides in general discussions.

carbonyl compounds and 1,2-ethanedithiol or 1,3-propanedithiol using $BF_3 \cdot Et_2O$ as the catalyst.[10] Dithiane **4-1e** and sulfides **4-1g** to **4-1i** are commercially available, **4-1j** was prepared by condensation between thiophenol and 1-iodopropane under basic conditions. The reactions are summarized in Scheme 25.

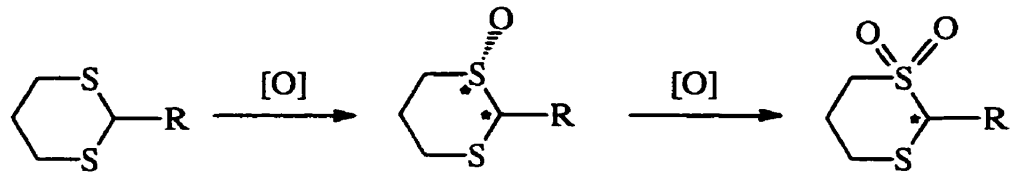


4-1a	n=0,	R_1=H,	R_2=H,	76%
4-1b	n=0,	R_1=Me,	R_2=H,	72%
4-1c	n=0,	R_1=Me,	R_2=Me,	96%
4-1d	n=0,	R_1=Ph,	R_2=H,	89%
4-1e	n=1,	R_1=H,	R_2=H,	--*
4-1f	n=1,	R_1=Ph,	R_2=H,	99%

4-1g	R_1=*t*-Bu,	R_2=me	--*
4-1h	R_1=*n*-Bu,	R_2=Me	--*
4-1i	R_1=Ph,	R_2=Me	--*
4-1j	R_1=Ph,	R_2=*n*-Pr	95%

Scheme 25

Oxidation of Dithianes



R=H, **4-1e** — **4-2e** — **4-3e**
R=Ph, **4-1f** — **4-2f** — **4-3f**

Scheme 26

The oxidations of dithianes are shown in Scheme 26. Purified enzyme- and *Acinetobacter*-catalyzed oxidations of 1,3-dithiane **4-1e** gave similar ratios of sulfoxide **4-2e** and sulfone **4-3e**;[8b] reactions with engineered *E. coli* gave a higher proportion of sulfoxide with lower optical purity, while 15C(pKR001) produced a smaller amount of

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* Commercially available materials

sulfoxide with higher optical purity and a larger proportion of sulfone. It appears that CHMO is able to further oxidize sulfoxide to sulfone, and the *(S)*-**4-2e** was preferentially removed. This was in agreement with the literature report,[8b] and was confirmed in a control experiment where racemic **4-2e** was oxidized to **4-3e**.

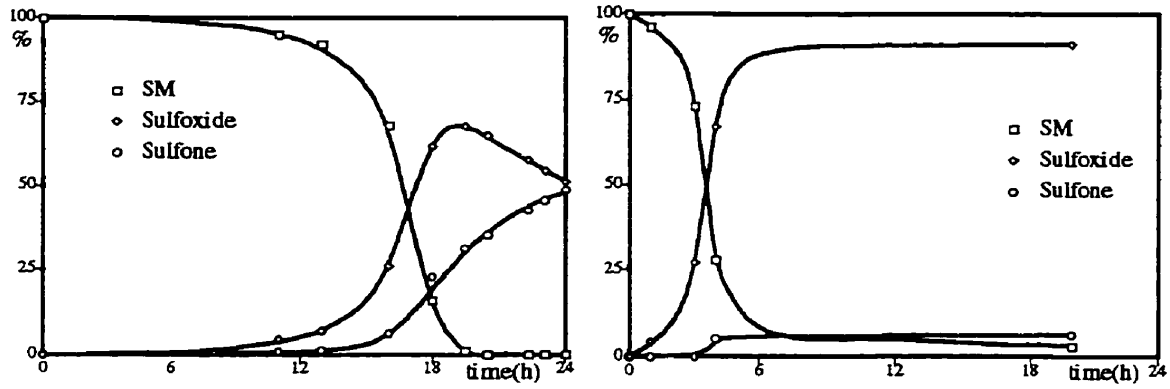
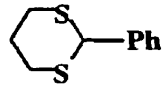


Figure 15: Oxidation of 1,3-dithiane (4-1e) by yeast (left) and *E. coli* (right)

In the oxidation of **4-1e** by bioengineered yeast or *E. coli*, a significant amount of sulfone **4-3e** was already formed in the early stage of the reaction (Figure 15), while in reactions catalyzed by isolated CHMO, sulfone was formed only when almost all the dithiane had been exhausted.[8a] This difference may occur because the transportation of the polar sulfoxide across the cell membrane is slow; thus the accumulation of sulfoxide in the cell favoured its subsequent oxidation to sulfone. The difference in sulfone ratios between yeast- and *E. coli*-catalyzed reactions was most likely the result of different membrane permeabilities towards the sulfoxide.

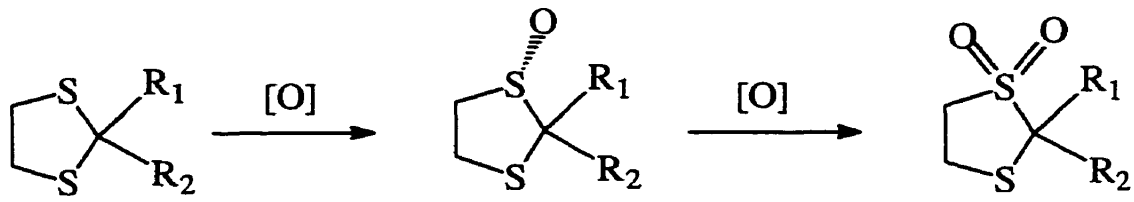
2-Phenyl-1,3-dithiane **4-1f** was oxidized by isolated CHMO to the *trans*-sulfoxide (d.e.>98%) with low enantioselectivity. A similar trend was observed in the oxidation with 15C(pKR001), but both the d.e. and e.e. of the product were lower than those obtained from the purified enzyme. The control experiments indicated that 15C's resident oxygenase independently catalyzes the transformation of **4-1f** to **4-2f** with the wrong enantio- and diastereoselectivity. The details of the control experiments are discussed in a later section. The *E. coli* overexpression system was expected to match the CHMO results more closely, but even here some difference was still observed. The results are summarized in Table 14.

Table 14: Oxidation of dithianes

substrate	biocatalyst	time (h)	GC ratio S : SO : SO_2	sulfoxide(%)[1] [*trans:cis*]	e.e. (%)	sulfone (%)[1]	Ref.
	CHMO	--	0 : 81 : 19	--	>98(*R*)	--	8b
	Acinetobacter	--	--	71	93(*R*)	5	8b
	15C(pKR001)	40	1 : 42 : 57	18	90(*R*)	19	--
	BL21(pMM04)	6	3 : 91 : 6	73	84(*R*)	--	--
	CHMO	--	0 : 100 : 0	-- [50:1]	28(1R,2R)	--	11
	UV4 *P.putida*	--	--	--	--(1S,2S)	--	
	15C(pKR001)	46	66 : 34 : 0	30 [9:1]	20(1R,2R)	0	12
	BL21(pMM04)	44	34 : 66 : 0	-- [19:1]	12(1R,2R)	0	
							--
							--

1. Isolated yield after purification by flash chromatography.

Oxidation of Dithiolanes



4-1a, R1=H, R2=H	**4-2a**	**4-3a**
4-1b, R1=Me, R2=H	**4-2b**	**4-3b**
4-1c, R1=Me, R2=Me	**4-2c**	**4-3c**
4-1d, R1=Ph, R2=H	**4-2d**	**4-3d**

Scheme 27

The oxidations of dithiolanes are shown in Scheme 27. Dithiolanes appeared to be somewhat toxic to yeast and *E. coli*, thus the addition of a stoichiometric amount of cyclodextrin was necessary. The most complicated situation was encountered in the reactions with 2,2-dimethyl-1,3-dithiolane **4-1c**, which formed an insoluble precipitate with β-cyclodextrin and evaporated rapidly in its absence. Switching to γ-cyclodextrin improved the final yield of the sulfoxide. Oxidation of 1,3-dithiolane **4-1a** and 2-phenyl-1,3-dithiolane **4-1d** were monitored by GC, and the results are shown in Figure 16.

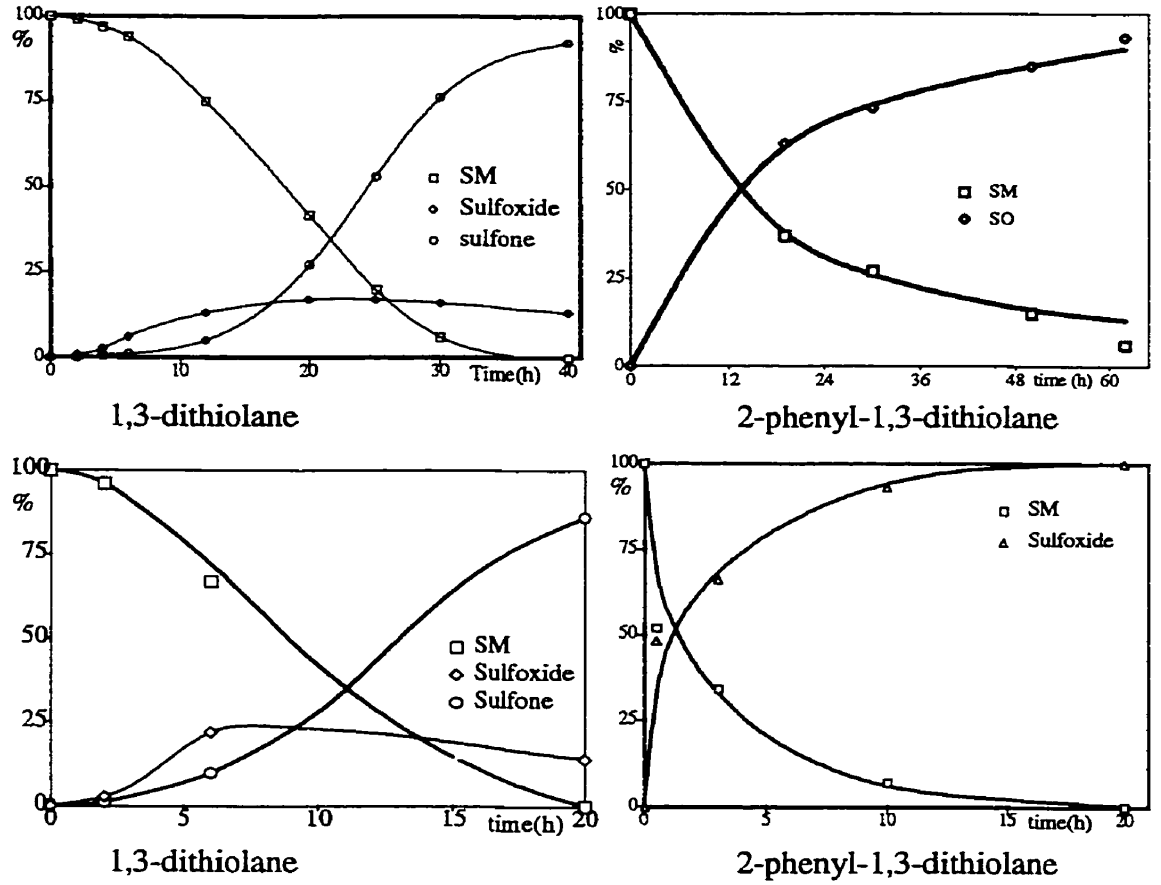


Figure 16: Oxidations of dithiolanes by yeast (upper) and *E. coli* (lower)

It is obvious that a large proportion of sulfone was formed in the oxidation of 1,3-dithiolane **4-1a** from the beginning of the reaction. Unlike in the oxidation of 1,3-dithiane, there did not seem to be much difference between yeast and *E. coli* catalyzed oxidations except that, as expected, the reaction mediated by *E. coli* was faster.

Both recombinant yeast and *E. coli* gave predominantly *trans*-sulfoxide; however, the enantiopreferences were different. *E. coli* produced 20% e.e. favouring one enantiomer, while the yeast gave 20% e.e. of the other. This difference is consistent with a

concomitant oxidation by native yeast oxygenase, with a preference for the opposite enantiomer of the 2-phenyl-1,3-dithiolane substrate to that of the CHMO.

In control experiments, racemic sulfoxides **4-2a** to **4-2c** were rapidly converted to the corresponding sulfones by yeast 15C(pKR001) and *E. coli* BL21(DE3)(pMM04) with relatively low stereoselectivity. On the other hand, a dithiolane with a bulky group in the 2-position (**4-1d**) escaped this further oxidation (Table 16). The host strains 15C and BL21(DE3) did not catalyze the oxidations of dithiolanes to sulfoxides or sulfoxides to sulfones, with the only exception being 2-phenyl-1,3-dithiolane **4-1d** which was slowly converted by 15C to *cis*-**4-2d** (66% d.e., 70% e.e.). The overlaid HPLC traces of **4-1d** oxidations are shown in Figure 17. Due to the low enantioselectivity in these reactions, no attempt has been made to establish the absolute configurations of *cis*- and *trans*-**4-2d**.

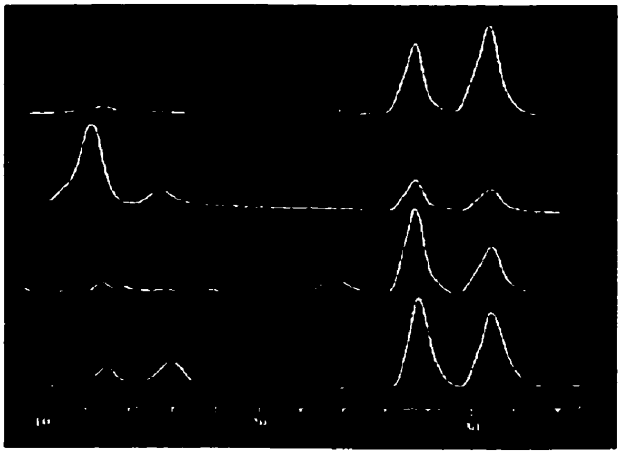
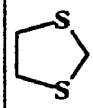
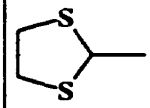
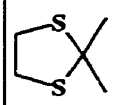
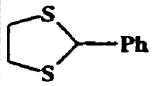


Figure 17: Overlaid HPLC traces of 2-phenyl-1,3-dithiolane-1-oxide from different oxidation conditions

In conclusion, the bioengineered yeast efficiently oxidizes 1,3-dithiolane and 1,3-dithiane to the corresponding sulfoxides. The subsequent oxidations to sulfones are competitively fast and, as a result, there is little accumulation of the sulfoxides.* In both cases the *(S)*- enantiomer is oxidized slightly faster than its antipode, leading to the *(R)*-enriched sulfoxides, albeit at low yields. The oxidations with BL21(DE3)(pMM04) are comparable to those performed with yeast but are faster and give less metabolite contamination. The results are summarized in Table 15.

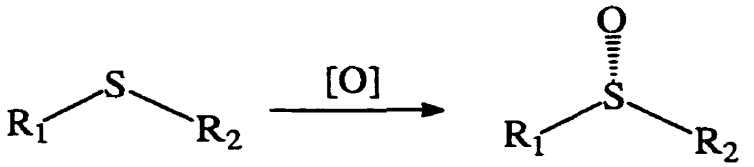
Table 15: Oxidation of dithiolanes

substrate	biocatalyst	time (h)	GC ratio S : SO : SO_2	sulfoxide[1] [*trans:cis*]	e.e. (%)	sulfone[1] (e.e.%)	Ref.
	CHMO	--	0 : 94 : 6	--	>98*(R)*	6	8b
	Acinetobacter	--	--	71	95*(R)*	16	8b
	15C(pKR001)	30	5 : 25 : 70	20	75*(R)*	45	--
	BL21(pMM04)	20	0 : 15 : 85	13	86*(R)*	47	--
	CHMO	--	0 : 96 : 6	-- [50:1]	50(1R,2R)/--	--	8a,b
	15C(pKR001)	28	1 : 22 : 77	16[10:1]	20(1R,2R)/63	15 (76)	--
	BL21(pMM04)	20	34 : 31 : 35	35[5:1]	40(1R,2R)/55	16 (73)	--
	CHMO	--	0 : 100 : 0	--	65*(S)*	--	8a
	15C(pKR001)	36	0 : 81 : 19	42	64*(S)*	--	--
	BL21(pMM04)	20	9 : 67 : 24	46	69*(S)*	6	--
	Hog liver FMO	--	0 : 100 : 0	-- [3:1]	100(1R,2R)/--	--	13
	15C(pKR001)	62	6 : 94 : 0	74 [32:1]	20(1R,2R)/--	0	--
	BL21(pMM04)	41	13 : 87 : 0	60 [40:1]	20(1R,2R)/68	0	--

1. Isolated yield (%) after purification by flash chromatography.

*Oxidations to sulfones were also observed in the reactions with 2-methyl-1,3-dithiolane **4-1b** and 2,2-dimethyl-1,3-dithiolanes **4-1c**.

Oxidation of sulfides



4-1g	R_1=*t*-Bu, R_2=Me	**4-2g**
4-1h	R_1=*n*-Bu, R_2=Me	**4-2h**
4-1i	R_1=Ph, R_2=Me	**4-2i**
4-1j	R_1=Ph, R_2=*n*-Pr	**4-2j**

Scheme 28

The oxidations of acyclic sulfides are outlined in Scheme 28, the results are summarized in Table 16. The oxidation of phenyl methyl sulfide **4-1i** by the engineered yeast gave an excellent isolated yield (95%) of optically pure sulfoxide **4-2i** (>99% e.e.). *t*-Butyl methyl sulfide **4-1g** was also oxidized with high enantioselectivity (97% e.e.) to the corresponding sulfoxide **4-2g**. In the latter case the isolated yield was significantly influenced by evaporation of the substrate. It was improved from 11% to 47% by performing the reaction in a sealed flask to minimize evaporation.

It appears that in order to achieve high enantioselectivity, a small group (methyl) and a large group (phenyl or *t*-butyl) are both necessary. When the difference in size between the two groups decreases, the enantioselectivity drops significantly. Thus when the *t*-butyl group in **4-1g** was changed to *n*-butyl (**4-1h**), the e.e. dropped from 97% to 74%; when the methyl group in **4-1i** was changed to *n*-propyl (**4-1j**), the e.e. dropped from >99% to only 25%. In general, methyl is the preferred "small" group, and sulfides with

longer chains are poor substrates. For example, the oxidation of phenyl *n*-propyl sulfide **4-1j** was very slow, even after prolonged incubation (120 hours as opposed to 24-36 hours for methyl sulfides) the conversion was still very low (10% conversion and 25% e.e. by GC).*

Table 16: Oxidation of sulfides

substrate	biocatalyst	time (h)	SM (GC %)	sulfoxide (isolated, %)	e.e. (%)	$[\alpha]_D^{25}$
Ph–S–	CHMO	–	–	100*	>99(*R*)	–
	Acinetobacter	–	–	97	85(*R*)	–
	15C(pKR001)	40	–	95	>99(*R*)	+166
	BL21(pMM04)	19	82	0*	–	–
Ph–S–*n*-Pr	*R.equi*[14]	–	–	–	–	–
	15C(pKR001)	120	20*	10*	25(*R*)	–
t-Bu–S–	CHMO	–	–	–	–	–
	15C(pKR001)	40	10*	47	97(*R*)	-14
	BL21(pMM04)	–	–	24	79(*R*)	–
n-Bu–S–	15C(pKR001)	23	0	53	74(*R*)	-70
	BL21(pMM04)	19	84	0	–	–

* Yields are based on GC integrations.

Complications encountered in yeast-mediated sulfur oxidations

There are certain differences between the results obtained from the yeast-mediated oxidations and those from the isolated enzyme- or *Acinetobacter*-catalyzed reactions. It is necessary to investigate the complexity in yeast-mediated oxidations in order to understand the reasons that caused such differences and to look for possible remedies.

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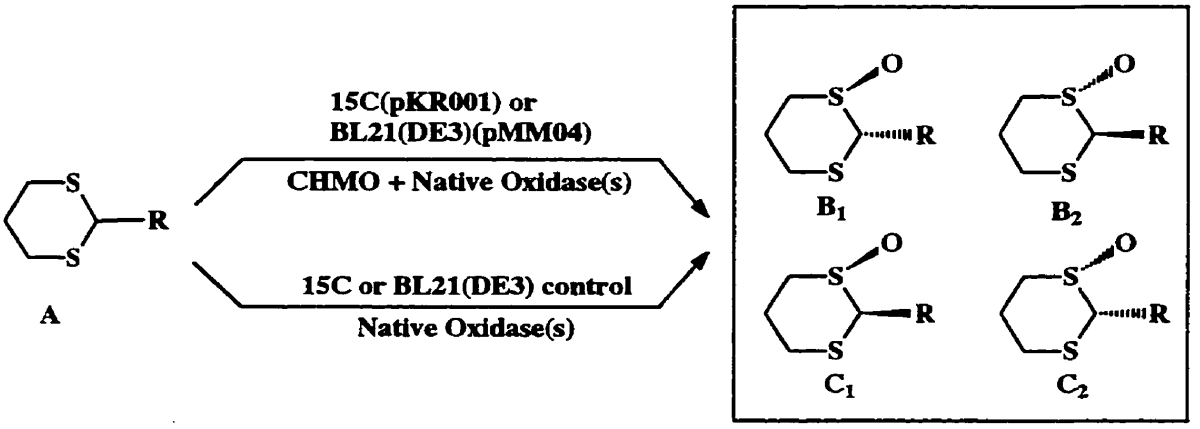
* Because of the low conversion and low e.e. in this reaction, no attempt was made to isolate the product or to improve the yield.

When a reaction is mediated by live microorganisms, the selectivity is often altered by competing enzymes in the same organism. In the yeast-mediated sulfur oxidations, this occurs when the host strain carries at least one native oxygenase that accepts the same substrate as CHMO, but converts it to a product with the opposite enantioselectivity (or different diastereoselectivity). To identify these cases, control reactions were performed for most of the substrates tested (Scheme 29).

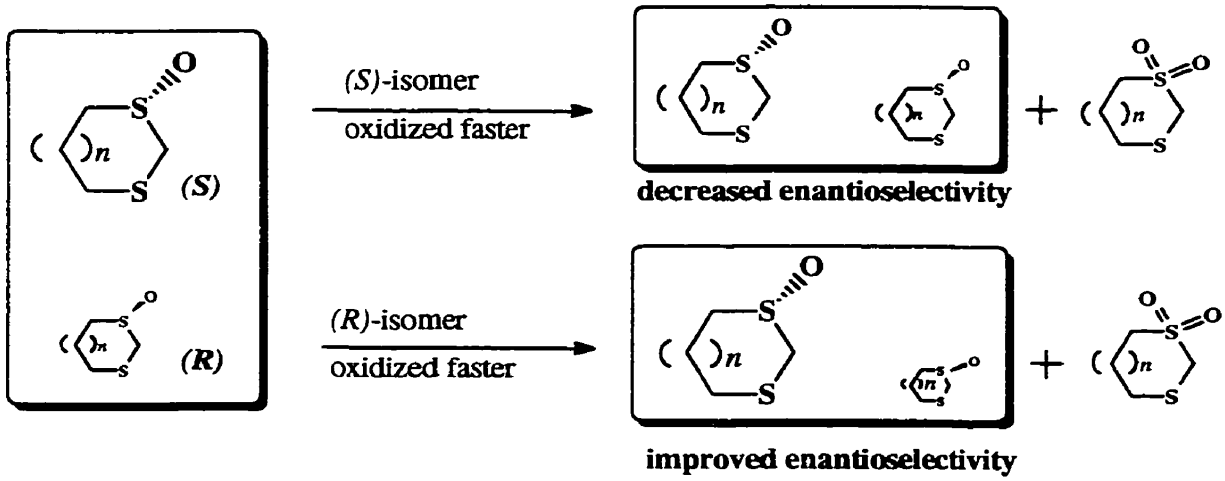


Scheme 29

Negative controls were performed with the carrier yeast strain 15C to see if native enzymes were involved in the oxidation.[15] A different CHMO expression system using *E. coli* as the host cell was bioengineered[16] and was used as a positive control. If the *E. coli* system gives similar results to those obtained from isolated CHMO, the differences between isolated enzyme and the oxidizing yeast may be attributed to the interference by native yeast enzymes. Although to the best of our knowledge there have been no literature reports that *E. coli* carries native enzymes for sulfur oxidations, a

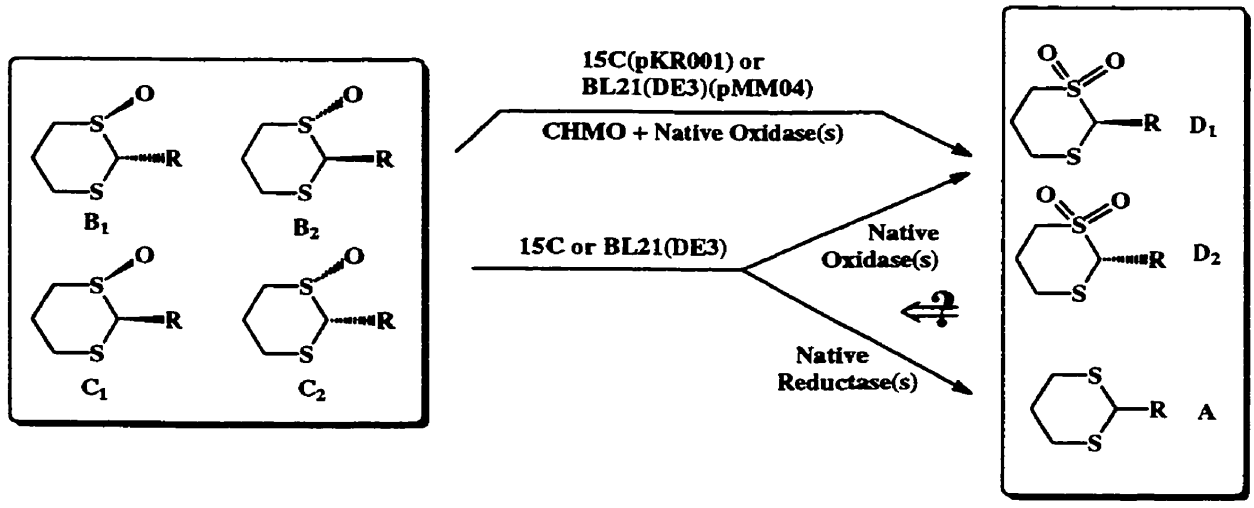
second negative control was performed with the carrier *E. coli* strain BL21(DE3). These control reactions showed the competing oxidations by native enzyme(s) did not occur except in the case of 2-phenyl-1,3-dithiolane **4-1d** 1,3-dithiane **4-1e**, and 2-phenyl-1,3-dithiane **4-1f**. The results are listed in Table 17.

Since sulfoxides can be further oxidized to sulfones by CHMO,[11] the composition of the final products would be the combined selectivities from the oxidation of sulfides to sulfoxides and of sulfoxides to sulfones (**Scheme 30**). When the second step has the same stereochemical preference as the first one, it will remove the major isomer and lead to lowered apparent selectivity; when the second step has the opposite stereochemical preferences, it will remove the minor isomer and "purify" the product. In both cases the overoxidization to sulfone will lower the sulfoxide yield.



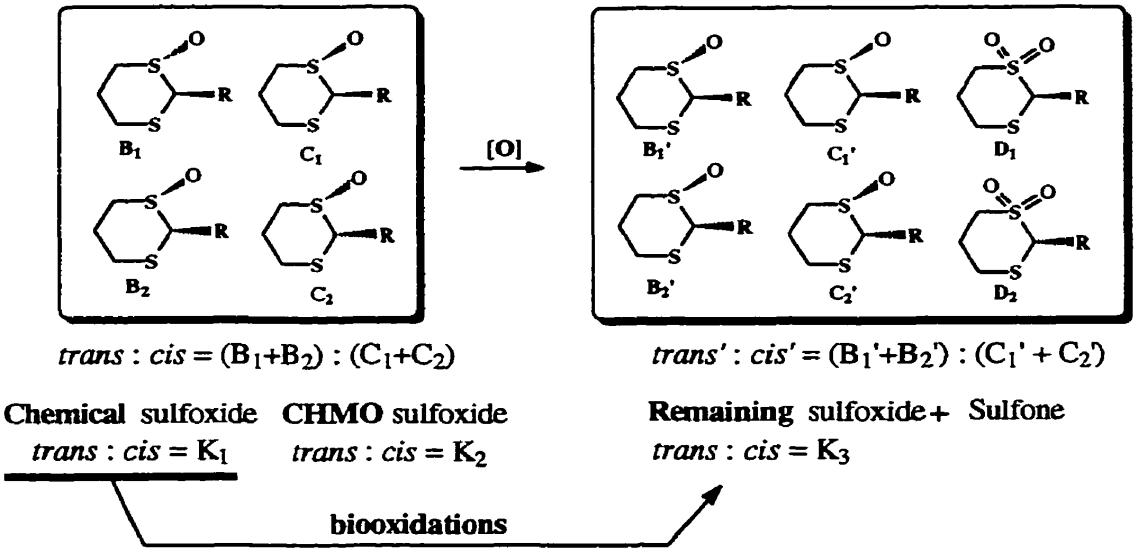
Scheme 30

In order to study the enantioselectivities in the second oxidation step, some of the substrates were chemically oxidized to *racemic* monosulfoxides[17] and subjected to yeast oxidation conditions to establish the stereoselectivity in the second oxidation step. Some racemic sulfoxides were also incubated with the host yeast and *E. coli* strains to clarify if native enzymes contributed to the oxidations of sulfoxides to sulfones. The same control experiments also served to see if yeast or *E. coli* have the ability to *reduce* a sulfoxide to a sulfide[18] (Scheme 31). Although possibilities exists that certain reductases may reduce sulfones to sulfoxides or sulfides, it is difficult to systematically test for such activity because monosulfones are not readily available from any chemical oxidations. Only in cases when the monosulfones were isolated from yeast oxidations in sufficient quantities (**4-3a** and **4-3e**) were these control experiments conducted. No sulfone reduction was observed in these two cases.



Scheme 31

Control experiments confirmed that both the bioengineered yeast and *E. coli* are capable of oxidizing the sulfoxides to sulfones, but the stereoselectivity is only low to moderate (Table 17). Which sulfoxide enantiomer gets preferentially oxidized depends on each substrates. For sulfoxides **4-2a**, **4-2c** and **4-2e**, the *(S)*-enantiomers were oxidized faster, while for **4-2b** the *(1R,2R)* isomer was oxidized faster. Sulfoxides having a bulky group at the 2-position (**4-2d**, **4-2f**) were not oxidized to sulfones. For 2-monosubstituted dithiolanes and dithianes, chemical oxidations gave a mixture of *cis*- and *trans*-isomers. By comparing the ratio of the two diastereomers from chemical oxidations and yeast oxidations, it is possible to give an estimate of CHMO's diastereoselectivity. The ratio of the two diastereomers in the first oxidation (sulfides to sulfoxides) and in the second step (sulfoxides to sulfones) can reveal whether higher selectivity was achieved in the first step or the second (Box 1).

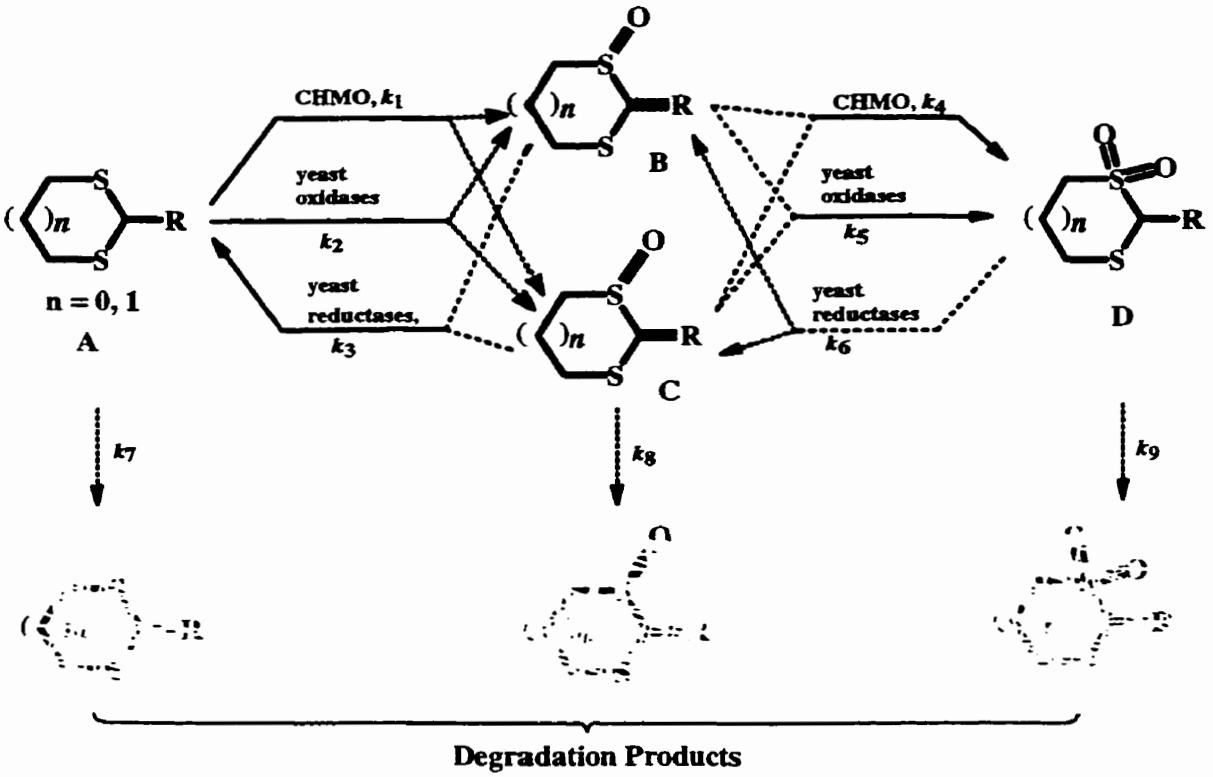


Assume the *trans:cis* ratio is K_1 in chemical oxidation and K_2 in enzymatic oxidation. If $K_1 < K_2$, it means the enzymatic reaction has the preference for *trans-* isomers, and *vice versa.* Furthermore, assume a sulfoxide from chemical oxidation, which has a *trans:cis* ratio of K_1 is partially oxidized in a biotransformation to sulfone, the remaining sulfoxide has *trans:cis* ratio of K_3. If $K_3 < K_1$, it means in the second oxidation step, the *trans*-isomer is preferentially removed, and *vice versa.* If $(K_2/K_1)>(K_3/K_1)$, higher selectivity is achieved in the first step; if $(K_2/K_1)<(K_3/K_1)$, higher selectivity is achieved in the second step.

Box 1: Deducing diastereoselectivity from control reactions

More complications can arise if the host yeast or *E. coli* degrades the sulfides, sulfoxides, and sulfones, or transforms those compounds into other products. There is no obvious control reaction to detect these possibilities, except from the decrease of

total mass of identifiable sulfur compounds, however it may be difficult to distinguish between mass lose due to degradation and that due to other processes such as evaporation. Scheme 32 summarizes the possible complexity discussed above.

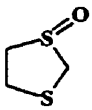
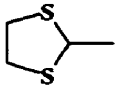
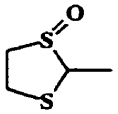
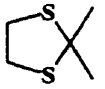
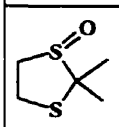
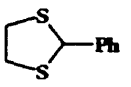
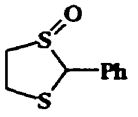
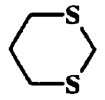
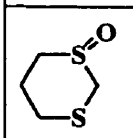
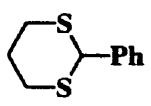


Scheme 32

In addition to the already very complicated situation, there can be other factors that affect the biooxidations of sulfur. For example, in all the reactions the starting materials need to be transported into the cells, and the products need to be transported out. There could be preferences in the rate of penetration across the cell membranes, both between enantiomers (or diastereomers) and between different sulfur species. Such preferences

will change the apparent selectivities and the product distribution of different species. Experiments to prove or disprove these possibilities are beyond the scope of this thesis.

Table 17: Control experiments for sulfur oxidations

substrate	biocatalyst	time (hours)	sulfides (GC %)	sulfoxide (GC %) [*trans:cis*]	e.e. (%) *trans / cis*	sulfone (GC %)
	15C BL21	50 48	100 100	0 0	-- --	0 0
	BL21(pMM04) 15C BL21	20 65 48	0 100 100	3 0 0	19(*R*) -- --	97 0 0
	15C BL21	53 48	100 100	0 0	-- --	0 0
	15C(pKR001) BL21(pMM04) 15C BL21	27 14 20 20	0 0 0 0	58 [8:1] 12[23:1] 100 [5:5:1] 100 [5:1]	13(S,S)/10 24(S,S)/-- -- --	42(35% e.e.) 88(36% e.e.) -- --
	15C BL21	36 48	100 100	0 0	-- --	0 0
	BL21(pMM04) BL21	24 48	0 31*	54 69	7(*R*) 43(*S*)	46 0
	15C BL21	46 48	81 100	19*[1:2] 0	7/70 0	0 0
	BL21	48	0	100	--	0
	15C BL21	120 48	82 100	1 0	-- --	17 0
	15C(pKR001) BL21(pMM04) 15C BL21	21 6 50 48	0 0 0 53*	37 23 92 47	20(*R*) 19(*R*) -- --	45 77 0 0
	15C BL21	48 48	92 100	8* 0	-- --	0 0

* Identity based on GC or HPLC retention time, not unambiguously established.

Conclusion

In conclusion, the present study shows that oxidations of sulfur carried out by engineered yeast *can* be highly enantioselective and efficient for a variety of substrates. For some substrates, oxidations with the recombinant yeast are highly enantioselective (1,3-dithiane **4-1e**, methyl phenyl sulfide **4-1i**, *t*-butyl methyl sulfide **4-1g**), or as enantioselective as the enzyme itself (2,2-dimethyl dithiolane **4-1c**). However for other substrates, the oxidations gave significantly lower selectivities. When the results are comparable to those obtained with the purified enzyme, the low cost, the easy access and experimental facility make designer yeast an attractive alternative to purified enzyme- or *Acinetobacter*-catalyzed transformations. The control experiments showed that resident yeast oxygenase(s) may in some cases compete for the dithiolane and dithiane substrates (for example, 2-phenyl-1,3-dithiolane **4-1d** and 2-phenyl-1,3-dithiane **4-1f**). However, oxidations by native enzymes were only observed to a significant extent for 2-phenyl substituted substrates, and even for these substrates the reactions were still very slow. It was expected that for most substrates tested, oxidations with native enzymes should not be a concern. No oxidation by native enzymes was observed in *E. coli*-mediated reactions.

Experimental problems and solutions

Yeast-mediated oxidations of sulfur compounds presented several experimental difficulties. Some corrective measures were applied to selected reactions that gave good results in preliminary tests. In most cases the isolated yields of the sulfoxides were

significantly enhanced. A systematic optimization of the reaction conditions was not performed at this time. The principal difficulties encountered and the solutions applied are outlined below.

Substrate toxicity

A few substrates appeared to be somewhat toxic both to yeast and *E. coli*, this is especially evident for dithiolanes.* The addition of one equivalent of β- or γ-cyclodextrin helped to reduce the toxicity by formation of inclusion complexes with the substrates which lowered the effective concentration of the starting materials. When γ-cyclodextrin was used, **4-1a** was oxidized within 20 hours.

Substrate evaporation

During prolonged reactions, significant quantities of starting materials may be lost because of evaporation. In several cases, loss of materials through evaporation was as fast as, or even faster than, the oxidation reaction itself, particularly when an insoluble and relatively volatile substrate formed a film on the surface of the reaction mixture. For example, in control experiments (substrate in water shaken at 30°C), methyl *t*-butyl sulfide **4-1g** and methyl *n*-butyl sulfide **4-1h** evaporated completely within 36 hours. The addition of cyclodextrins helped to slow down the evaporation process, but this route is not problem-free because certain substrates can form insoluble precipitates with cyclodextrins (for example, 2,2-dimethyl-1,3-dithiolane **4-1c** precipitated with β-

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*For example, no reaction was observed after 1,3-dithiolane **4-1a** was incubated with *E. coli* BL21(DE3)(pMM04) for 43 hours.

cyclodextrin). When this happens, the reactions are very slow, can not be monitored effectively by GC or HPLC, and give low isolated yields. Furthermore, the addition of cyclodextrin does not completely eliminate evaporation. In control experiments (substrate + β-cyclodextrin in water, shaken at 30°C), 64% of 2-methyl-1,3-dithiolane **4-1b** and 60% of 2,2-dimethyl-1,3-dithiolane **4-1c** remained after 36 hours, and only 14% *t*-butyl methyl sulfide **4-1g** remained after 29 hours. Eventually, to minimize the losses caused by evaporation, some reactions (**4-1g, 4-1h**) were carried out in baffled flasks covered with aluminum foil and sealed with parafilm®. To ensure an adequate supply of oxygen, the size of the reaction flasks had to be at least doubled. These modifications effectively improved the isolated yields.

Difficulties in reaction monitoring

During the reactions, GC was extensively used to monitor the oxidations. However in almost all the reactions tested, some lose of the total mass was observed when conducting GC analysis based on the internal standard method. The problems associated with GC monitoring may result from the variations in extraction efficiency and the response coefficient of the GC detector (FID) to the starting materials and the products.

Some corrective measurement can be applied to address these problems, such as constructing calibration curves and using a known amount of the starting material and the product to check the extraction efficiencies, however, these methods are tedious and inconvenient.[19] Because the current work is only a preliminary study to examine the

scope and limitations of yeast and *E. coli*-mediated oxidations, no additional corrective measurement were employed. For this reason, the GC yields reported in this work were treated as qualitative ratios which showed the degree of conversion, and the progress of the reactions; the correct ratio of each component was based on the isolated materials.

Contamination with metabolites

During the oxidations, especially those that needed prolonged fermentations, both yeast and *E. coli* generated a significant quantities of metabolites. The major extractable metabolite produced by yeast was isolated and identified to be 2-phenylethanol. This compound was observed in the reactions with the engineered yeast 15C(pKR001), the carrier strain 15C, and commercial baker's yeast. This phenomenon imposed certain experimental difficulties, because the metabolite partially overlapped with the product during flash chromatography. Either the chromatography needed to be repeated using different solvent system, or some fractions contaminated with metabolite had to be discarded. This affected the isolated yield in some reactions.

Experimental

General procedures for synthesizing dithiolanes and dithianes [9]

The aldehyde or ketone (20mmol) and 1,2-ethanedithiol (1.7mL, 20mmol) were dissolved in 40mL CH_2Cl_2. The solution was cooled in an ice-water bath and boron trifluoride etherate (1mL) was added dropwise. After the addition, the cold bath was removed and the reaction mixture was vigorously stirred for 1 hour at room temperature. The mixture was poured into a mixture of saturated $NaHCO_3$ and ice, the

organic layer was washed thoroughly with saturated $NaHCO_3$, and the aqueous phase was extracted with CH_2Cl_2. The combined organic phases were dried over anhydrous $MgSO_4$ and the solvent was removed on a rotary evaporator. The residue was purified when necessary by vacuum distillation or flash chromatography on silica gel using mixtures of hexane and ethyl acetate as the eluent to give the final product. Dithianes can be synthesized in a similar manner using 1,3-propanedithiol.

1,3-Dithiolane **4-1a** Paraformaldehyde (0.6g, 20mmol) and 1,2-ethanedithiol (1.7mL, 20mmol) were reacted according to general procedures. Vacuum distillation (80°C/2mmHg) gave **4-1a** as a colourless oil, 1.6g (76%). IR ν_{max} (neat): 2973(m), 2927(vs), 2842(w), 1420(s), 1289(s), 867(s), 690(m) cm^{-1}. ^1H NMR δ: 3.90(s, 2H), 3.19(s, 4H) ppm. ^{13}C NMR δ: 38.0, 34.2 ppm.

2-Methyl-1,3-dithiolane **4-1b** Acetaldehyde (1.5mL, 20mmol) and 1,2-ethanedithiol (1.7mL, 20mmol) were reacted according to general procedures. Vacuum distillation (76-85°C/18mmHg) gave **4-1b** as a colourless oil, 1.73g (72%). IR ν_{max} (neat): 2960(s), 2927(vs), 2861(m), 1453(s), 1282(s), 860(m) cm^{-1}. ^1H NMR δ: 4.59(1H, q, J=6.4), 3.28(2H, m), 3.20(2H, m), 1.59(3H, d, J=6.5) ppm. ^{13}C NMR δ: 53.4, 48.1, 39.1, 24.7 ppm.

2,2-Dimethyl-1,3-dithiolane **4-1c** Acetone (1.5mL, 1.2g, 20mmol) and 1,2-ethanedithiol (1.7mL, 1.9g, 20mmol) were reacted according to general procedures. Vacuum distillation (62-72°C/6mmHg) gave **4-1c** as a colourless oil, 2.6g (96%). IR ν_{max}

(neat): 2965(m), 2960(s), 2927(vs), 2861(w), 1446(m), 1157(w), 1111(w), 683(w) cm^{-1}. ^1H NMR δ: 339(4H, s), 1.80(6H, s) ppm. ^{13}C NMR δ: 62.1, 40.2, 34.1 ppm.

2-Phenyl-1,3-dithiolane **4-1d** Benzaldehyde (2mL, 20mmol) and 1,2-ethanedithiol (1.7mL, 20mmol) were reacted according to general procedures. Vacuum distillation (105-110°C/1mmHg) gave **4-1d** as a colourless oil, 3.24g (89%) which solidified upon standing to give white needles. IR ν_{max} (neat): 3065(w), 3026(m), 2973(w), 2927(m), 2835(w), 1677(m), 1499(s), 1453(vs), 1289(m), 690(vs) cm^{-1}. ^1H NMR δ: 7.53(2H, m), 7.34(3H, m), 5.63(1H, s), 3.52-3.43(2H, m), 3.38-3.28(2H, m) ppm. ^{13}C NMR δ: 140.2, 128.4, 127.9, 127.8, 56.2, 40.1 ppm.

2-Phenyl-1,3-dithiane **4-1f** Benzaldehyde (5.1mL, 50mmol) and 1,3-propanedithiol (5.0mL, 50mmol) were reacted according to general procedures to give **4-1f** as white crystals, 9.8g (>99%). IR ν_{max} ($CHCl_3$): 3065(m), 2993(w), 2954(w), 2901(m), 1440(m), 1269(vs), 748(vs), 709(s) cm^{-1}. ^1H NMR δ: 7.48-7.44(2H, m), 7.35-7.25(3H, m), 5.15(1H, s), 3.07-2.98(2H, m), 2.90-2.84(2H, m), 2.16-2.08(2H, m), 1.96-1.84(1H, m) ppm. ^{13}C NMR δ: 139.0, 128.6, 128.3, 127.6, 51.3, 31.9, 25.0 ppm.

Phenyl propyl sulfide **4-1j** K_2CO_3 (1.5g, 11mmol) was suspended in anhydrous ethanol (10mL) under nitrogen and cooled in an ice bath. Thiophenol (1mL, 1.1g, 10mmol) was added, followed by *n*-propyl iodide (1mL, 10mmol). After stirring at room temperature for 1 hour, the mixture was filtered through a sintered glass funnel and the solid was washed with ethanol. The filtrate was concentrated by rotary evaporation. The residue was dissolved in CH_2Cl_2 and passed through a small silica plug. The silica plug was

washed with CH_2Cl_2 and the solvent was removed by rotary evaporation to give **4-1j** as a colourless oil, 1.44g (95%). IR ν_{max} (neat): 3092(w), 3059(w), 2967(s), 2927(m), 2875(m), 1598(m), 1486(s), 1440(s), 1091(m), 1032(m), 749(vs), 696(vs) cm^{-1}. ^1H NMR δ: 7.34-7.30(2H, m), 7.29-7.24(2H, m), 7.18-7.13(1H, m), 2.89(2H, t, J=7.2), 1.67(2H, sextet, J=7.3), 1.02(3H, t, J=7.3) ppm. ^{13}C NMR δ: 136.9, 128.93, 128.86, 128.83, 125.7, 125.6, 35.6, 22.5, 13.4 ppm.

General procedures for chemical oxidations[17]

The sulfur compound (10mmol) was dissolved in 50mL methanol and cooled in a water bath around 15°C. $NaIO_4$ (2.35g in 10mL warm water, 11mmol) was added at such a rate that the temperature did not exceed 20°C. After the addition, the mixture was stirred for 30-90 minutes at room temperature until TLC showed complete disappearance of the starting material. The mixture was evaporated to almost dryness, the residue was diluted with distilled water and extracted with ethyl acetate or CH_2Cl_2. The combined extracts were dried over anhydrous Na_2SO_4 and the solvent was removed on a rotary evaporator to give the sulfoxide, which can be further purified by flash chromatography on silica gel using 2:1 petroleum ether and acetone as the eluent.

1,3-Dithiolane-1-oxide **4-2a** and 1,3-dithiolane-1-sulfone **4-3a** Yeast-mediated oxidation of **4-1a** (100μL) according to general procedures in presence of 0.8g γ-cyclodextrin followed by flash chromatography on silica gel using 1:1 petroleum ether and ethyl acetate, then acetone as the eluent gave **4-2a** 30mg (26%), **4-3a** 59mg (45%). *E. coli*- mediated oxidation of **4-1a** (50μL) according to general procedures in presence

of 0.5g γ-cyclodextrin followed by flash chromatography on silica gel using 1:1 petroleum ether and ethyl acetate, then acetone as the eluent gave **4-2a** 25mg (43%). IR ν_{max} (neat): 2980(w), 2933(w), 1670(m), 1032(s) cm^{-1}. ^1H NMR δ: 3.95(1H, d, J=12.5), 3.85(1H, dd, J_1=12.5, J_2=1.2), 3.62(1H, td, J_1=11.5, J_2=5.1), 3.47(1H, dm, J_1=14), 3.37(1H, ddd, J_1=12.0, J_2=7.2, J_3=2.4), 2.71(1H, dq, J_1=11.6, J_2=6.8) ppm. ^{13}C NMR δ: 57.1, 55.2, 31.5 ppm. **4-3a** 34mg (52%). IR ν_{max} (neat): 3164(w), 3006(w), 2947(w), 2263(s), 1326(s), 1263(m), 1182(m), 1121(s), 914(vs), 740(vs) cm^{-1}. ^1H NMR δ: 3.83(2H, s), 3.30(2H, t, J=6.4), 3.19(2H, t, J=6.4) ppm. ^{13}C NMR δ: 52.0, 48.4, 25.3 ppm. Chemical oxidation of **4-1a** (0.5g) according to general procedures gave **4-2a** as light brown oil, 350mg (61%).

2-Methyl-1,3-dithiolane-1-sulfoxide **4-2b** and 2-methyl-1,3-dithiolane-1-sulfone **4-3b**

Yeast-mediated oxidation of **4-1b** (100μL) according to general procedures in the presence of 0.7g β-cyclodextrin gave **4-2b** (19mg, 16%) and **4-3b** (20mg, 15%) as pale yellow oils. **4-2b**: IR ν_{max} (neat): 2973(w), 2927(m), 2861(w), 1446(m), 1400(w), 1052(vs), 828(w) cm^{-1}. ^1H NMR δ: 3.98(1H, q, J=6.8), 3.40-3.25(2H, m), 3.17-3.06(2H, m), 1.58(3H, d, J=6.8) ppm.. ^{13}C NMR δ: 56.0, 51.2, 22.9, 14.6 ppm. **4-3b**: IR ν_{max} (neat): 2991(w), 2939(w), 146(m), 1308(vs), 1269(m), 1163(w), 1117(s), 874(w), 742(w) cm^{-1}. ^1H NMR δ: 3.98(1H, q, J=6.8), 3.32(2H, m), 3.10(2H, m), 1.56(3H, d, J=6.8) ppm.. ^{13}C NMR δ: 56.0, 51.1, 22.9, 14.6 ppm. *E. coli* mediated oxidation of **4-1b** (50μL) according to general procedures in presence of 0.7g γ-cyclodextrin gave **4-2b**, 20mg (35%) and **4-3b**, 10mg (16%). Chemical oxidation of **4-**

1b (0.44g, 5mmol) according to general procedures gave **4-2b** as a yellow sticky oil, 0.13g (24%).

2,2-Dimethyl-1,3-dithiolane-1-oxide **4-2c** and 2,2-dimethyl-1,3-dithiolane-1-sulfone **4-3c**

Yeast-mediated oxidation of **4-1c** (100μL) according to general procedures gave **4-2c** as colourless oil, 47mg (42%). IR ν_{max} (neat): 2973(m), 2921(m), 2868(w), 1052(vs) cm^{-1}. ^1H NMR δ: 3.59(1H, m), 3.23(3H, m), 1.63(3H, s), 1.52(3H, s) ppm. ^{13}C NMR δ: 71.3, 54.2, 30.5, 26.6, 21.6 ppm. **4-3c** was observed on GC (12%), but was not isolated in this reaction. *E. coli*-mediated oxidation of **4-1c** (100μL) according to general procedures gave **4-2c** and **4-3c** as light yellow oils, **4-2c** 52mg (46%) and **4-3c** 7mg (6%). **4-2c** IR ν_{max} (neat): 2993(w), 2940(w), 2868(w), 1460(m), 1308(vs), 1262(m), 1177(m), 1144(s), 1104(vs), 736(w), 591(m) cm^{-1}. ^1H NMR δ: 3.41(2H, t, J=7.2), 3.10(2H, t, J=7.2), 1.67(6H, s) ppm. ^{13}C NMR δ: 62.7, 49.6, 24.6, 20.8 ppm. **4-3c**: IR ν_{max} (neat): 2993(m), 2940(m), 2868(w), 1308(vs), 1262(m), 1144(m), 1104(vs) cm^{-1}. ^1H NMR δ: 3.41(2H, t, J=7.2), 3.10(2H, t, J=7.2), 1.67(6H, s) ppm. ^{13}C NMR δ: 62.7, 49.6, 24.6, 20.8 ppm. Chemical oxidation of **4-1c** (0.67g, 5mmol) according to general procedures gave **4-2c** as a light yellow oil, 0.35g (47%).

2-Phenyl-1,3-dithiolane-1-oxide **4-2d** Yeast-mediated oxidation of **4-1d** (50μL) according to general procedures gave **4-2d** as a sticky pale yellow oil (40mg, 74%). IR ν_{max} (neat): 3065(w), 3026(w), 2927(w), 2367(m), 2342(m), 1052(vs), 703(m) cm^{-1}. ^1H NMR δ: 7.51-7.47 (2H, m), 7.41-7.31(3H, m), 3.85(1H, td, J_1=11.3, J_2=5.5), 3.61(1H, qd, J_1=7.0, J_2=2.1), 3.36(1H, dddd, J_1=13.5, J_2=5.5, J_3=2.2, J_4=0.9), 2.91(1H,

dq, J_1=11.3, J_2=7.0) ppm. ^{13}C NMR δ: 133.1, 128.99, 128.97, 128.5, 77.8, 53.2, 32.4 ppm. *E. coli* mediated oxidation of **4-1d** (100μL) according to general procedures gave **4-2d** as a pale yellow oil, 68mg (60%), with the recovery of 30mg starting material (30%). Chemical oxidation of **4-1d** (0.36g) according to general procedures gave **4-2d** as light yellow oil, 0.35g (88%, 11:1 trans/cis according to GC).

1,3-Dithiane-1-oxide **4-2e** and 1,3-dithiane-1-sulfone **4-3e** Yeast-mediated oxidation of **4-1e** (0.1g) according to general procedures followed by chromatography using 1:1 petroleum ether and ethyl acetate, then acetone as the eluent gave **4-2e** (20mg , 18%) and **4-3e** (24mg, 19%) as light brown solids. **4-2e**: IR ν_{max} (neat): 2967(m), 2908(m), 1664(m), 1433(m), 1019(m), 933(m) cm^{-1}. ^{1}H NMR δ: 3.98(1H, d, J=12.3), 3.62(1H, d, 12.8), 3.29(1H, m), 2.67-2.43(4H, m), 2.57(1H, m) ppm. ^{13}C NMR δ: 52.8, 50.4, 28.2, 27.1 ppm. **4-3e**: IR ν_{max} (neat): 2980(m), 2940(w), 2914(w), 1288(vs), 1115(vs), 851(m) cm^{-1}. ^{1}H NMR δ: 3.88(2H, t, J=1.0), 3.10(2H, m), 2.70(2H, m), 2.57(2H, m) ppm. ^{13}C NMR δ: 52.8, 51.2, 28.9, 27.5 ppm. *E. coli*-oxidation of **4-1e** (0.1g) according to general procedures followed by flash chromatography on silica gel using 1:1 petroleum ether and EtOAc, then acetone as the eluent gave **4-2e** 83mg (73%). **4-3e** was observed on GC (*ca.* 6%), but was not isolated from this reaction.

2-Phenyl-1,3-dithiane-1-oxide **4-2f** Yeast-oxidation of **4-1f** (0.1g) was performed according to general procedures and was monitored by HPLC. No attempt was made to isolate the product. *E. coli*-oxidation of **4-1f** (0.1g) according to general procedures gave **4-2f** as a light brown sticky oil, 15mg (13%). IR ν_{max} (neat): 3033(w), 2967(m),

2921(m), 2868(w), 1427(m), 1045(vs), 700(s) cm^{-1}. Chemical oxidation of **4-1f** (0.4g) according to general procedures gave **4-2f** as a white powder, 0.42g (97%). Spectroscopic data were recorded from the diastereomeric mixture (ca. 9:1 *trans:cis* according to NMR), major peaks are reported. IR ν_{max} ($CHCl_3$): 3026(w), 2965(w), 2913(m), 2842(w), 1453(m), 1040(s), 699(s) cm^{-1}. ^1H NMR δ: 7.44-7.37(5H, m), 4.56(1H, s), 3.57(1H, dm, J_1=12.8), 2.89(1H, ddd, J_1=14.2, J_2=12.5, J_3=2.6), 2.76(1H, td, J_1=13.2, J_2=2.7), 2.69(1H, dtd, J_1=14.2, J_2=3.4, J_3=1.4), 2.53(1H, m), 2.38(1H, m) ppm. ^{13}C NMR δ: 133.3, 129.3, 129.1, 128.7, 69.7, 54.8, 31.4, 29.5 ppm.

t-Butyl methyl sulfoxide **4-2g** Yeast-mediated oxidation of **4-1g** (100μL) was performed according to general procedures in a 500mL flask without cyclodextrin. The flask was covered with aluminum foil and sealed with parafilm®. The reaction was allowed to proceed for 2 days without opening the flask. Flash chromatography was performed on a silica gel column using a gradient from 2:1 petroleum ether and acetone to acetone as the eluent to give **4-2g** as a pale yellow oil, 56mg (47%), e.e.>98%. IR ν_{max} (neat): 2960(s), 2934(m), 2875(w), 1368(m), 1045(vs) cm^{-1}. ^1H NMR δ: 2.34(3H, s), 1.21(9H, s) ppm. ^{13}C NMR δ: 52.5, 31.5, 22.4 ppm. Chemical oxidation of **4-1g** (1.04g, 10mmol) according to general procedures gave **4-2g** as a colourless oil, 1.16g (97%). IR ν_{max} (neat): 2970(s), 2940(m), 2920(m), 2870(m), 1170(m), 1050(vs) cm^{-1}. ^1H NMR δ: 2.29(3H, s), 1.16(9H, s) ppm. ^{13}C NMR δ: 52.4, 31.4, 22.3 ppm.

n-Butyl methyl sulfoxide **4-2h** The same method as in **4-2g** was used to oxidize **4-1h** (100μL) to give **4-2h** as a light yellow oil, 61mg (53%), e.e.74%. IR ν_{max} (neat):

2973(s), 2927(m), 2868(m), 1473(w), 1433(w), 1032(vs) cm^{-1}. Chemical oxidation of **4-1h** (1.04g, 10mmol) according to general procedures gave **4-2h** as a yellow oil, 1.08g (90%). IR ν_{max} (neat): 2966(s), 2933(s), 2881(s), 1472(w), 1420(w), 1410(w), 1070(m), 1038(vs) cm^{-1}. ^1H NMR δ: 2.65-2.50(2H, m), 2.44(3H, s), 1.62(pd, J_1=6.7, J_2=0.9), 1.46-1.27(2H, m), 0.84(3H, t, J=7.3) ppm. ^{13}C NMR δ: 54.1, 38.3, 24.3, 21.7, 13.4 ppm.

Phenyl methyl sulfoxide **4-2i**[•] Yeast-mediated oxidation of **4-1i** (100μL) according to general procedures in the presence of 0.5g γ-cyclodextrin gave **4-2i** as a pale yellow oil, 101mg (90%), e.e. >99%. IR ν_{max} (neat): 3055(m), 2993(m), 2914(w), 1091(vs), 1047(vs) cm^{-1}. ^1H NMR δ: 7.65(2H, dd, J_1=8.2, J_2=1.9), 7.57(3H, m), 2.72(3H, s) ppm. ^{13}C NMR δ: 130.7, 129.1, 123.1, 43.7 ppm.

Phenyl propyl sulfoxide **4-2j** Chemical oxidation of **4-1j** (0.3g, 2mmol) according to general procedures gave **4-2j** as a yellow oil, 0.24g (72%). IR ν_{max} (neat): 3065(m), 2967(s), 2934(m), 2875(m), 1486(m), 1453(s), 1091(vs), 1019(vs), 755(vs), 690(vs) cm^{-1}. ^1H NMR δ: 7.61-7.57(2H, m), 7.52-7.43(3H, m), 2.81-2.67(2H, multiplet of 20 peaks), 1.84-1.70(1H, m), 1.69-1.57(1H, m), 1.02(3H, t, J=7.3) ppm. ^{13}C NMR δ: 144.0, 130.9, 129.1, 124.0, 59.2, 15.9, 13.2 ppm.

[•]The reference sample was purchased from Aldrich.

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1988(29)435.], methylstyryl sulfide [Fauve, A., Renard, M.F., Veschambre, H., Madesclaire, M., Roche, D., *Biocatalysis*, 1991(4)265.], and methyl tolyl sulfide.[2]

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[18] Although there has been no evidence that native enzymes in *E. coli* were involved in any of the oxidations tested, control reactions did suggest that stereoselective *reductions* of sulfoxides to sulfides occurred in the case of 2,2-dimethyl-1,3-dithiolane-1-oxide **4-2c** and 1,3-dithiane-1-oxide **4-2e**. This may be responsible for the differences between results obtained from the engineered *E. coli* and those from *Acinetobacter* or isolated CHMO. These observations are not unprecedented since it has been known that *E. coli* harbours several different enzymes capable of reducing sulfoxides to sulfides, such as *E. coli* anaerobic dimethylsulfoxide reductase and *E. coli* biotin sulfoxide reductase. For details about these two enzymes, see (a) Wooton, J.C., Nicolson, R.E., Cock, J.M., Walters, D.E., Burke, J.F., Doyle, W.A., Bray, R.C., *Biochim. Biophys. Acta*, 1991(1057)157; (b) Bilous, P.T., Cole, S.T., Anderson W.F., Weiner, J.H., *Mol. Microbiol.*, 1988(2)785; (c) Trieber, C.A., Rothery, R.A., Weiner, J.H., *J. Biol. Chem.*, 1994(269)7103.

[19] In order to obtain accurate values in the biooxidations, a calibration curve for FID response should be constructed for each individual compound, and each compound should be tested for extraction efficiency from the actual cell culture under different

concentrations. In order to minimize random human and instrument errors, each test point need to be repeated for at least 3 times. In addition to the large number of experiments needed to obtain these calibration coefficient, many of the testing materials are not readily available, especially the monosulfones, which can not be obtained from direct chemical oxidations. The use of representative compounds to determine those correction coefficient will inevitably introduce errors and round all the efforts to qualitative results. Even if calibration were made for each compound, the non-controllable variations in cell growth may still introduce fluctuations in the experimental readings.

Chapter Five: Yeast-Mediated Oxidations of Substrates with Functionalized Side Chains

Introduction

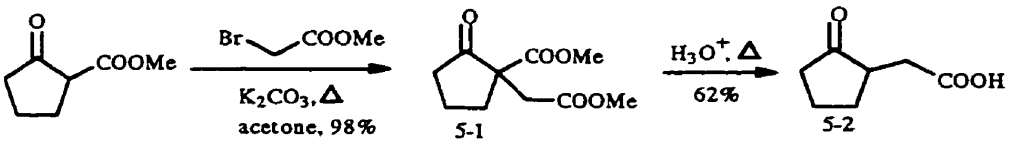
In Chapters Three and Four, the bioengineered yeast reagent was used to oxidize various cyclopentanones and sulfur-containing compounds possessing simple hydrocarbon side chains. To facilitate further derivatizations of the chiral products, and to examine the functional group compatibilities of this bio-oxidant, several cyclopentanones and thiocompounds with functionalized side chains were synthesized and were subjected to biotransformations. Preliminary tests with the yeast and *E. coli* reagents were performed, while systematic investigation and rigorous control experiments have not been possible. The results are summarized in this chapter.

Results and discussions

Synthesis of substrates

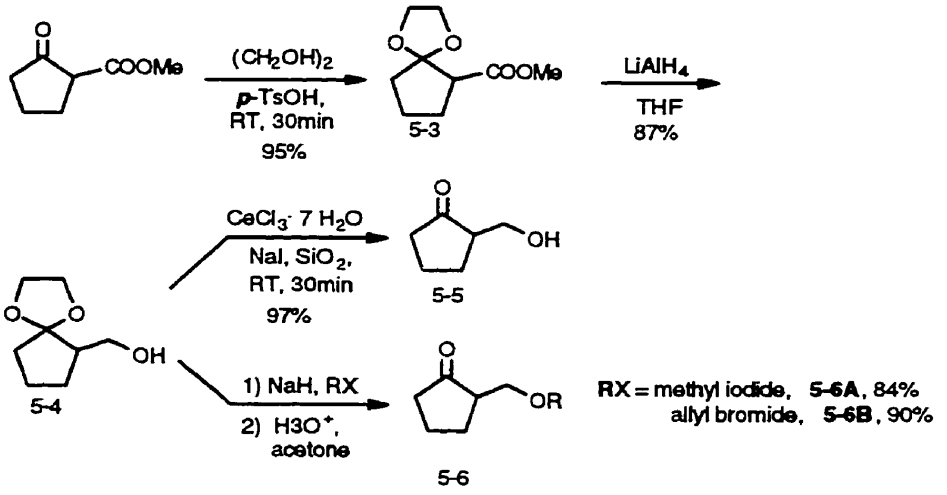
Functionalized cyclopentanones

The synthesis of (2'-oxocyclopentyl)acetic acid **5-2** paralleled that for simple alkyl side chains. Coupling of methyl cyclopentanone-2-carboxylate and methyl bromoacetate followed by acidic hydrolysis and decarboxylation afforded the desired product in good yield (Scheme 33).



Scheme 33

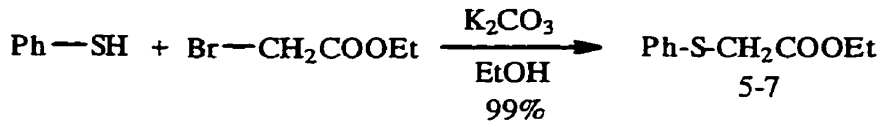
2-Oxocyclopentyl-1-methanol **5-5** and 2-alkoxymethylcyclopentanones **5-6** were synthesized in 3 and 4 steps respectively from methyl cyclopentanone-2-carboxylate. The keto group was protected as an ethylene ketal and the ester group was reduced by $LiAlH_4$ to give the hydroxyketal **5-4**. Hydrolysis of the ketal protective group with mild Lewis acid catalyst preserved the hydroxyl group and yielded hydroxyketone **5-5**. Alkylation of **5-4** followed by hydrolysis with dilute aqueous acid gave the alkoxyketone **5-6** (Scheme 34).



Scheme 34

Functionalized sulfides

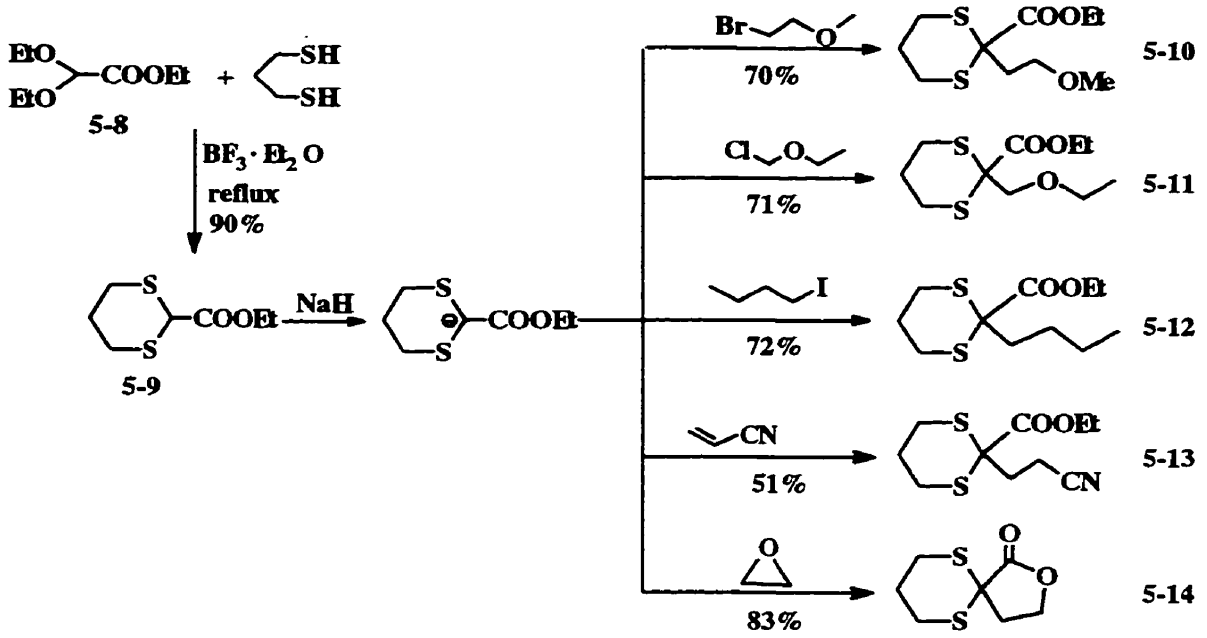
Ethyl thiophenylacetate **5-7** was synthesized by the coupling of ethyl bromoacetate and thiophenol under basic conditions. The desired product was isolated in a quantitative yield (Scheme 35).



Scheme 35

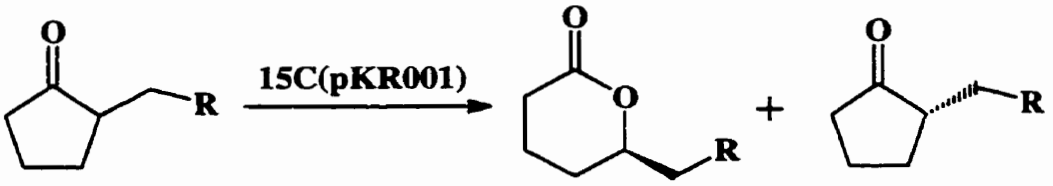
Functionalized 1,3-dithianes

Ethyl 1,3-dithiane-2-carboxylate **5-9** was synthesized by transketalization between commercially available diethoxy acetic acid ethyl ester **5-8** and 1,3-propanedithiol using $BF_3 \cdot Et_2O$ as a Lewis acid catalyst. Treatment of **5-9** with NaH gave the stabilized carbanion intermediate which was trapped by the subsequent addition of suitable electrophiles to give several 2,2-disubstituted dithianes (Scheme 36).


EtO
EtO
COOEt
5-8
SH
SH
BF3 · Et2 O
reflux
90%
S
S
COOEt
5-9
NaH
S
S
COOEt
Br
O
70%
S
S
COOEt
OMe
5-10
Cl
O
71%
S
S
COOEt
O
5-11
I
72%
S
S
COOEt
5-12
CN
51%
S
S
COOEt
CN
5-13
O
83%
O
S
O
S
5-14

Scheme 36

Oxidation of 2-functionalized cyclopentanones



R = OH,	5-5	5-15	5-16
R = COOH,	5-2	5-17	5-18
R = OMe,	5-6A	5-19	5-20
R = $OCH_2CH{=}CH_2$,	5-6B	5-21	5-22

Scheme 37

There have been reports that cyclic ketones with unprotected hydroxyl groups[1,2] were oxidized to the corresponding lactones with isolated CHMO. It was also established in Chapter Two that 4-hydroxycyclohexanone was oxidized successfully by the engineered yeast. The oxidations of cyclopentanones substituted with polar side chains at the 2-position appeared to parallel these reactions (Scheme 37). By extrapolation from 4-hydroxycyclohexanone and 2-hydroxymethylcyclopentanone,* these reactions are generally slower than those with non-polar side chains, and the conversions were not complete even after prolonged reaction periods. The difficulty in transporting these polar substrates across cell membranes could be partially responsible for the low reaction rates.† When the hydroxy group in **5-5** was protected as its ether derivatives (**5-6A** and

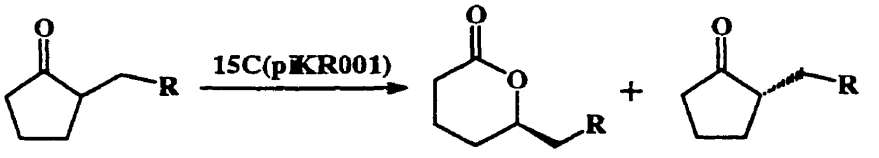
---

* (2'-oxocyclopentyl)acetic acid **5-2** is too polar to be monitored by GC and lacks an effective chromophore to be monitored on HPLC; therefore the kinetic data for its oxidation are not available.

† Slow mass transportation of polar compounds across the cell membrane was also observed in the oxidation of sulfur compounds, see Chapter Four.

5-6B), the oxidations became faster, which supported the polarity argument to some extent. In addition to the reactions being slow and incomplete, the enantioselectivities for substrates with oxygenated side chains were generally low. The hydroxyketone **5-5** was oxidized to lactone **5-15** with an estimated[3] 34% e.e. at 24% conversion while prochiral 4-hydroxycyclohexanone **2-2** was oxidized with only 12% e.e. Although substrates with alkoxy side chains reacted more rapidly than ketones with free hydroxyl groups, their enantioselectivity was lower compared to simple alkyl substituted ketones with a similar chain length. The results from these reactions are summarized in Table 18.

Table 18: Oxidation of 2-functionalized cyclopentanones



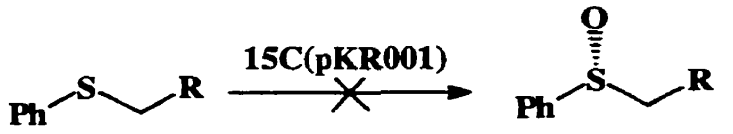
R = OH,	5-5	5-15	5-16
R = COOH,	5-2	5-17	5-18
R = OMe,	5-6A	5-19	5-20
R = $OCH_2CH=CH_2$,	5-6B	5-21	5-22

Substrate	R	Time (hours)	Ketone[1] % (e.e. %) $[\alpha]_D$	Lactone[1] % (e.e. %) $[\alpha]_D$
5-5	OH	96	20(11)	26(34)[2]
5-2	COOH	96	not determined	not determined
5-6A	OMe	36	66[3](7)[4] [-17, c1.3]	14[3] (44)
5-6B	$OCH_2CH=CH_2$	36	19 (81)[4] [-56, c1.9]	33 (93) [-14, c2.2]

(1) Isolated yield of chromatographically purified products; (2) The lactone decomposed on GC; the e.e. value was estimated using the method described in [3]; (3) GC yields based on the internal standard method; (4) The ketone was not resolved on chiral GC; its e.e. was estimated using the method described in [3].

Oxidation of functionalized sulfur compounds

Oxidation of sulfides



R = COOEt, 5-7 — 5-24
R = $CH_2CH_2OCH_3$, 5-23 — 5-25

Scheme 38

It has been concluded that in the oxidation of simple hydrocarbon sulfides one large group and one small group are both necessary to obtain good activity and enantioselectivity. The best substrates are methyl sulfides, and any group longer than methyl is not desired.* When sulfides **5-7** and **5-23** were subjected to yeast oxidation conditions, they both failed to give the oxidized product (Scheme 38). It is not sure at this stage whether the polarity of the side chains played any role in this failure, but judging from their chain length, neither good reactivity nor high enantioselectivity should be expected.

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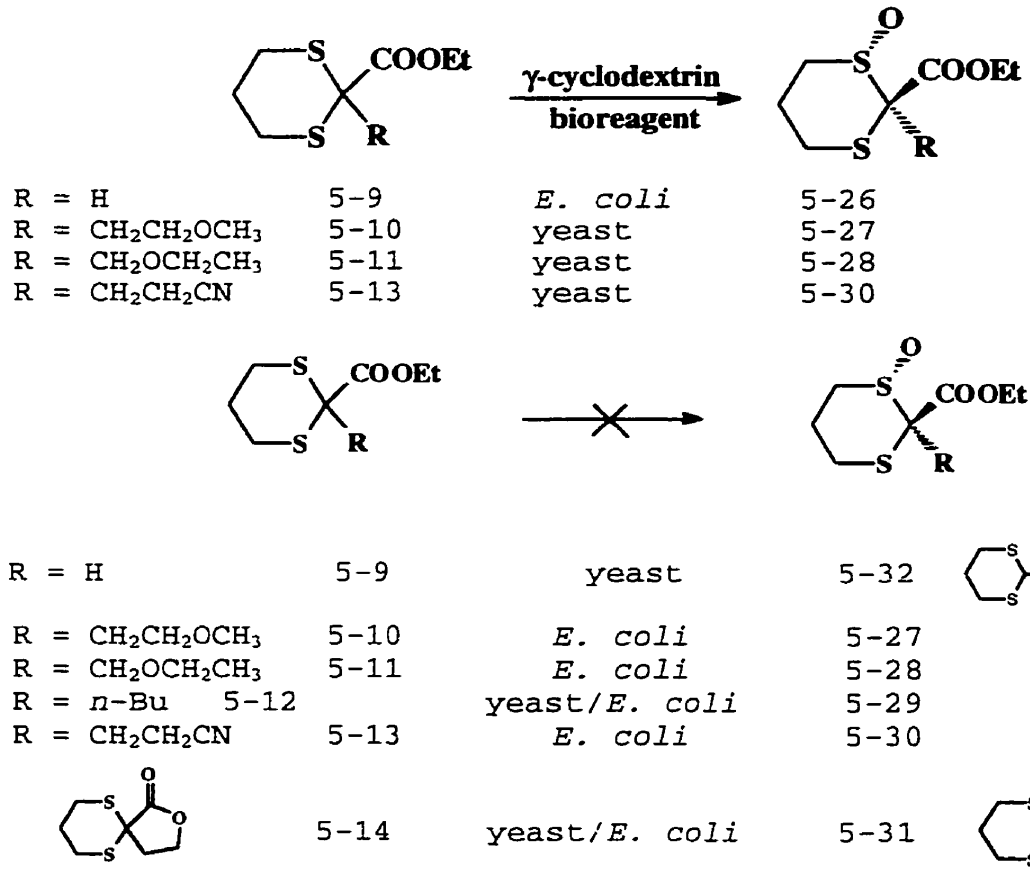
* For detailed discussion, see Chapter Four.



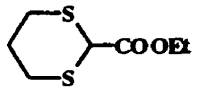
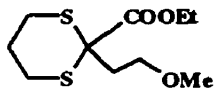
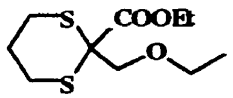
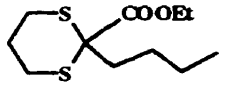
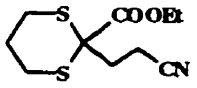
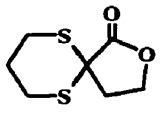
Scheme 39

The oxidations of functionalized dithianes are highly dependent on the nature of the individual substrate and the bioreagent used. The reactions are outlined in Scheme 39. When **5-9** was incubated with the yeast reagent for as long as 90 hours, 71% of the starting material was recovered together with 17% of hydrolyzed material (**5-32**) and no evidence of oxidation; when this compound was incubated with the bioengineered *E. coli*, 37% of the sulfoxide (**5-26**) was formed within 15 hours in addition to 10% sulfone and 12% of recovered starting material. On the other hand, **5-10** and **5-11** were successfully oxidized by the yeast reagent while *E. coli* failed to produce any desired sulfoxide and most of the starting material was recovered after 48 hours.

Interestingly, neither yeast nor *E. coli* was successful in oxidizing **5-12**, even though this compound closely resembles **5-10** and **5-11**. This may suggest that an ether linkage is necessary for this type of substrate to fit into the active site of CHMO. Therefore, there might be an amino acid residue at the active site that can make a hydrogen bond with the etherial oxygen. This hydrogen bonding postulation could be indirectly supported by the reactivity of cyclopentanones and cyclohexanones bearing oxygenated substituents. Since the oxidations of both **5-10** and **5-11** showed excellent enantio- and diastereoselectivities, which could be explained by a favourable arrangement of the functional groups in the active site, the difficulty to oxidize **5-13** and the failure in oxidizing **5-14** might be a result from the rigidity and unfavorable shapes of these compounds. The results are summarized in Table 19.

As an internal control reaction, a trace amount (5%) of cyclohexanone had been added together with the substrates in all the reactions. Its prompt and complete conversion to caprolactone indicated the normal cell growth and activity of yeast and *E. coli* were not inhibited by any of these thiocompounds. Thus the difference in their reactivities was most likely due to the difference in cell membrane permeabilities. In control experiments, compounds **5-10** and **5-11** were incubated with the carrier yeast and with commercial baker's yeast and showed no reaction, which demonstrated that the native enzymes are not responsible for the oxidations of **5-10** and **5-11**.

Table 19: Oxidation of functionalized dithianes

Entry	Substrate		reagent[1]	Time (h)	SM[2] (%)	SO[3] % (e.e.%) [*trans:cis*]
1	Ph-S-$CH_2CH_2OCH_3$	**5-23**	A	130	35	0
2	Ph-S-$CH_2COOCH_2CH_3$	**5-7**	A	150	40	0
3		**5-9**	A B	90 15	71 (hydrolysis 17%) 12	0 37 [2:1]
4		**5-10**	A B C D	130 48 110 110	not determined 60 >90[4] >90[4]	63 (99) [50:1] not determined 0 0
5		**5-11**	A D	130 110	not determined 86	64 (99) [19:1] 0
6		**5-12**	A B D	130 48 110	>90[4] >90[4] >90[4]	0 0 0
7		**5-13**	A	130	78	ca. 8
8		**5-14**	A	130	ND	0

1. A = 15C(pKR001), B = BL21(DE3)(pMM04), C = 15C, D = commercial baker's yeast **2.** GC results. **3.** Sulfoxide, isolated after chromatography. **4.** GC ratio exceeded 100% according to internal standard calculations, probably due to evaporation of the medium during prolonged incubations.

Conclusions

Preliminary experiments showed that cyclopentanones bearing oxygenated side chains are acceptable substrates for the bioengineered yeast 15C(pKR001). Substrates with polar side chains such as an unprotected hydroxyl group can be oxidized, but the reaction is slow, incomplete, and the enantioselectivity is low. Cyclopentanones with less polar side chains, such as alkoxides, are better substrates. The reactions are faster,

but the enantioselectivities are still lower than those of simple alkyl substituted cyclopentanones with similar chain lengths.

Dithianes with an ester side chain at the 2-position can be oxidized by CHMO, but the selectivity of cell membrane may in some cases retard the oxidation. 2,2-Disubstituted dithianes with one ester chain and one ether chain appeared to be good substrates, both the enantioselectivity and the diastereoselectivity are very high. However similar compounds with no ether group in the side chain or with rigid substituents did not seem to be suitable substrates.

Experimental

Synthesis of 2-functionalized cyclopentanones

1,4-Dioxa-spiro[4.4]nonane-6-carboxylic acid methyl ester **5-3** Methyl cyclopentanone-2-carboxylate (5.0mL, 40mmol) was added to a solution of *p*-toluene sulfonic acid (5.6g, 40mmol) in ethylene glycol (20mL) and stirred at room temperature for 30 minutes. The mixture was poured into 50mL 1M KOH solution saturated with NaCl and extracted with 200mL ether in 4 portions. Combined extracts were washed once with brine and dried over anhydrous Na_2SO_4. The solvent was removed on a rotary evaporator to give **5-3** as a colourless oil, 4.73g (75%). IR ν_{max} (neat): 2950(s), 2890(m), 1700(vs), 1480(m), 1350(m), 1210(s), 1040(m) cm^{-1}. ^1H NMR δ: 4.05-4.00(1H, m), 3.97-3.88(3H, m), 3.70(3H, s), 2.92(1H, t, J=7.7), 2.12(1H, m), 1.98-1.79(4H, m), 1.67(1H, m) ppm. ^{13}C NMR δ: 172.8, 118.3, 65.1, 64.5, 52.2, 51.7, 51.6, 36.7, 26.9, 22.0 ppm.

1,4-Dioxa-spiro[4.4]non-6-yl-methanol **5-4** 1,4-Dioxa-spiro[4.4]nonane-6-carboxylic acid methyl ester (3.8g in 10mL THF, 20mmol) was added to a vigorously stirred suspension of $LiAlH_4$ (0.8g, 21mmol) in THF (60mL) at 0°C. The mixture was stirred for 1.5 hours and allowed to warm up to room temperature. The reaction was quenched by pouring into an ice-cold saturated tartaric acid solution and extracted with 200mL ethyl acetate in 5 portions. The combined extracts were dried over anhydrous Na_2SO_4 and the solvent was removed on a rotary evaporator to give **5-4** as a colourless oil, 2.81g (87%). IR ν_{max} (neat): 3500(m, br), 2950(s), 2890(s), 2390(s), 2280(m), 1460(m), 1390(m), 1330(m), 1160(s), 1100(m), 1020(s) cm^{-1}. ^1H NMR δ: 3.96-3.84(4H, m), 3.66-3.54(2H, multiplet of 8 peaks), 2.64(1H, br), 2.34-2.05(1H, multiplet of 8 peaks), 1.84-1.76(1H, m), 1.74-1.46(5H, m) ppm. ^{13}C NMR δ: 119.0, 64.5, 64.0, 62.5, 47.0, 35.6, 25.6, 21.3 ppm.

2-Hydroxymethylcyclopentanone **5-5**[41] $CeCl_3 \cdot 7H_2O$ (1.86g, 5mmol) and KI (0.3g, 1.8mmol) were mixed with 18g silica gel (200-425 mesh) in 30mL acetone and ground with a mortar to mix well. 1,4-Dioxa-spiro[4.4]non-6-yl-methanol (**5-4**, 0.96g, 6mmol) was added and the mixture was stirred at room temperature for 30 minutes. The silica was filtered off and was washed with acetone. The solvent was removed on a rotary evaporator and the residue was purified by flash chromatography using 3:1 hexane and ethyl acetate as the eluent to give **5-5** as a colourless oil, 0.67g (97%). IR ν_{max} (neat): 3427(s, br), 2966(s), 2877(s), 1734(vs), 1404(m), 1154(m), 1054(m). ^1H NMR (acetone-d6) δ: 3.73-3.64(3H, including an overlapping OH peak, m), 2.26-2.12(3H, m),

2.10-1.95(2H, m), 1.96-1.75(2H, m) ppm. ^{13}C NMR (acetone-d6) δ: 219.5, 61.6, 51.7, 39.0, 27.1, 21.4 ppm.

6-Methoxymethyl-1,4-dioxa-spiro[4.4]nonane NaH (2g, 50% dispersion in mineral oil, excess) was added to a 100mL 2-neck round bottom flask and the system was flushed with nitrogen. The NaH was washed 3 times with THF, and was finally suspended in 10mL THF. MeI (2mL, excess) was added, followed by 1,4-dioxa-spiro[4.4]non-6-yl-methanol (**5-4**, 1.58g in 40mL THF, 10mmol) with vigorous stirring. After reacting overnight, the mixture was poured onto ice cubes and extracted with 100mL ethyl acetate in 3 portions. The combined extracts were washed once with 0.2M KOH, once briefly with 0.5M HCl and once with brine. After drying over anhydrous K_2CO_3, the solvent was removed on a rotary evaporator to give the title product as a colourless oil, 1.11g (89%). IR ν_{max} (neat): 2950(vs), 2870(vs), 1450(s), 1380(s), 1320(s), 1200(s), 1100(vs), 750(s) cm^{-1}. The crude product was used in the next step without further characterizations.

6-Allyloxymethyl-1,4-dioxa-spiro[4.4]nonane This compound was synthesized with the same protocol using allyl bromide instead of MeI. The crude product was used in the next step without further purification.

2-Methoxymethylcyclopentanone **5-6A** 6-Methoxymethyl-1,4-dioxa-spiro[4.4]nonane (0.8g, 4.7mmol) was dissolved in 10mL acetone. In a separate flask 30mg of $PdCl_2$ was dissolved in 1mL acetone and 0.5mL of acetonitrile. The two solutions were combined and 1mL TFA was added. The mixture was stirred at room temperature for 3 hours, poured into brine and extracted with 100mL ethyl acetate in 3 portions. The combined extracts were dried over anhydrous Na_2SO_4 and the solvent was removed on a

rotary evaporator. The residue was purified by flash chromatography using 10:1 hexane and ethyl acetate as the eluent to give **5-6A** as a colourless oil, 0.56g (94%). IR ν_{max} (neat): 2970(s), 2920(s), 2870(s), 1730(s), 1150(m), 1120(m) cm^{-1}. ^1H NMR δ: 3.53(2H, m), 3.30(3H, s), 2.34-1.96(5H, m), 1.80(2H, m) ppm. ^{13}C NMR δ: 219.5, 71.5, 59.0, 49.4, 38.6, 27.1, 20.8 ppm.

2-Allyloxymethylcyclopentanone **5-6B** Crude 6-allyloxymethyl-1,4-dioxa-spiro[4.4]-nonane was dissolved in a mixture of 20mL acetone and 5mL 2M HCl and stirred at room temperature for 2 hours. The acetone was removed by rotary evaporation and the residue was extracted with CH_2Cl_2. The combined extracts were dried with anhydrous Na_2SO_4 and the solvent was removed by rotary evaporation. The crude product was purified by flash chromatography using 6:1 hexane and ethyl acetate as the eluent to give **5-6B** as a colourless oil, 0.35g (90% from **5-4**). IR ν_{max} (neat): 3081(w), 2967(s), 2877(s), 1737(vs), 1647(w), 1152(s), 1088(s), 926(s) cm^{-1}. ^1H NMR δ: 5.84-5.73(1H, m), 5.20-5.04(2H, m), 3.87(2H, dt, J_1=5.5, J_2=1.4), 3.57-3.48(2H, multiplet of 8 peaks), 2.31-2.10(3H, m), 2.12-2.00(1H, m), 2.00-1.91(1H, m), 1.88-1.76(1H, m), 1.76-1.65(1H, m) ppm. ^{13}C NMR δ: 219.3, 134.5, 116.6, 71.9, 68.9, 49.2, 38.5, 27.0, 20.7 ppm.

2-Hydroxymethyltetrahydropyran-2-one **5-15** prepared as described in general procedures was purified by flash chromatography on silica gel using 2:1 petroleum ether and acetone as the eluent. IR ν_{max} (neat): 3394(m, br), 2947(m), 2881(w), 1723(vs), 1249(s), 1058(s) cm^{-1}. ^1H NMR δ: 4.42-4.35(1H, multiplet of 12 peaks), 3.76(1H, dd, J_1=12.1, J_2=3.2), 3.66(1H, dd, J_1=12.3, J_2=5.5), 2.64-2.54(1H, m), 2.48-

2.39(1H, m), 2.00-1.90(1H, m), 1.90-1.81(2H, m), 1.81-1.64(2H, m) ppm. ^{13}C NMR δ: 171.4, 81.0, 64.9, 29.6, 23.6, 18.3 ppm.

6-Methoxymethyltetrahydropyran-2-one **5-19** prepared as described in general procedures was purified by flash chromatography on silica gel using 5:1 petroleum ether and acetone followed by 3:1 petroleum ether and acetone as the eluent. IR ν_{max} (neat): 2940(m), 2881(m), 2815(w), 1736(vs), 1249(s), 1071(s) cm^{-1}. ^{1}H NMR δ: 4.46-3.90(1H, m), 3.55-3.47(1H, m), 3.36(3H, s), 3.28-3.18(1H, m), 2.62-2.53(1H, m), 2.43-2.34(1H, m), 1.98-1.87(2H, m), 1.74-1.62(2H, m) ppm. ^{13}C NMR δ: 172.6, 79.0, 74.4, 59.4, 29.6, 24.5, 18.3 ppm.

6-Allyloxymethyltetrahydropyran-2-one **5-21** prepared as described in general procedures was purified by flash chromatography on silica gel using 5:1 petroleum ether and acetone followed by 3:1 petroleum ether and acetone as the eluent. IR ν_{max} (neat): 3078(w), 2947(m), 2876(m), 1726(vs), 1453(m), 1347(m), 1240(s), 1062(s), 932(m) cm^{-1}. ^{1}H NMR δ: 5.91-5.80(1H, multiplet of 12 peaks), 5.25(1H, dq, J_1=17.3, J_2=1.5), 5.16(1H, qd, J_1=10.4, J_2=1.2), 4.46-4.39(1H, multiplet of 12 peaks), 4.01(2H, J_1=5.5, J_2=1.5), 3.56(2H, multiplet of 8 peaks), 2.60-2.52(1H, m), 2.48-2.39(1H, m), 1.98-1.88(2H, m), 1.87-1.76(1H, m), 1.75-1.63(1H, m) ppm. ^{13}C NMR δ: 171.2, 134.2, 117.3, 79.1, 72.5, 71.8, 29.6, 24.6, 18.2 ppm. MS m/e 171(M+1, 0.2), 114(48), 99(58), 71(100), 55(28). $[\alpha]_D^{25} = -14.1°$ ($CDCl_3$, c2.2).

General procedures for synthesizing 2-functionalized 1,3-dithianes

NaH (50% dispersion in mineral oil) was put in a 100mL 3-neck round bottom flask equipped with a refluxing condenser, a pressure equalizing dropping funnel, and a

nitrogen balloon. The system was flushed 3 times with nitrogen, and NaH was washed 3 times with THF. A solution of 1,3-dithiane-2-carboxylic acid ethyl ester (**5-9**, 10mmol in 20mL THF) was added and the mixture was heated to approximately 70°C and stirred until a homogeneous gray-greenish suspension was obtained. The mixture was cooled in an ice-water bath and appropriate electrophile in THF was added slowly with vigorous stirring. When the reaction had finished, the mixture was poured into ice water and extracted with 100mL ethyl acetate in 3 portions. The combined extracts were dried with anhydrous Na_2SO_4 and the solvent was removed on a rotary evaporator. The residue was purified by flash chromatography using mixtures of petroleum ether and ethyl acetate as the eluent to give the product.

2-(2'-Methoxyethyl)-1,3-dithiane-2-carboxylic acid ethyl ester **5-10** NaH (1g, 20mmol), **5-9** (1.92g in 10mL THF, 10mmol) and 2-bromoethyl methyl ether (2.0g in 10mL THF, 16mmol) were reacted at 50°C for 4 hours according to general procedures. The mixture was poured into distilled water and the pH was adjusted to acidic range with 2M HCl. The mixture was extracted with 100mL petroleum ether in 3 portions. The combined extracts were dried with anhydrous $MgSO_4$ and the solvent was removed on a rotary evaporator. The residue was purified by flash chromatography using 8:1 petroleum ether and ethyl acetate as the eluent to give **5-10** as a light yellow oil, 1.75g (70%). IR ν_{max} (neat): 2990(s), 2840(s), 2900(s), 2840(m), 2820(m), 1730(vs), 1420(m), 1220(s), 1115(s), 1020(m) cm^{-1}. ^1H NMR δ: 4.25(2H, q, J=7.0), 3.60(2H, t, J=7.0), 3.31(3H, s), 3.30(2H, td, J_1=12.2, J_2=2.75), 2.67(2H, m), 2.32(2H, t, J=6.7),

2.15(1H, m), 1.87(1H, m), 1.33(3H, t, J=7.0) ppm. ^{13}C NMR δ: 171.0, 68.1, 61.9, 60.4, 58.8, 38.2, 27.8, 24.6, 14.2 ppm.

2-(2'-Ethoxymethyl)-1,3-dithiane-2-carboxylic acid ethyl ester **5-11** NaH (4g, excess), chloromethyl ethyl ether (3mL, 32mmol), and **5-9** (3.84g in 20mL THF, 20mmol) were reacted at refluxing temperature for 1 hour according to general procedures. The crude product was purified by flash chromatography using 10:1 petroleum ether and acetone as the eluent to give **5-11** as a colourless oil, 3.57g (71%). IR ν_{max} (neat): 2973(m), 2934(m), 2901(m), 2868(w), 1729(vs), 1440(w), 1229(vs), 1117(s), 1071(m), 1019(w) cm^{-1}. ^{1}H NMR δ: 4.22(2H, q, J=7.0), 3.77(2H, s), 3.52(2H, q, J=7.0), 3.20(2H, ddd, J_1=14.5, J_2=11.8, J_3=2.7), 2.65(2H, ddd, J_1=14.4, J_2=5.0, J_3=3.2), 2.09(1H, multiplet of 13 peaks), 1.92-1.79(1H, m), 1.27(3H, t, J=7.2), 1.13(3H, t, J=7.0) ppm. ^{13}C NMR δ: 170.0, 74.5, 67.4, 61.8, 53.0, 27.0, 24.7, 14.7, 14.0 ppm.

2-*n*-Butyl-1,3-dithiane-2-carboxylic acid ethyl ester **5-12** NaH (0.8g, excess), **5-9** (1.92g, 10mmol), and *n*-BuI (2mL, excess) were reacted at room temperature overnight according to general procedures to give **5-12** as a pale yellow oil, 1.78g (72%). IR ν_{max} (neat): 2960(m), 2930(m), 2870(w), 1725(vs), 1465(w), 1420(w), 1240(m), 1200(s), 1120(m), 1025(m) cm^{-1}. ^{1}H NMR δ: 4.20(2H, q, J=7.2), 3.24(2H, m), 2.60(2H, ddd, J_1=14.3, J_2=4.3, J_3=3.2), 2.1(1H, dm, J_1=14.0), 1.96-1.91(2H, m), 1.87-1.71(2H, m), 1.46-1.33(2H, m), 1.28(1H, t, J=7.7), 1.27(3H, t, J=7.2), 0.85(3H, t, J=7.5) ppm. ^{13}C NMR δ: 170.9, 61.6, 52.6, 38.6, 27.8, 26.4, 24.7, 22.8, 14.1, 13.7 ppm.

2-(2'-Cyanoethyl)-1,3-dithiane-2-carboxylic acid ethyl ester **5-13** NaH (1g, 20mmol), **5-9** (1.92g, 10mmol) and acrylonitrile (1mL in 10mL THF, 15mmol) were reacted for

30 minutes at 0°C according to general procedures. The product was purified by flash chromatography using 8:1 petroleum ether and ethyl acetate as the eluent to give **5-13** as a sticky, colourless oil, 1.24g (51%). IR ν_{max} (neat): 2980(m), 2934(m), 2894(m), 2829(w), 2256(m), 1736(vs), 1427(m), 1223(vs), 1183(s), 1052(m) cm^{-1}. ^1H NMR δ: 4.28(2H, q, J=7.0), 3.35(2H, m), 2.77-2.62(4H, m), 2.38(2H, t, J=8.9), 2.18(1H,m), 1.74(1H, m), 1.35(3H, t, J=7.0) ppm. ^{13}C NMR δ: 170.0, 119.1, 62.2, 60.3, 33.8, 27.5, 24.0, 14.1, 13.0 ppm.

2-Oxa-6,10-dithia-spiro[4.5]decan-1-one **5-14** NaH (0.5g, 50% dispersion in mineral oil, 10mmol) was placed in a 100mL 3-neck round bottom flask equipped with a pressure equalizing dropping funnel and a nitrogen inlet tube. The system was flushed with nitrogen and the NaH was washed 3 times with THF. **5-9** (1.92g in 20mL THF, 10mmol) was added and the flask was heated to 35-40°C for 20 minutes until a homogeneous gray-greenish suspension was obtained. In another 50mL 3-neck round bottom flask equipped with a pressure equalizing dropping funnel and a nitrogen balloon put 0.8g NaH (50% dispersion in mineral oil, 17mmol). The system was flushed 3 times with nitrogen and the NaH was washed 3 times with THF. 10mL THF was added and the flask was cooled to -15°C in an ice-salt bath. 2-Bromoethanol (1.25g in 50mL THF, 10mmol) was added slowly. After stirring at -15°C for 30 minutes, this solution was transferred to the first flask via cannula over a period of 5 minutes at -15°C. The flask was kept at -15°C to 0°C for 30 minutes, and then warmed up to room temperature over 30 minutes. The mixture was poured into ice water and extracted with 100mL ether in 3 portions. The solvent was removed on a rotary

evaporator and the residue was purified by flash chromatography using 8:1 petroleum ether and ethyl acetate as the eluent to give **5-14** as a pale yellow oil, 1.59g (83%). IR ν_{max} (neat): 2987(s), 2934(s), 1736(vs), 1295(s), 1144(vs), 1032(s) cm^{-1}. ^1H NMR δ: 4.16(2H, q, J=6.8), 3.34(2H, ddd, J_1=14.0, J_2=11.2, J_3=2.8), 2.54(2H, ddd, J_1=13.6, J_2=5.6, J_3=3.2), 2.07(1H, m), 1.96(1H, m), 1.25(2H, t, J=6.8) ppm. ^{13}C NMR δ: 169.7, 61.5, 60.2, 39.9, 25.8, 24.9, 13.9 ppm.

Ethyl thiophenylacetate **5-7** K_2CO_3 (1.5g, 11mmol) was suspended in anhydrous ethanol (10mL) under nitrogen and cooled in an ice bath. Thiophenol (1mL, 1.1g, 10mmol) was added, followed by ethyl bromoacetate (1.1mL, 1.67g, 10mmol). After stirring at room temperature for 1 hour, the mixture was filtered through a sintered glass funnel and the solid was washed with ethanol. The filtrate was concentrated by rotary evaporation. The residue was dissolved in CH_2Cl_2 and passed through a small silica plug, and the silica was washed with CH_2Cl_2. The solvent was removed by rotary evaporation to give **5-7** as a colourless oil, 1.78g (>99%). IR ν_{max} (neat): 3065(w), 2987(m), 2947(w), 2908(w), 1749(vs), 1275(s), 1150(s), 1038(s), 749(s) cm^{-1}. ^1H NMR δ: 7.43-7.39(2H, m), 7.32-1.20(3H, m), 4.16(2H, q, J=7.2), 3.63(3H, s), 1.22(3H, t, J=7.2) ppm. ^{13}C NMR δ: 169.6, 134.9, 130.0, 129.0, 126.9, 61.5, 36.7, 14.0 ppm.

2-(2'-Methoxyethyl)-1-oxo-1,3-dithiane-2-carboxylic acid ethyl ester **5-27** 2-(2'-methoxyethyl)-1,3-dithiane-2-carboxylic acid ethyl ester (**5-10**, 200μL, 0.86mmol) was oxidized with 15C(pKR001) according to general procedures. The product was purified by chromatography using 4:1 petroleum ether and ethyl acetate as the eluent to give **5-27** as a colourless oil, 130mg (61%), together with some recovered starting material

(74mg, 37%). IR ν_{max} (neat): 2993(m), 2927(m), 2815(w), 1723(s), 1229(s), 1124(s), 1038(s) cm^{-1}. ^1H NMR δ: 4.40-4.28(2H,m), 3.66-3.60(2H, m), 3.32(3H, s), 3.31-3.21(2H, m), 3.13-3.06(1H, m), 2.85-2.75(1H, m), 2.49-2.40(2H, m), 2.32-2.20(2H, m), 1.35(3H, t, J=7.2) ppm. ^{13}C NMR δ: 166.8, 67.4, 66.7, 62.6, 58.6, 47.5, 34.1, 26.7, 26.6, 14.1 ppm. Chemical oxidation was performed overnight on 0.25g **5-10** (1mmol) according to general procedures. Flash chromatography using 8:1 petroleum ether and acetone, then 2:1 petroleum ether and acetone as eluent gave 95mg of product (36%) as a colourless oil.

2-Ethoxycarboxy-2-ethoxymethyl-1,3-dithiane-1-oxide **5-28** 2-Ethoxycarboxy-2-ethoxy-methyl-1,3-dithiane (**5-11**, 25μL) was oxidized by yeast according to general procedures to give **5-28** as colourless oil, 17mg(64%). IR ν_{max} (neat): 2978(m), 2928(m), 2871(w), 1718(s), 1225(m), 1116(m), 1056(m) cm^{-1}. ^1H NMR δ: 4.33(2H, m), 4.30(2H, s), 3.50(2H, m), 3.38(2H, m), 3.13(1H, m), 2.42(2H, m), 2.30(1H, m), 1.33(3H, t, J=7.0), 1.20(3H, t, J=7.0) ppm. ^{13}C NMR δ: 166.0, 69.8, 67.8, 63.8, 62.7, 47.7, 27.8, 26.5, 14.8, 14.2 ppm. Chemical oxidation: **5-11** (0.25g) was oxidized according to general procedures to give **5-28** as colourless sticky oil, 168mg (63%) as a mixture of two diastereomers, the spectroscopic data were too complex to assign.

2-(2'-Cyanoethyl)-1-oxo-1,3-dithiane-2-carboxylic acid ethyl ester **5-30** 2-(2'-cyano-ethyl)-1,3-dithiane-2-carboxylic acid ethyl ester (**5-13**, 100μL, 0.41mmol) was oxidized in 200mL YPG in presence of 0.4g γ-cyclodextrin according to the general procedure. The crude product was purified by chromatography using 4:1 petroleum ether and ethyl acetate, then ethyl acetate as the eluent to give a small amount of product (9mg, 8%) as

a white crystal together with the recovered starting material (80mg, 78%). The major IR peaks were in agreement with those obtained from chemically oxidized product. Since the total conversion was low, further characterization of the product was not performed. IR ν_{max} (neat): 2967(m), 2927(m), 2875(w), 1729(m), 1664(s), 1446(m), 1249(m), 1052(m) cm^{-1}. Chemical oxidation was performed on 0.25g starting material (1mmol) according to general procedures. Flash chromatography using 2:1 petroleum ether and acetone as the eluent gave 214mg product (80%) as a sticky, slightly cloudy oil. HPLC barely resolved the two diastereomers(approximately 8:1 ratio), the enantiomers were not resolved. Spectroscopic data were obtained from the mixture of two diastereomers. Some peaks overlapped, characteristic peaks were reported where possible. IR ν_{max} (neat): 2986(m), 2934(m), 2263(w), 1729(vs), 1440(m), 1242(s), 1196(s), 1065(s) cm^{-1}. ^{1}H NMR δ: 4.28(2H, q, J=7.2), 3.37-3.19(2H, m), 3.09(1H, m), 2.97(1H, m), 2.72(1H, dd, J1=14.7, J2=6.5), 2.61(2H, m), 2.42-2.30(2H, m), 2.27-2.12(1H, m) ppm. ^{13}C NMR δ: 165.9, 118.2, 67.9, 63.2, 47.8, 30.5, 27.3, 26.6, 14.0, 12.7 ppm.

6-Oxo-2-oxa-6,10-dithia-spiro[4.5]decan-1-one **5-31** 2-oxa-6,10-dithiaspiro[4.5]decan-1-one (100μL, 0.53mmol) was subjected to the yeast-oxidation conditions. No identifiable product was isolated. Chemical oxidation was performed on 0.19g starting material (1mmol) according to general procedures. Flash chromatography using 2:1 petroleum ether and acetone as the eluent gave 130mg product as a colourless oil (63%). HPLC resolved the two diastereomers, but was unable to resolve the enantiomers. Integration of the partially overlapping peaks indicated an approximately

5:1 ratio of the two diastereomers. Spectroscopic data were obtained from the mixture. Some peaks overlapped, characteristic peaks were reported. IR ν_{max} (neat): 2987(m), 2927(m), 1743(vs), 1433(m), 1302(s), 1256(s), 1157(s), 1058(s), 861(w) cm^{-1}. ^1H NMR δ: 4.38-4.24(2H, m), 3.58(1H, dt, J_1=12.6, J_2=2.9), 3.37(1H, ddd, J_1=13.5, J_2=7.9, J_3=3.1), 3.24(1H, 1H,m), 1.31(2H,t, J=7.2) ppm. ^{13}C NMR δ: 165.8, 76.6, 63.0, 46.5, 28.0, 24.2, 14.0 ppm.

1-Oxo-1,3-dithiane-2-carboxylic acid ethyl ester **5-26** 1,3-Dithiane-2-carboxylic acid ethyl ester (**5-9**, 100μl, 0.52mmol) was subjected to the yeast oxidation conditions in presence of 0.5g γ-cyclodextrin according to general procedures. The product was purified by chromatography using 3:1 petroleum ether and acetone, then acetone as the eluent. Most starting material was recovered (79mg, 77%), a small amount product was isolated (19mg, 17%) as light brown crystals. Spectroscopic data showed it was the hydrolyzed 1,3-dithiane-2-carboxylic acid. IR ν_{max} (neat): 3085(m, br), 3026(m, br), 2980(m), 2829(m, br), 2690(m), 2578(m), 1703(vs), 1414(s), 1308(s), 1177(s), 927(m) cm^{-1}. ^1H NMR δ: 4.17(1H, s), 3.42(2H, m), 2.60(2H, m), 2.25-1.93(2H, m) ppm. ^{13}C NMR δ: 176.1, 39.2, 25.6, 24.7 ppm. *E. coli*-mediated oxidation of 5-9 (100μL) according to general procedures gave **5-26** as a sticky oil (40mg, 37%). NMR indicated a mixture of *cis/trans*-isomers, spectroscopic data were recorded from the mixture. IR ν_{max} (neat): 2987(m), 2927(m), 1736(vs), 1427(m), 1315(s), 1262(s), 1163(s), 1052(s), 867(w) cm^{-1}. ^1H NMR δ: 4.29(1H, br, overlapped two singlet), 4.27(2H, overlapped two sets of quartet, J=7.2), 3.55(1H, td, J_1=12.5, J_2=2.7), 3.35(1H, multiplet of 16 peaks), 3.22(1H, multiplet of 7 peaks), 3.01(1H, m), 2.80(2H, m), 2.64-2.42(3H, m),

2.32(1H, dt, J_1=14.0, J_2=4.8), 2.26-2.17(1H, m), 2.17-2.04(1H, m) ppm. ^{13}C NMR δ: 166.1, 165.7, 64.3, 62.9, 55.3, 50.4, 46.5, 28.0, 26.4, 24.2, 14.1, 13.9 ppm. Chemical oxidation was performed on 1.92g starting material (10mmol) according to general procedures. Flash chromatography using 2:1 petroleum ether and acetone as the eluent gave 1.54g product (74%) as sticky, slightly cloudy oil, which solidified upon standing.

.(2-Methoxy-ethanesulfinyl)benzene **5-25** Chemical oxidation on **5-23** (1.68g, 10mmol) according to general procedures gave **5-25** as a colourless oil, 1.84g (>99%). IR ν_{max} (neat): 3065(m), 2995(m), 2930(m), 2895(m), 2830(m), 2740(w), 1480(m), 1450(s), 1120(vs), 1090(vs), 1050(vs), 750(vs), 695(s) cm^{-1}. ^{1}H NMR δ: 7.57-7.54(2H, m), 7.46-7.38(3H, m), 3.81-3.75(1H, multiplet of 13 peaks), 3.56-3.50(1H, multiplet of 11 peaks), 3.29(3H, s), 2.90(2H, dd, J_1=11.3, J_2=5.0) ppm. ^{13}C NMR δ: 143.9, 130.9, 129.1, 123.7, 65.1, 58.8, 58.0 ppm.

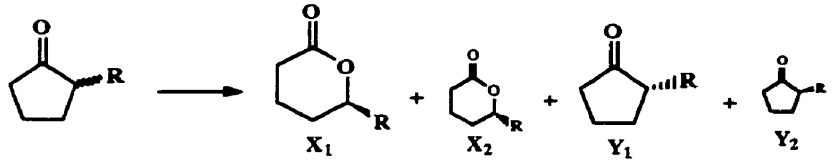
Benzenesulfinyl acetic acid ethyl ester **5-24** Chemical oxidation of **5-7** (0.4g, 2mmol) according to general procedures and chromatography using 4:1 petroleum ether and ether followed by 3:1 petroleum ether and acetone as the eluent gave **5-24** as a colourless oil, 0.37g (86%). IR ν_{max} (neat): 3050(m), 2980(s), 2930(m), 1740(vs), 1470(m), 1440(s), 1390(m), 1360(s), 1270(vs), 1180(s), 1120(s), 1090(vs), 1050(vs), 1020(s), 750(s), 690(s) cm^{-1}. ^{1}H NMR δ: 7.65-7.60(2H, m), 7.50-7.45(3H, m), 4.08(2H, q, J=7.2), 3.70(2H, AB quartet, J=13.7), 2.10(3H, s), 1.14(3H, t, J=7.0) ppm. ^{13}C NMR δ: 164.5, 143.0, 131.6, 129.2, 124.0, 61.8, 61.5, 13.8 ppm.

References

[1] (a) Donoghue, N.A., Norris, D.B., Trudgill, P.W., *Eur. J. Biochem.*, 1976(63)175; (b) Norris, D.B., Trudgill, P.W., *Eur. J. Biochem.*, 1976(63)193; (c) Taschner, M.J., Black, D.J., *J. Am. Chem. Soc.*, 1988(110)6892.

[2] Taschner, M.J., Black, D.J., Chen, Q.Z., *Tetrahedron Asymmetry*, 1993(4)1387.

[3] If the ketone cannot be directlt resolved by chiral GC, but the e.e. of the corresponding lactone is available, the optical purity of the ketone can be estimated from the following calculation: Assume a racemic ketone is partially oxidized, and the proportions of the enantiomeric pairs of the lactone and remaining ketone are X_1, X_2, Y_1, Y_2 (normalized to $X_1+X_2+Y_1+Y_2=100$, and the racemic starting material is by defination a 50:50 mixture).



The e.e. of the remaining ketone can be calculated as:

$$e.e. = \frac{(50 - X_1) - (50 - X_2)}{(50 - X_1) + (50 - X_2)} = \frac{X_2 - X_1}{100 - X_1 - X_2}$$

Similarly, if the ketone can be resolved but not the lactone, the e.e. of the lactone can be calculated from the ketone:

$$e.e. = \frac{(50 - Y_1) - (50 - Y_2)}{(50 - Y_1) + (50 - Y_2)} = \frac{Y_2 - Y_1}{100 - Y_1 - Y_2}$$

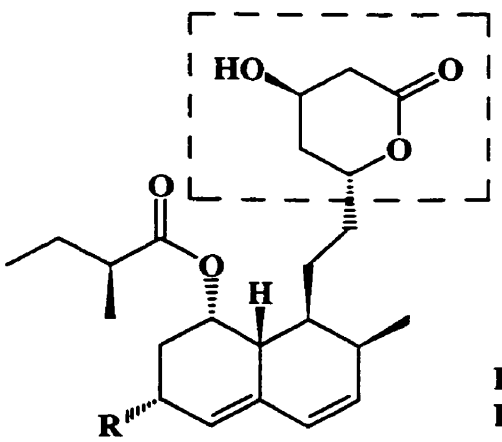
In this calculation it is assumed that there is no competing reactions for the substrate or the product, and that substrate racemization is negligible under the reaction conditions.

[4] Marcantoni, E., Nobili, F., Bartoli, G., Bosco, M., Sambri, L., *J. Org. Chem.*, 1997(62)4183.

Chapter Six: Attempted Synthesis of Mevinolin Analogues

Introduction

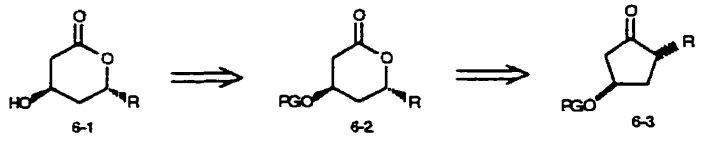
Mevinolin and compactin are two representative compounds of the mevinic acid family (Figure 18). They share the common δ-valerolactone pharmacophore, and both of them are effective hypocholesterolemic agents that inhibit **3-hydroxy-3-methylglutaryl-CoA-reductase** (HMGCoA reductase), the rate-limiting enzyme in the human *de novo* cholesterol biosynthetic pathway.[1] The synthesis of a chiral β-hydroxy-δ-valerolactone backbone is a key step in the total synthesis of mevinic acid derivatives. Several methods have been reported for their synthesis, however many of them suffer from long synthetic routes and low overall yields.[2]



R = H, compactin
R = CH_3, mevinolin

Figure 18: Mevinic acid family

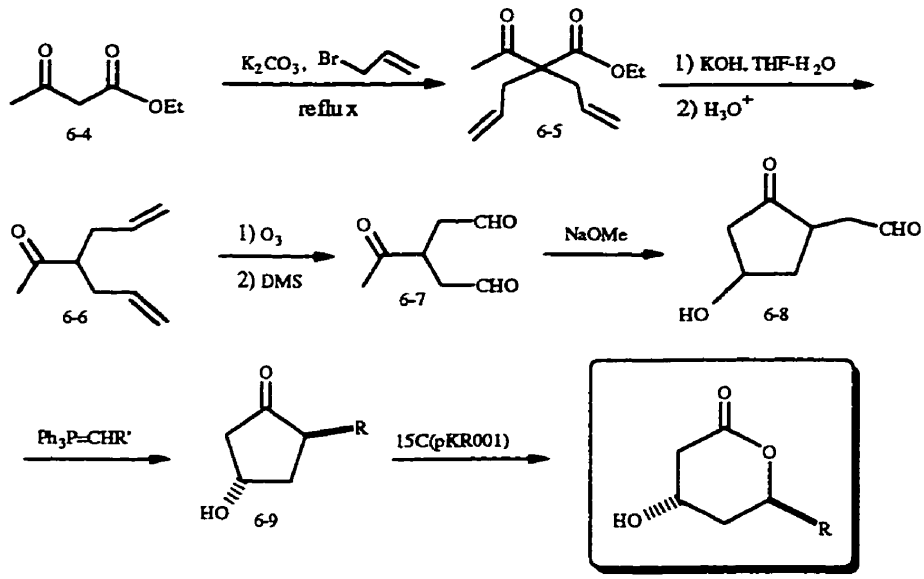
A shorter route for the synthesis of mevinolin analogues was designed using the oxidizing yeast reagent to introduce the chirality in the final product (Scheme 40). The key step in this route is to synthesize the *trans*-disubstituted ketone **6-3**. Three methods towards its synthesis were briefly explored, preliminary results are reported in this chapter.



Scheme 40

Cyclization-Wittig coupling-oxidation route

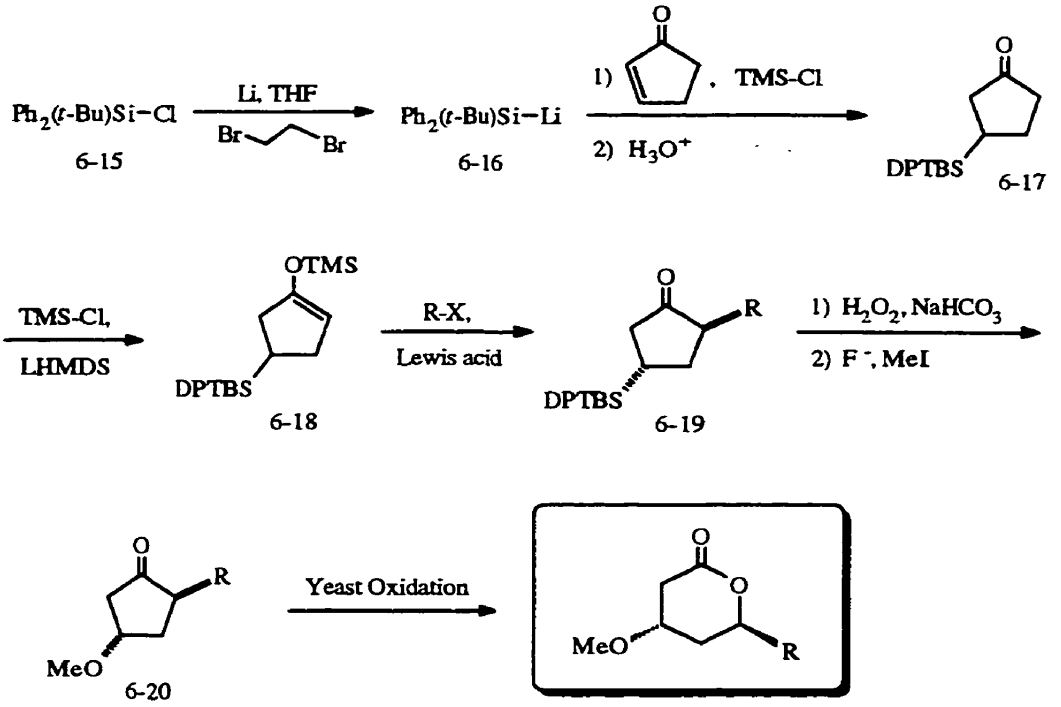
The designed synthesis is illustrated in Scheme 41. Ethyl acetoacetate **6-4** could be doubly substituted with an excess of allyl bromide to give **6-5**. After decarboxylation, the double bonds in **6-6** could be cleaved by ozonolysis[31] to give a dialdehyde **6-7**. A base catalyzed annulation would give hydroxyketoaldehyde **6-8**. The remaining aldehyde group could be used to introduce the desired side chain by Wittig reaction to give ketone **6-9**, the direct precursor of mevinic acid analogues.



Scheme 41

The dialkylated ester **6-5** was successfully obtained in over 90% isolated yield on a 0.2mol scale. In the subsequent decarboxylation step the same problems as those encountered in the synthesis of 2-allylcyclopentanone (**3-3D**) were experienced. Finally the ester was hydrolyzed in a THF-DME-water biphase system by KOH using THAC as a phase transfer catalyst. Although the yield was moderate, sufficient material was obtained for a preliminary test. Ozonolysis was carried out using standard conditions,[3] but the product resembled polymeric materials on NMR, and multiple products were seen on TLC. It is possible that **6-6** or **6-7** might be unstable in its isolated form. No further attempt was made to isolate or identify the complex products.

Silylation-alkylation-oxidation route



Scheme 42

The second route employed for the synthesis of **6-3** is outlined in Scheme 42. A bulky silyl group could be attached to the 3-position by Michael addition of a silyl Gilman reagent to cyclopentenone. Because of the bulky silyl group, alkylation of ketone **6-17** could be controlled to occur in the less hindered side and the resulting 2,4-disubstituted ketone **6-19** should have a *trans* configuration. Oxidation of the silyl group to an alcohol followed by protection would give ketone **6-20** which could be oxidized by the yeast reagent to give mevinolin analogues.

The formation of silyl lithium reagent usually requires the use of HMPA as solvent.[4] Because of its carcinogenic nature,[5] it is more desirable to use less toxic solvents such

as THF or DME as the reaction medium. In either of these two solvents, TMSCl failed to react with lithium, even when finely dispersed lithium metal and activating agents like iodine and 1,2-dibromoethane were used. Considering that substituents on the silicon atom capable of delocalizing a negative charge would stabilize the product and facilitate the formation of the silyl lithium reagent, DPTBS (diphenyl-*t*-butylsilyl, **6-15**) was used instead of the more common TMS or TBDMS as the silyl component. The increased steric bulkiness of this silyl reagent should also have a better directing effect in the subsequent alkylation step. When DPTBSCl and finely dispersed lithium metal were used in combination with 1,2-dibromoethane as the activating agent, silyl lithium reagent **6-16** was successfully obtained in THF solution with high yield.

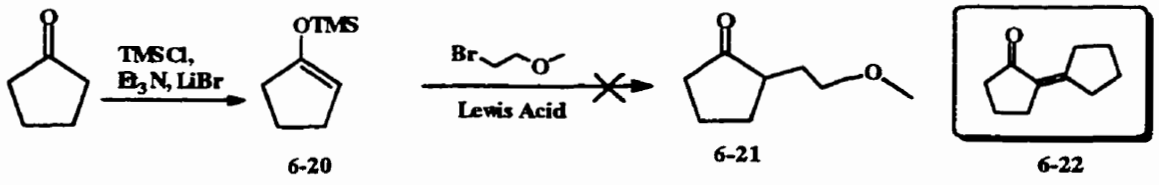
The Michael addition step was performed using the protocols developed in the synthesis of 3-alkyl substituted cyclopentanones (Chapter Three), with one modification that 2 equivalents of DMS were added to fulfill the ligand requirement in the cuprate reagent.[6] When 1 equivalent of DPTBSLi was reacted with 2-cyclopentenone in the presence of 1 equivalent of CuI, 1 equivalent of TMSCl, and 2 equivalents of DMS, 3-(diphenyl-*t*-butylsilyl)cyclopentanone **6-17** was obtained in 60% isolated yield from DPTBSCl.

In the next step, the formation of silyl enol ether can give two possible regioisomers. By using a strong, bulky base like HMDSLi under strictly kinetic conditions, it should be possible to obtain preferentially silyl enol ether with the double bond in the less hindered

side.[7] If a mixture of regioisomers were obtained, it should be possible to separate the regioisomers by chromatography.

The key step in this series is the alkylation of the silyl enol ether. The alkylation step has stereoelectronic requirements for the axial-addition of the alkyl group.[8c] The sterically bulky silyl group should occupy the quasi-equatorial position, which will guide the approach of the electrophile and give the correct *trans*- configuration between the silyl and R groups. There are two modes for the alkylation of silyl enol ethers. One is formal anionic alkylation in the presence of F^- and S_N2-active substrates,[9, 10] the other is Lewis acid-catalyzed alkylation.[8] The anionic alkylation method calls for the use of commercially unavailable anhydrous fluoride salts.[7, 10] In the latter mode, S_N1-active compounds such as tertiary alkyl halides are the preferred substrates. However in the subsequent yeast oxidation step, overly bulky substituents like *t*-butyl are not suitable. It is preferable to use a linear substituent which is at least 4 atoms long and has a derivatizable group for future transformations. 2-Methoxyethyl ether was chosen because the β-ether link can provide certain S_N1 activity via an anchimeric effect by the formation of an oxonium intermediate,[7a] and has been proven to be acceptable in yeast-mediated oxidations (Chapter Five). In addition, the methyl ether can be conveniently cleaved by treatment with TMSI or suitable Lewis acids to allow further derivatizations.[9]

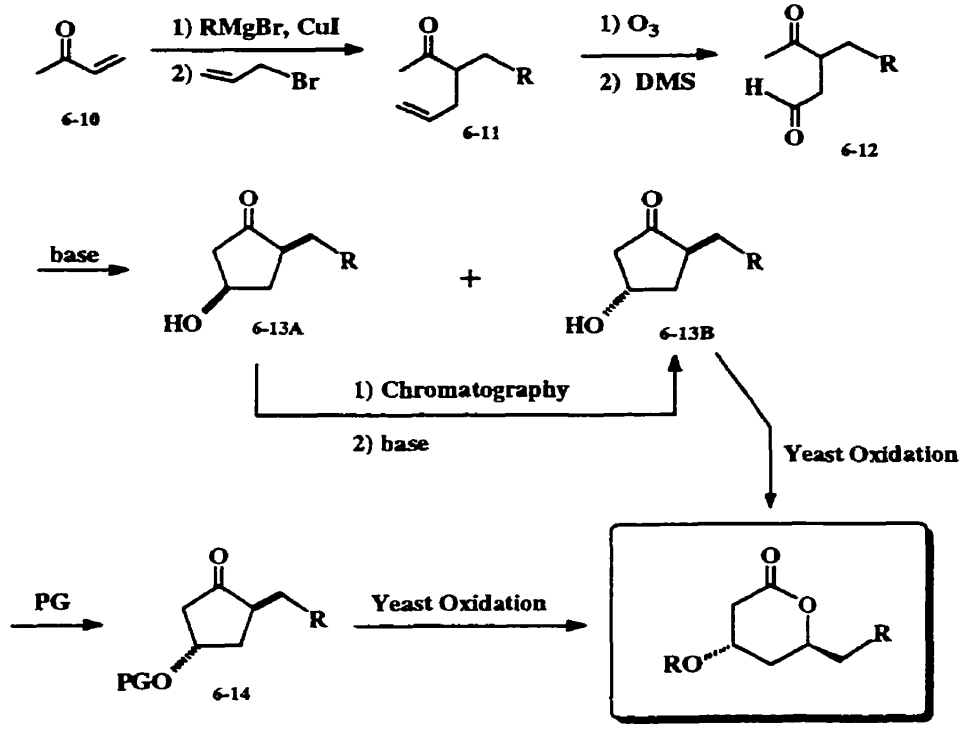
The alkylation reaction between 1-(trimethylsiloxy)cyclopentanone and 2-bromoethyl methyl ether was used as a model reaction (Scheme 43). Under the conditions tested, this reaction failed to give the desired product in reasonable yield; instead, the aldol condensation-dehydration product **6-22** was recovered as the major product (24% isolated). It was reported in the literature that alkylations of cyclopentanone derivatives are not nearly as effective as corresponding reactions with cyclohexanones and the yield with even the most active substrates, like allyl bromide or benzyl chloride, were in the 60-70% range.[10] With these facts, no further attempts were made along this route.



Scheme 43

Michael addition-alkylation-cyclization-oxidation route

This route is similar to the first one, except that the substituents are introduced in a more convenient way (Scheme 44). The first substituent was introduced by Michael addition of a suitable Grignard reagent to 3-buten-2-one (**6-10**) in the presence of a cuprous catalyst. The intermediate enolate was trapped with allyl bromide to give the 3-substituted hexenone derivative **6-11**.[11] The terminal double bond was converted to an aldehyde by ozonolysis, and base-catalyzed annulation would give the hydroxyketone **6-13**, which could either be oxidized directly or as its hydroxy-protected equivalent **6-14**. This strategy was successfully carried out to give the substituted ketoaldehyde **6-12**, but suitable conditions for the following annulation step have not been found.



Scheme 44

Conclusion

A short and convenient route has been proposed for the synthesis of mevinolin analogues. Three methods have been briefly tested for the synthesis of a key intermediate, 2,4-disubstituted cyclopentanone (**6-3**). Some preliminary experiments suggested that the synthesis besed on Scheme 42 and Scheme 44 hold the most promise. Although the key intermediate **6-3** has not been obtained within the time frame of this thesis, and it has not been possible to test the enantio- and diastereoselectivities of yeast-mediated oxidations of **6-3**, the simplicity of asymmetrical Baeyer-Villiger oxidation strategy is such that future work towards the synthesis of **6-3** should be continued.

Experimental

2-Acetyl-2-allylpent-4-enoic acid ethyl ester **6-5** Anhydrous K_2CO_3 (120g, 0.87mol) was placed in a 500mL 3-neck round bottom flask fitted with a mechanical stirrer, a refluxing condenser, and a thermometer. Dry acetone (300mL, dried with and distilled from anhydrous $CaSO_4$) was added followed by ethyl acetoacetate **6-4** (26g, 0.2mol), and allyl bromide (70g, 0.58mol). The mixture was vigorously stirred and slowly refluxed for 20 hours until GC showed complete conversion of the starting material. The organic phase was filtered through a sintered glass funnel and the filter cake was washed extensively with acetone. The combined solutions were concentrated by rotary evaporation, and the residue was purified by flash chromatography using 100:1 then 10:1 petroleum ether and ethyl acetate as the eluent to give **6-5** as a colourless oil, 37.75g

(90%). IR ν_{max} (neat): 3085(m), 2987(s), 2940(m), 1740(vs), 1722(vs), 1644(s), 1216(vs), 1157(s), 927(s).

3-Allyl-5-hexen-2-one **6-6** 2-Acetyl-2-allyl-pent-4-enoic acid ethyl ester **6-5** (4.2g, 20mmol) was added to a mixture of KOH (4.5g, 80mmol), distilled water (80mL), THF (80mL), DME (50mL) and THAC (0.2g). The mixture was gently refluxed with vigorous stirring under nitrogen overnight until GC showed complete conversion. The mixture was acidified with 2M HCl and extracted with ether. The combined extracts were dried with anhydrous K_2CO_3 and the solvent was removed by rotary evaporation. The residue was purified by flash chromatography using 100:1 petroleum ether and ethyl acetate as the eluent to give **6-6** as a colourless oil, 1.74g (62%). IR ν_{max} (neat): 3085(m), 2987(s), 2940(m), 1723(vs), 1460(s), 1377(s), 1216(vs), 1157(s), 927(s) cm^{-1}. ^1H NMR δ: 5.73-5.63(2H, multiplet of 10 lines), 5.04-4.99(4H, m), 2.61(1H, multiplet of 9 lines), 2.36-2.28(2H, m), 2.22-2.14(2H, m), 2.10(3H, s) ppm. ^{13}C NMR δ: 210.9, 135.3, 117.0, 52.1, 41.4, 35.2 ppm.

General procedures for ozonolysis

The olefinic substrate was dissolved in 40mL CH_2Cl_2 and cooled to -80°C. A stream of O_2/O_3 mixture was bubbled through the solution while maintaining the temperature below -70°C until a blue colour persisted. The cold bath was removed and a stream of nitrogen was flushed over the surface until the blue colour of solution had faded. DMS (3 equivalents per double bond) was added and the mixture was stirred for 2 days at room temperature under nitrogen. Low boiling point components were removed by rotary evaporation, and the residue was purified by flash chromatography using mixtures

of petroleum ether and ethyl acetate or acetone as the eluent to give the aldehyde product.

Attempted synthesis of 3-acetyl-pentanedial **6-7** 3-Allyl-4-penten-2-one **6-6** (1.2g, 8.7mmol) was subjected to ozonolysis conditions according to general procedures and the product was purified by chromatography using 10:1 petroleum ether and ethyl acetate as the eluent. The product showed complex peaks in both ^{1}H and ^{13}C NMRs, which is typical for polymeric materials. No further attempt was made to characterize the product.

3-Allyl-2-octanone **6-11** CuI (1.9g, 10mmol) and LiI (2.7g, 20mmol) were placed in a 250mL 3-neck round bottom flask. The system was flame dried and flushed with nitrogen. A 1:1 mixture of THF and DME (100mL) was added at room temperature and the system was stirred until a clear solution was obtained. The solution was cooled to -80°C and BuLi (2.2M in hexane, 9.2mL, 20mmol) was added via a dropping funnel over 20 minutes. The mixture was stirred for another 20 minutes and the temperature was allowed to warm up to -40°C until a gray-green mixture was obtained. The mixture was re-cooled to -80°C, and 3-buten-2-one (**6-10**, 0.83mL, 10mmol) in 1:1 mixture of THF and DME (10mL) was added over 10 minutes. The mixture was stirred for another 20 minutes and the temperature was allowed to warm to -30°C. The mixture was re-cooled to -80°C and allyl bromide (5mL, excess) in 1:1 mixture of THF and DME (10mL) was added in one shot. The cold bath was removed and the temperature was allowed to warm up to -30°C until a yellow-green mixture was obtained. The reaction was quenched by addition of 40mL saturated $NaHCO_3$ and extracted with 300mL

petroleum ether in 4 portions. The combined extracts were dried with anhydrous $MgSO_4$ and the solvent was removed on a rotary evaporator. The residue was purified by flash chromatography on silica gel using 20:1 petroleum ether and ethyl acetate as the eluent to give **6-11** as a pale yellow oil, 1.03g (81%). IR ν_{max} (neat): 3080(w), 2960(s), 2940(vs), 2860(m), 1715(vs), 1640(w), 1350(w), 1170(w), 1000(w), 920(m) cm^{-1}. ^1H NMR δ: 5.73-5.61(1H, m), 5.03-4.91(2H, m), 2.55-2.44(1H, m), 2.33-2.24(1H, m), 2.19-2.11(1H, m), 2.09(3H, s), 1.61-1.52(1H, m), 1.44-1.34(1H, m), 1.30-1.16(6H, m), 0.84(3H, t, J=6.8) ppm.

3-Acetyl-octanal **6-12** 3-Allyl-2-octanone (**6-11**, 1.0g, 6.1mmol) was subjected to ozonolysis conditions according to general procedures and the product was purified by flash chromatography using 10:1 petroleum ether and acetone as the eluent to give **6-12** as a light yellow oil, 0.48g (46%). IR ν_{max} (neat): 2960(s), 2940(s), 2860(s), 2740(w), 1720(br, s), 1470 (w), 1390(w), 1355(w), 1170(w) cm^{-1}. ^1H NMR δ: 9.66(1H, s), 3.01-2.93(1H, m), 2.87(1H, ddd, J_1=18.1, J_2=9.6, J_3=0.5), 2.42(1H, dd, J_1=18.1, J_2=3.6), 2.17(3H, s), 1.60-1.50(1H, m), 1.40-1.30(1H, m), 1.28-1.16(6H, m), 0.81(3H, t, J=6.5) ppm. ^{13}C NMR δ: 210.5, 200.6, 45.7, 44.7, 31.6, 31.0, 29.2, 26.5, 22.3, 13.9 ppm.

Lithiodiphenyl-*t*-butyl silane **6-16** Lithium (30% dispersion in mineral oil, 0.8g, excess) was placed in a 50mL 3-neck round bottom flask fitted with a pressure equalizing dropping funnel, a reflux condenser and a thermometer. The system was flame dried and flushed with nitrogen. Lithium was washed three times with THF, and DPTBSCl (2.6mL, 10mmol) was dissolved in 10mL THF in the dropping funnel. A 1-2mL portion of this solution was introduced and the mixture was warmed with a heat gun. When

refluxing had started, a few drops of 1,2-dibromoethane were added and the system was slowly agitated with a magnetic stirrer. As soon as exothermic reaction had started, the stirring speed was increased and the remaining DPTBSCl solution was added at such a rate as to maintain vigorous but controllable refluxing. After the addition, the blood-red solution was stirred at room temperature overnight under nitrogen to give **6-16**.

3-(Diphenyl-*t*-butylsilyl)-cyclopentanone **6-17** CuI (0.9g, 4.7mmol) and LiI(0.7g, 5.2mmol) were placed in a 100mL 3-neck round bottom flask fitted with a pressure equalizing dropping funnel and a thermometer. The system was flame dried and flushed with nitrogen. THF (20mL) was added and the mixture was stirred at room temperature until a clear solution was obtained. The solution was cooled to -80°C and DPTBSLi solution (**6-16**) was added via the dropping funnel at such a rate to keep the temperature below -40°C. After stirring at -80 to -40°C for 1 hour, the solution was cooled to -80°C and TMSCl (1.5mL, 11.8mmol) was added followed by 2-cyclopentene-1-one (0.9mL in 10mL THF, 10.8mmol). After stirring at -100 to -80°C for 1 hour, DMS (1.5mL, 20mmol) was added and stirring was continued for 1 hour. The reaction was quenched with saturated NH_4Cl and the mixture was extracted with 300mL petroleum ether in 4 portions. The combined extracts were washed with $NaHCO_3$ and NH_3/NH_4Cl and the aqueous washings were back-extracted once with 100mL petroleum ether. The combined organic layers were dried with anhydrous $MgSO_4$ and the solvent was removed by rotary evaporation. The residue was purified by flash chromatography using 20:1 petroleum ether and ethyl acetate as the eluent to give **6-17** as a sticky oil, 1.90g (60%). IR ν_{max} (neat): 3090(m), 3060(m), 2975(s), 2950(s), 2900(m), 2870(s),

1750(vs), 1475((w), 1430(s), 1160(m), 1115(s), 820(m), 710(s) cm^{-1}. ^1H NMR δ: 7.62-7.58(4H, m), 7.42-7.32(6H, m), 2.50-2.38(1H, m), 2.34-2.20(1H, m), 2.16-1.87(4H, m), 1.78-1.61(1H, m), 1.10(9H, s) ppm. ^{13}C NMR δ: 220.7, 136.5, 133.0, 129.4, 127.7, 41.3, 38.7, 28.4, 25.6, 20.2, 18.6 ppm.

1-Trimethylsiloxycyclopentene **6-20** TMSCl (14mL, 0.1mol), Et_3N (17mL, 0.12mol), and cyclopentanone (8.8mL, 0.1mol) were dissolved in 40mL THF in a 100mL round bottom flask under nitrogen. Anhydrous LiBr (8.0g, 0.1mol, dried at 140°C/0.05mmHg for 12 hours) was dissolved in THF (20mL) and slowly added to the flask via a dropping funnel. After stirring at room temperature overnight under nitrogen, the mixture was poured onto ice cubes and extracted with 400mL petroleum ether in 5 portions. The combined extracts were dried with anhydrous Na_2SO_4 and the solvent was removed by rotary evaporation. The residue was fractionally distilled at 150-151°C to give **6-20** as a colourless oil, 9.16g (65%). IR ν_{max} (neat): 3070(w), 2960(s), 2900(m), 2850(s), 1640(s), 1340(s), 1250(s), 920(s), 890(s), 860(s), 840(s) cm^{-1}. ^1H NMR δ: 4.60(1H, t, J=1.5), 2.24(4H, m), 1.83(2H, pentet, J=7.9), 0.18(9H, s) ppm. ^{13}C NMR δ: 155.0, 102.1, 33.5, 28.7, 21.3, 0.0 ppm.

Attempted synthesis of 2-(2-methoxyethyl)-cyclopentanone **6-21** 1-Trimethylsiloxy-cyclopentene **6-20** (0.2mL, 1.1mmol) and 2-bromoethyl methyl ether (0.1mL, 1.1mmol) were dissolved in CH_2Cl_2 (2mL) under nitrogen and cooled in an ice bath. $SnCl_4$ (1mL, 1M in heptane) was added and the solution was stirred for 2 hours. After quenching with saturated $NaHCO_3$, the mixture was extracted with CH_2Cl_2. The combined extracts were dried with anhydrous Na_2SO_4 and the solvent was removed by rotary evaporation. The residue was purified by flash chromatography to give a colourless oil, 17mg, which was shown to be bicyclopentylidine-2-one (**6-22**). ^1H NMR δ: 2.79-2.72(2H, m), 2.51(2H, multiplet of 11

lines), 2.28(4H, t, J=7.7), 1.89(2H, quintet of doublets, J_1=7.7, J_2=0.7), 1.74-1.62(4H, m) ppm. ^{13}C NMR δ: 207.4, 158.6, 127.9, 39.8, 34.3, 32.5, 29.5, 26.9, 25.2, 20.1 ppm.

References

[1] (a) Ref. 2c in Chapter Three; (b) Wakil, S.J., *Lipid Metabolism*, Academic Press, NY., 1970, page 371.

[2] For examples, see Ref. 2 in Chapter Three and the footnote on page 50.

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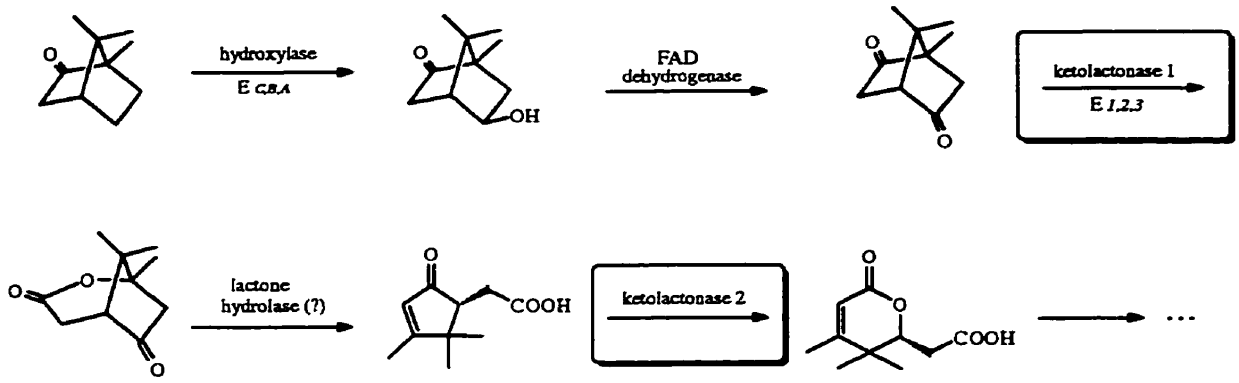
Chapter Seven: Cloning of Diketocamphane Monooxygenase

Introduction

The recombinant yeast strain constructed in this lab[1] overexpresses cyclohexanone monooxygenase from *Acinetobacter sp.* (NCIB 9871). It catalyses asymmetric Baeyer-Villiger oxidations of racemic 2- and 3-substituted cyclic ketones to *(S)*-lactones and *(R)*-ketones. In order to obtain *(R)*-lactones, the *(R)*-enriched ketones need to be oxidized chemically to the corresponding lactones; unfortunately at present it is not possible to obtain *(S)*-ketones from yeast-mediated reactions. Therefore it is highly desirable to clone into yeast another Baeyer-Villiger enzyme that has the opposite stereoselectivity, since this will allow different stereoproducts to be formed from the same racemic ketone substrate simply by switching the yeast reagent used.

Bacteria species *Pseudomonas* have the ability to degrade various aliphatic and aromatic hydrocarbons,[2] this feature has been used in remediations of contaminated industrial sites[3] and in the synthesis of novel compounds.[2,4] In 1959 Gunsalus *et al.* isolated the strain *Pseudomonas putida* (ATCC 17453) capable of growth on camphor as the only carbon source.[5] Since that initial report, many studies have been conducted on camphor degradation by this bacterium strain,[6] including the metabolic pathway, the enzymes involved, and some genetic basis of this process.



Scheme 45

Pseudomonas putida can metabolize both D- and L-camphor, and there is no significant preference for either isomer. According to the established mechanism,[4a, 4g] there are two Baeyer-Villiger oxidation steps involved in camphor degradation (Scheme 45). Further investigation showed that two enzymes with strict but complimentary stereoselectivities are involved in the first Baeyer-Villiger step, rather than one non-selective enzyme.[4a] Both enzymes have been isolated, and were designated as 2,5-diketocamphane-1,2-monooxygenase[7] (2,5-DCMO) and 3,6-diketocamphane-1,6-monooxygenase[8] (3,6-DCMO). Oxidations by isolated enzymes of monocyclic and bicyclic ketones, acyclic sulfides, and cyclic thiocompounds showed that 2,5-DCMO generally exhibits good stereoselectivities, but has opposite enantiomeric preferences to that of CHMO.[9] Therefore it appears to be an excellent candidate for overexpression in yeast. In addition, DCMO enzymes may have different substrate acceptabilities from that of CHMO, which would extend the scope of yeast-mediated reactions. For this reason, it is desirable to clone both 2,5-DCMO and 3,6-DCMO into yeast. The target in

this study was to identify the genes for *Pseudomonas putida* monooxygenases and to initiate the cloning of these genes.

Methods in cloning *Pseudomonas* genes

Isolation of *CAM* plasmid

Plasmids are small, cyclic, double strand DNA molecules. Their replication and expression are independent of the chromosome. Plasmids provide various functions that the host organisms lack, such as resistance to antibiotics and the ability to metabolize unusual substrates, they can be isolated from the host cells. When preparing cell culture for plasmid isolation, it is important to select only the desired strain and inhibit the growth of any contaminating or mutated cells to ensure the homogeneity of the isolated DNA. For this reason, *Pseudomonas putida* (ATCC 17453) was grown in mineral medium supplemented with camphor instead of the usual rich media. Because camphor is an unusual energy source, this ensured only cells with *CAM* plasmid would grow, any contaminating cells or mutants that had lost the plasmid would not.

The *CAM* plasmid in *Pseudomonas putida*, where all the genes coding for camphor degrading enzymes reside, has been isolated.[10] It is approximately 160M dalton in size, which accounts for ca. 240K base pairs. Because of its large size, the usual procedures optimized for isolating small plasmids[11] failed. A special protocol was adapted for

isolating the *CAM* plasmid.[12] The main differences between these two methods are outlined in Table 20.

Table 20: Comparison between plasmid isolation protocols

normal protocol	special protocol
disrupt and lyse cells with SDS/NaOH	disrupt and lyse cells with NaOH and half amount of SDS
add KOAc/HOAc (to neutralize cell lysate, to adjust the ionic strength and to precipitate chromosomal DNA/proteins at the same time.)	add Tris-HCl to neutralize cell lysate add the remaining SDS add NaCl to adjust the ionic strength and to precipitate debris
add ethanol to precipitate DNA	add PEG to precipitate DNA

The separation of plasmid DNA from proteins and chromosomal DNA is based on the fact that under suitable conditions, supercoiled DNA remains in solution while denatured DNA and proteins bind SDS and precipitate. In the usual protocol for plasmid isolation, NaOH/SDS treatment lyses the cells and uncoils all the DNA molecules. During the neutralization step, small plasmid DNA re-coils quickly and resumes its native supercoiled form, while the much larger chromosomal DNA does not renature under these conditions and precipitates with SDS and protein debris[8a]. Large plasmids, however, cannot be isolated in this manner, because they do not recoil fast enough upon neutralization, and are removed with the chromosomal DNA. In the special protocol, NaOH/SDS disruption is followed by a neutralization step with Tris-HCl to allow sufficient time for the plasmid to recoil, while chromosomal DNA is even larger and remains in a denatured form. When more SDS is added and the ionic strength adjusted with NaCl, chromosomal DNA and proteins are precipitated. After removing those debris, plasmid DNA is usually recovered by ethanol precipitation. For large plasmids,

PEG is more suitable because it does not precipitate single strand DNA, which can result from mechanical shearing of the large plasmids during the isolation processes. With these modifications, *CAM* plasmid was successfully isolated in high yield and purity.

Cloning methods

camR hypothesis

Several genes on the *CAM* plasmid related to the early part of camphor degradation have been cloned and sequenced. These include a regulatory region *camR*,[4f] P-450cam hydroxylase complex gene *camCAB*,[4c,13] and the 5-exo-hydroxycamphor dehydrogenase gene *camD*.[4g,14] It has been proven that *camABCD* genes are negatively regulated by *camR*, i.e., *camABCD* genes are expressed when *camR* is inactivated by binding with camphor. Since all enzymes in this metabolic pathway are related to camphor degradation and are inducible by camphor, it is possible that the expression of other genes in this metabolic pathway are also controlled by camphor. Though these genes may spread over many locations on the plasmid, from the view point of gene conservation, the regulatory mechanisms should be the same as *camR*, both in terms of sequence and in terms of relative locations and orientations of the control regions. Since the *camR* sequence is known, it should be possible to identify adjacent segments by Southern Blot method[15] and to identify the complete genes for 2,5- and 3,6-DCMO. This approach is referred to as the "*camR* hypothesis" (Figure 19).

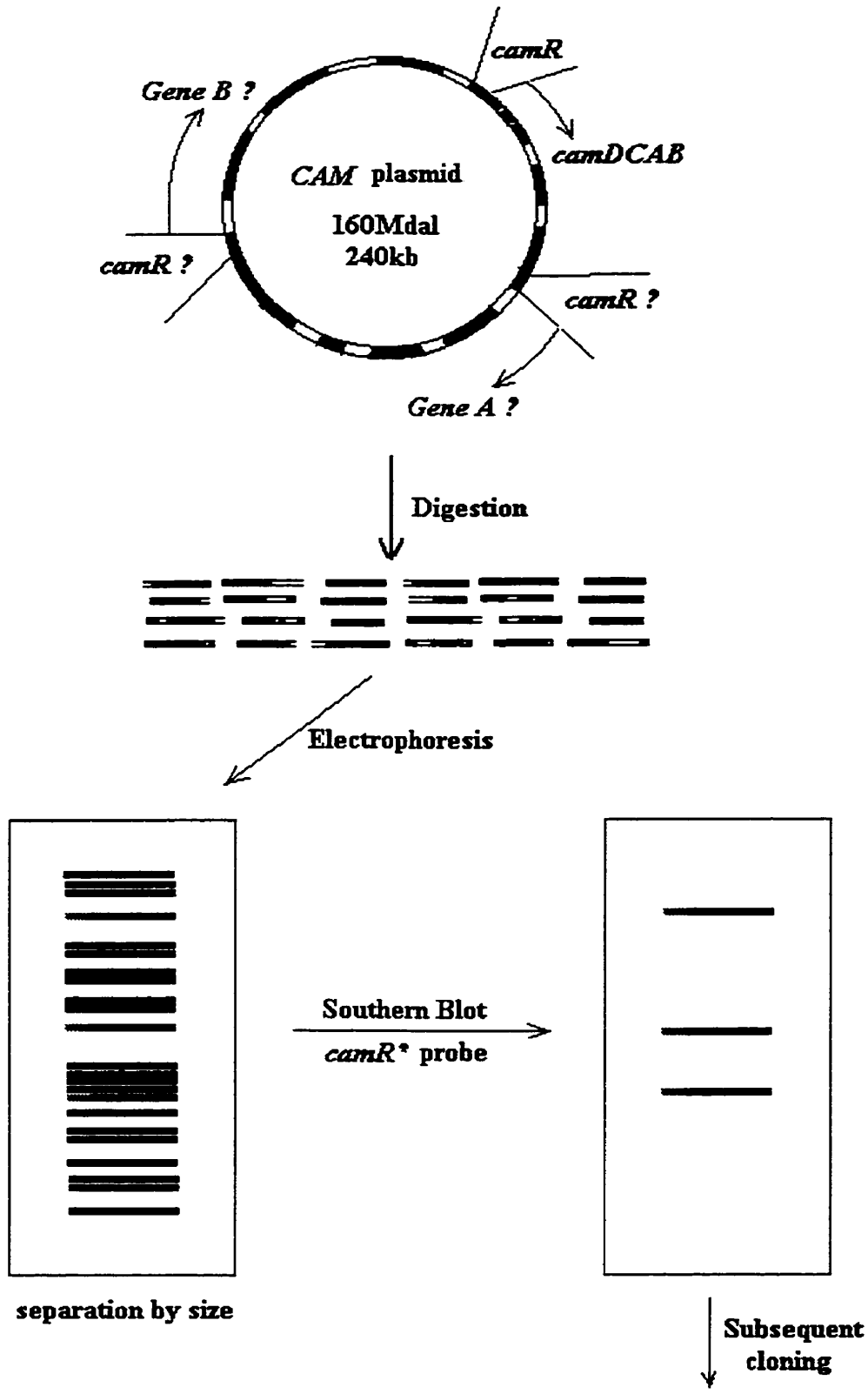


Figure 19: *camR* hypothesis

Southern Blot analysis is the basis of this approach. In the original protocol, radioactively labeled probes and autoradiographical methods were used for the detection of matching sequences. This detection method, however, poses several difficulties: the operator is exposed to radioactive materials, hazardous radioactive waste must be treated, experimental times are lengthy, and detection sensitivity is insufficient for short probes. In recent years some alternative methods based on ELISA (enzyme linked immuno absorbent assay) have been developed. These new methods are generally faster, easier to perform, safer for the operator and the environment, and are often more sensitive, especially for short probes. The Genius® kit (Boehringer Mannheim Biochemicals), a DNA labeling and detection kit based on ELISA method, was used in this work. Digoxygenin (DIG) is used to label the DNA probe, an antibody against DIG is covalently linked to an enzyme that can catalyze a colour reaction. When the antibody binds to DIG-labeled probe, the coupled enzyme catalyzes a colour reaction to show the location of the matching fragment. The principle of this detection kit is illustrated in Figure 20.

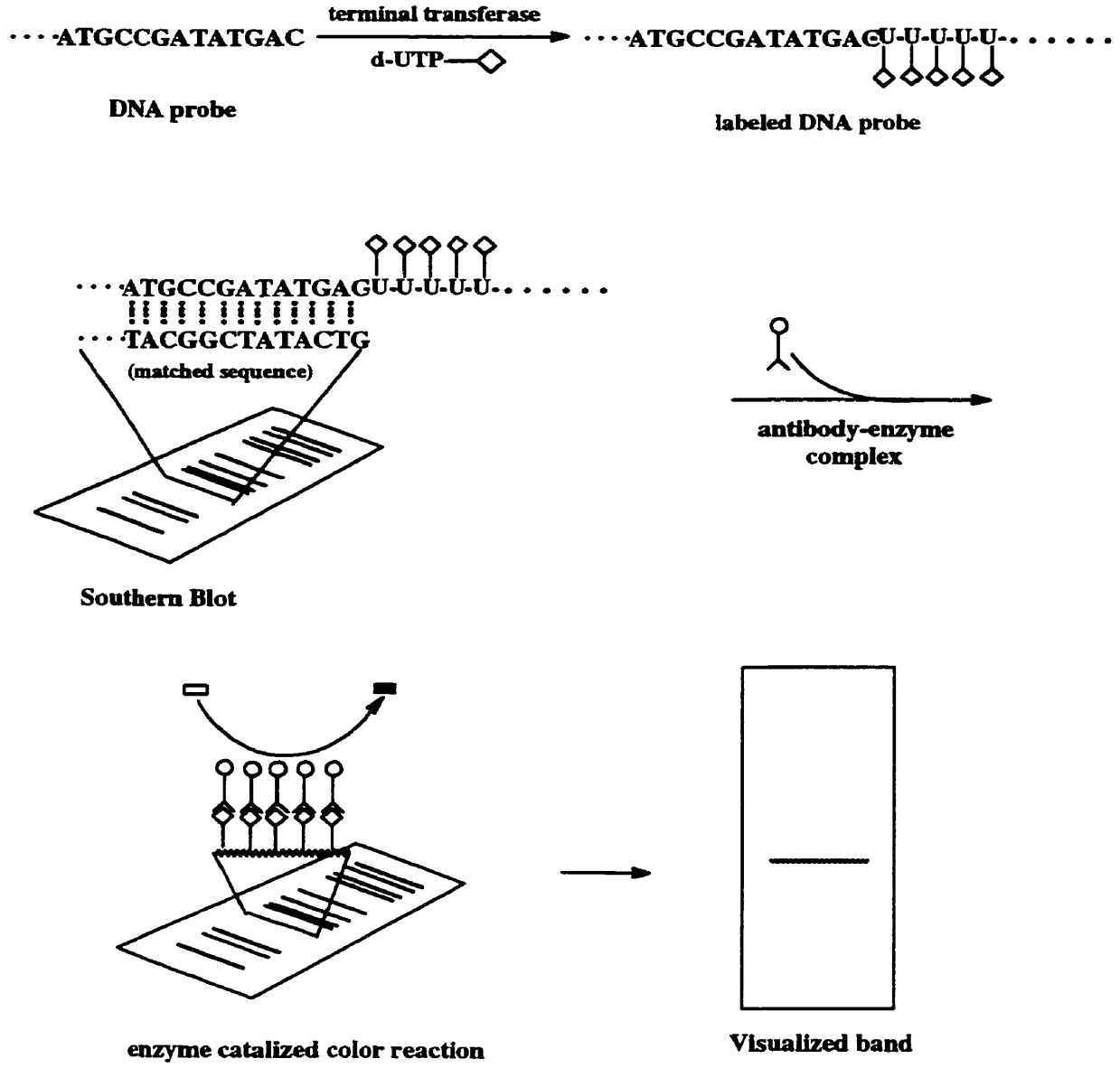


Figure 20: ELISA-based DNA labeling and detection

Two 18 deoxyribonucleotide oligomers corresponding to the camphor binding region in *camR*[41] (forward sequence, ***Binding I***, and reverse sequence, ***Binding II***) were ordered from Integrated DNA Technologies (Coralville, IA). 3′-Tailing with DIG-dUTP by terminal transferase was used to label the two DNA probes.[16] According to the technical bulletin from Boehringer Mannheim, terminal transferase will add a 300-500 base poly-DIG-U tail to a single strand DNA under standard conditions. Since this is too long for an 18-base probe, 0.1x diluted solutions were used in the expectation that this would bring the tailing length to below 30 bases. If one considers the lowered labeling efficiency at lower concentrations, even shorter tailings could be expected. Tailing reaction product was tested by spotting serial dilutions on nitrocellulose membrane and subjecting it to standard fitting, hybridization, and visualization procedures. The concentration of labeled probe was determined to be ca. 35pg/μL and the visualization sensitivity was ca. 0.25pg/μL.

The *CAM* plasmid has to be fragmented before any blotting test. Two major methods are available for this purpose. The first one is fragmentation by mechanical shearing. This method can provide truly random fragments, however they can have protruding ends, retreating ends and blunt ends,* and there is no control over the terminal sequences. These will introduce many difficulties in subsequent cloning steps. The

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* Protruding end means that the 3' strand is longer than the 5' strand; retreating ends means that the 3' strand is shorter than the 5' strand; blunt end means the 3' and 5' strands are of the same length.

second fragmentation method employs restriction endonuclease digestion. This method fragments DNA molecules at specific nucleic acid sequences as determined by the enzyme(s). Because the distribution of the restriction sites may not be even throughout the DNA molecule, this method does not produce truly random fragments. On the other hand, this method generates defined termini suitable for subsequent cloning. In the first trial, fragmentation with restriction enzymes was used.

The following criteria were used in choosing the restriction enzymes: (1) restriction sites within the polylinker region of the cloning vector pUC19 to facilitate subsequent closing. (2) 1 or 2 restriction sites within *camABCD* regions to ensure the plasmid will be fragmented, but not into too small fragments. (3) no restriction sites in *camR* region, so the *camR* sequence would be conserved in the fragments. In addition, some enzymes that have suitable restriction site(s) in the *camABCD* region but not in the polylinker region were also tested. Table 21 summarizes the conditions tested for the digestion.

Table 21: Restriction digestion of CAM plasmid

single enzyme	digestion buffer*	enzyme combinations	digestion buffer*
EcoR I	H	*BamH* I, *EcoR* I	B
Bgl II	M	*MLu* I, *Sal* I	H
Pvu II	M	*MLu* I, *Sca* I	H
Sal I	H	*Sal* I, *Sca* I	H
Apa I	A	*Not* I, *Sal* I	H
BamH I	B	*EcoR* I, *Bgl* II	H
Cla I	H	*Bgl* II, *Hind* III	M
EcoR V	B	*BamH* I, *Hind* III	B
MLu I	H	*EcoR* I, *Sal* I	H
Not I	H	*Bgl* II, *Sal* I	H
Sac I	A	*BamH* I, *Bgl* II	B
Sca I	H	*Nde* I, *Not* I	H
Acc I	A	*Xho* I, *Nde* I	H
Stu I	B	*EcoR* I, *Nde* I	H
Xho I	H	*Bgl* II, *Nde* I	H
Xmn I	NEB2 + BSA	*EcoR* I, *Not* I	H
Nco I	H	*Bgl* II, *Not* I	H

* Either Boehringer Mannheim SuRE/Cut buffer system or NEB buffer system was used. For single-enzyme reactions, the optimum buffer was used; for combinations, if two enzymes have different optimum buffers, an alternative buffer which retained over 50% of maximum activity was used.

The mixtures from restriction enzyme digestion were analyzed by electrophoresis in agarose gel containing ethidium bromide. The DNA fragments were visualized by UV irradiation and recorded by a photographic method. The electrophoreses were routinely conducted at 86V for 1.5 hours to obtain fast, preliminary results. Under this voltage, the migratory velocity was very high but the resolving power was limited. The digestion mixtures that showed most fragments in the desired 1-4Kb size range were further separated by electrophoresis at 50V for 6-8 hours to obtain better resolution, and were used in Southern Blot analysis.

The protocol for Southern Blot was adapted for use with Genius Kit.[8a,12,17] Several runs using digestion mixtures from different restriction enzymes and combinations of enzymes showed only one band in each case, which indicated the *camR* sequence appeared only once in the *CAM* plasmid, therefore the original "*camR* hypothesis" had to be revised, there must be different regulatory sequences for different genes, which can be activated either by camphor or by its degradation intermediates. Based on the assumption of genetic code conservation, those regulatory sequences may still have a certain degree of homology, but slight sequence variations allow different genes to be activated at different substrate concentrations.

In order to detect homologous genes with small variations in the sequences, the washing stringency in the Southern Blot had to be lowered. This included lowering the washing temperature, increasing the salt concentration in the washing buffer, and shortening the

washing time. Table 22 summarizes the conditions tested. Under extremely nonstringent conditions (entry 5), many faint bands were visible, however the signals were too weak to unambiguously distinguish imperfectly matched fragments from randomly unmatched bands. These results suggested that genes coding for different camphor-degrading enzymes are under different controls, therefore those enzymes may not be specific for camphor degradation, rather for degradation of a much broader spectrum of unnatural substrates present in environment.

Table 22: Washing conditions tested in Southern Blot analysis

entry	washing conditions
1	68°C, 0. 1x SSC, 0. 1% SDS, 2x15min
2	55°C, 0. 1x SSC, 0. 1% SDS, 2x15min
3	55°C, 1x SSC, 1% SDS, 2x15min
4	37°C, 1x SSC, 1% SDS, 1x20min
5	25°C, 1x SSC, 1% SDS, 3x30min

Shotgun Method

The first attempt to search for DCMO genes based on the "*camR* hypothesis" did not lead to the desired results. Due to the lack of sequence data about *CAM* plasmid or DCMO genes, there was no direct method to select and clone these genes. Although the first 20 amino acid sequences for both 2,5-DCMO and 3,6-DCMO have been reported, and it is theoretically possible to obtain the nucleic acid sequence from the amino acid

sequence, the degeneration of codons makes this method difficult and expensive*. In view of that the shotgun method was more appropriate.

The basic principle of this method is to sequence the whole DNA and then look for the gene of interest. This method showed increased application following the latest advances in automated sequencing, mostly uses variants of Sanger chain termination method.[18] Limited by the capacity of the instrument, 3 - 4 kb can be sequenced for a double strand DNA, and larger DNA molecules have to be fragmented into this range. Taking into account that some overlapping is necessary in order to find the connecting fragments, about 2. 5-3. 5 Kb of useful sequence can be obtained from each fragment.

Massive subcloning and sequencing are needed in order not to miss a given gene of interest. Since the average size for a bacterial gene is 1-2kb, and there are no introns in bacterial DNA, it can be assumed that ca. 200 genes are coded in the 240kb *CAM* plasmid, and each subcloned fragment contains one gene. To calculate the number of fragments needed, the following assumptions are made: (1) the *CAM* plasmid is randomly fragmented; (2) all the pieces are ligated into cloning vectors with equal efficiency; (3) all the plasmids are transformed into *E. coli* with equal efficiency; (4) all

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* There are 64 possible base triplets, 61 of them code for the 20 aminoacids. This means for most aminoacids, there are more than one code word. It is impossible to determine which one is actually used in a particular gene. Statistic method helps to make a guess of the most probable codon, but even then the probability to hit the right sequence is low. For example, the nucleicacid sequence for the first 7 aminoacids in 2,5-DCMO, Pro-Tyr-Met-Arg-Pro-Gly-Arg, can have 4608 combinations, the chance to hit the right sequence using the statistically most probable codons based on known sequences for *Pseudomonas putida* is only 2.5%.

the colonies bearing the fragments grow equally well; (5) there are unlimited colonies available. If one colony is sequenced, there is a 1/200 chance to hit a particular gene of interests, and a (1-1/200) chance to miss that gene. Since this can be considered as an independent probability case, if a second colony is taken, there is still a 1/200 chance to hit that particular gene, and a (1-1/200) chance to miss it. The probability of still missing that particular gene after two sequencings becomes $(1-1/200)^2$, and the degree of confidence that a particular gene is not missed becomes $[1-(1-1/200)^2]$. When more colonies are sequenced, the chance of still missing that particular gene will decrease further and the confidence of not missing that gene will increase. Table 23 shows the relationship between the number of DNA fragments sequenced and the degree of confidence that can be achieved.

Table 23: Relationship between confidence and sequenced fragments

fragments	possibility of getting (each)	possibility of missing (total)	confidence
1	1/200	$(1-1/200)^1$	0.005
2	1/200	$(1-1/200)^2$	0.01
. . .	. . .	. . .	. . .
10	1/200	$(1-1/200)^{10}$	0.05
. . .	. . .	. . .	. . .
100	1/200	$(1-1/200)^{100}$	0.39
. . .	. . .	. . .	. . .
1000	1/200	$(1-1/200)^{1000}$	0.993
. . .	. . .	. . .	. . .
n	1/200	$(1-1/200)^n$	$1-(1-1/200)^n$

Based on this analysis, 600 sequencings are necessary to get 95% confidence level; 920 for 99%; and 1100 for 99.5%. However these are only qualitative results, the actual number of sequencings needed may be several times higher, because the assumptions

made will not always be true. Despite the massive work involved in the shotgun method, there are attractive advantages, the major one being the possibility of discovering a large number of new genes. If one takes 2kb as the average gene length in bacteria, the enzymes involved in camphor degradation need only about 40kb, which is less than 20% of the *CAM* plasmid. The rest of the plasmid can contain a large number of genes, many of which are unknown and may code for enzymes that catalyze useful reactions. Sequencing the entire plasmid will open a door to a variety of new yeast reagents.

In order to use the shotgun method, *CAM* plasmid was first fragmented by restriction endonuclease digestion. *Sau3AI* was selected because the resulting fragments would have suitable termini which could be directly ligated to *BamHI* digested pUC19 vector. *Sau3AI* is a four-base pair restriction enzyme, statistically its restriction site can occur in every 4^4 base pairs. If the *CAM* plasmid were subjected to full digestion, the average fragment size would have been 256 bp; if partial digestion were performed, longer segment could be expected, and there would be some overlapping between those segments. This would permit re-assembling of the original DNA by analyzing the overlapping sequences. By varying the amount of enzyme used and the reaction time, partial digestion conditions were found that gave mostly 1-4kb fragments.

The fragments were purified by agarose gel electrophoresis and were ligated into pUC19 vector. This vector carries a gene for ampicillin resistance, which allows the selection of successfully transformed cells. It also has a polylinker region that codes for a small

protein capable of activating a native enzyme in carrier *E. coli* strain *XL1 Blue*. The activated enzyme can hydrolyze 5-bromo-4-chloro-3-indonyl-β-D-galactopyranoside (X-Gal) to give a blue coloured colony. When a foreign DNA fragment is inserted into the polylinker region, it no longer produces the activating protein, therefore the colony is white. This colour test distinguishes colonies that carry foreign DNA fragments from those that only have the re-ligated pUC19 vector.

Electroporation was used to transform *E. coli* with the religated plasmids which contained fragments from *CAM* plasmid. The transformed bacteria were grown on LB-Agar plates containing ampicillin, X-Gal and IPTG. White colonies were grown in liquid LB medium containing ampicillin to obtain enough cells for the DNA isolation.

Shotgun method requires the isolation of plasmids from hundreds of colonies, thus the traditional method for plasmid isolation was not suitable. The Wizard® miniprep kit (Promiga Biotechnologies) was used for faster DNA isolation. This kit used a special resin to absorb plasmid DNA from the cell lysate. After the removal of debris by centrifugation and buffer washings, plasmid DNA was released through elution with water or TE buffer. By this method, only 20 minutes were required to finish one miniprep, and 12 minipreps could be performed at the same time; in comparison, the traditional method would take two hours for each miniprep, and no more than 4 parallel operations would be possible.

All isolated plasmids were checked by linearization with *SacI* and gel electrophoresis to ensure they contained suitable DNA inserts. Correct plasmids were submitted to a sequencing laboratory in the University of Florida(Gainesville, FL) for automated sequencing. Since residual salt would interfere with the sequencing reactions, an extra washing step with 1:1 water and ethanol was added to ensure the absence of such impurities. After approximately 200 minipreps, the plasmid isolation was switched to the Quantum® kit (Bio-Rad Laboratories Ltd.), which gave higher yields and better quality products.

All sequences obtained were checked by on line BLAST (Basic Local Alignment Search Tool) search, and fragments that showed good sequence homology to genes coding for useful enzymes were selected for further examinations. At the end of this project, more than 600 fragments had been isolated and sequenced, which corresponded to ca. 95% confidence level. Although the desired 2,5- and 3,6-DCMO gene were still not found, 112 fragments showed various degree of sequence homology to reported genes coding for potentially useful enzymes. A partial list of fragments homologous to redox enzyme genes is given in Table 24. Further cloning work will be conducted by Dr. J. Stewart's group in the University of Florida.

Table 24: Partial list of redox genes obtained from shotgun method

clone	gene	score*
32-4E	*Pseudomonas aeruginosa* pyruvate dehydrogenase	$1x10^{-89}$
35-4B	Electron transfer flavoprotein-ubiquinone oxidoreductase homolog	$4.\ 1x10^{-76}$
39-5D	*E. coli* NADH dehydrogenase	$1.\ 1x10^{-46}$
33-6E	*Pseudomonas aeruginosa* cyanide insensitive terminal oxidase	$3.\ 0x10^{-23}$
53-2H	*H. influenzae* acyl carrier protein and 3-ketocarrier protein reductase	$4.\ 83x10^{-23}$
55-4D	*Pseudomonas putida* ketoglutarate semialdehyde dehydrogenase	$7.\ 9x10^{-24}$
54-4G	*E. coli* dicarboxylate transport protein	$6.\ 3x10^{-33}$
54-4I	*E. coli* kup system potassium uptake protein	$3.\ 0x10^{-60}$
55-4G	*Pseudomonas putida* b-ketoadipate:succinyl-coA transferare	$1.\ 7x10^{-43}$
39-2D	*Pseudomonas* fluoresence rho gene	$8.\ 2x10^{-105}$
42-3E	*Pseudomonas aeruginosa* Tn501 gene *tnpA* for transposase	$6.\ 5x10^{-67}$

* possibility of sequence homology in random DNA fragments

Experimental Section

List of Abbreviations

Ac	acyl
Amp	ampicillin
BSA	bovine serum albumin
DTT	dithiothreitol
EB	ethidium bromide
EDTA	ethylenediaminetetraacetic acid, disodium salt
IPTG	isopropyl-β-D-thiogalactopyranoside
RNAse	ribonuclease
SDS	sodium dodecyl sulfate
Tris	tris(hydroxymethyl)aminomethane, free base
X-Gal	5-bromo-4-chloro-3-indonyl-β-D-galactopyranoside

Concentration of solutions are expressed as multiples from the original solution. For example, 10x indicates the solution is 10 times concentrated from the original solution, 0.1x means the solution is 1:10 diluted.

Preparation of solutions

All chemicals and culture media were purchased from Fisher Scientific and Sigma. Water was purified by a Milli-Q ultra pure water system. All chemicals were dissolved in ca. 90% of the total amount of water at room temperature. When necessary, the pH

of the solutions was measured using a Fisher ACCUMET 25 pH meter and adjusted by titrating with 10% NaOH and 1:1 HCl. Water was added to adjust the volumes to desired values. All solutions were autoclaved at 15psi for 15 minutes unless otherwise noted. Agar plates were poured when the solutions had cooled to 40-50°C and were let to stand at room temperature for at least 24 hours to set before storing in a 4°C refrigerator.

TBE (Tris-Borate-EDTA buffer, 5x, Tris 0.45M, boric acid 0.45M, EDTA 10mM)

Tris	54g
boric acid	27.5g
EDTA	3.92g
water	1L
pH	8.0

TE (Tris-EDTA buffer, 0.1x, Tris 10mM, EDTA 1mM)

Tris	1.21g
EDTA	0.37g
water	1L
pH	8.0

Phosphate buffer (0.01M)

Na_2HPO_4, heptahydrate	1.34g
KH_2PO_4	0.68g
water	500mL
pH	7.0

PEG (42%)

polyethyleneglycol 6000	21g
phosphate buffer(0.01M, pH 7.0)	50mL

GTE (Glucose-Tris-EDTA buffer, glucose 50mM, Tris 25mM, EDTA 10mM)

glucose	9g
Tris	3.03g
EDTA	3.72g
water	1L
pH	8.0

KOAc-HOAc (KOAc 3M, HOAc 2M)

KOAc	49.1g
water	100mL
take 60mL of this solution, add	
HOAc, glacial	11.5mL
water	28.5mL
pH	<5.2

SDS-NaOH (SDS 10%, NaOH 2M)

NaOH	8g
water	100mL
SDS	10g
water	100mL
kept separate and mixed prior to use	

NaCl (4M)

NaCl	23.4g
water	100mL

LiCl (4M)

LiCl	8.5g
water	50mL

SSC (20x, NaCl 3M, sodium citrate 0.3M)

NaCl	87.75g
sodium citrate	44.11g
water	500mL
pH	7.0

SSC (10x, NaCl 1.5M, sodium citrate 0. 15M)

NaCl	43.83g
sodium citrate	22.06g
water	500mL
pH	7.0

TS (Tris 0.1M, NaCl 0.15M)

Tris	6.05g
NaCl	4.39g
water	500mL
pH	7.5

TSM (Tris 0.1M, NaCl 0.1M, $MgCl_2$ 0.05M)

Tris	6.05g
NaCl	2.93g
$MgCl_2$, hexahydrate	5.08g
water	500mL
pH	9.5

EDTA (0.5M)

EDTA	18.6g
water	100mL
NaOH, pellets	adjust pH to 8.0

EDTA (0.25M)

EDTA	9.30g
water	100mL
pH	8.0

Lysozyme (10mg/mL)

Lysozyme	10mg
EDTA (0.25M, pH 8.0)	1mL

sterilized by filtration, stored at -20°C in 50mL aliquots

NaOAc-HOAc (0.01M)

NaOAc	82mg
water	100mL
adjust pH with HOAc	5.2

Sucrose-Tris (sucrose 25%, Tris 0.01M)

sucrose	125g
Tris	0.60g
water	500mL
pH	7.0

Tris-HCl (2M)

Tris	24.2g
water	100mL
pH	8.0

RNAse (1mg/mL)

RNAse	1mg
NaOAc-HOAc (0.01M)	1mL
Tris-HCl (2M)	50mL

sterilized by filtration, stored at -20°C in 50mL aliquots

EB (10mg/mL)

ethidium bromide	100mg

water	10mL

TTB (terminal transferase buffer, 10x)

cacodylic acid	1.932g
Tris-HCl (2M)	1.5mL
adjust pH to	7.2
DTT (1M in 0.01M NaOAc buffer)	10mL
add water to	10mL

sterilized by filtration and stored at -20°C in 1mL aliquots

Denaturing solution (NaOH 0.5M, NaCl 1.5M)

NaOH	10.0g
NaCl	43.88g
water	500mL

Neutralizing buffer (Tris-HCl 1M, NaCl 1. 5M)

Tris	60.5g
NaCl	43.88g
pH	7.4
water	500mL

NaCl (0.1M)

NaCl	2.93g
water	500mL

Wizard I (Tris 50mM, EDTA 10mM, RNAse 100µg/mL)

Tris	0.61g
EDTA	0.37g

add water to	100mL
pH	7.5

autoclaved, then add RNAse (10mg in 1mL water, sterilized by filtration)

Wizard II (NaOH 0.2M, SDS 1%)

NaOH	0.80g
SDS	1.00g
water	100mL

Wizard III (KOAc 1.32M)

KOAc	12.94g
water	100mL
adjust pH with HOAc	4.8

Wizard IV (NaCl 0.2M, Tris 20mM, EDTA 5mM, EtOH 1.4 volume)

NaCl	2.34g
Tris	0.48g
EDTA	0.37g
water	200mL
ethanol	280mL

Trace Mineral Stock [19]

$MgCl_2$, hexahydrate	5.08g
$CaCl_2$, dihydrate	0.294g
$FeSO_4$, heptahydrate	0.56g
$ZnSO_4$, hexahydrate	0.144g
$MnSO_4$, tetrahydrate	0.11g
$CuSO_4$, anhydrous	16mg
$CoCl_2$, hexahydrate	23.7mg
H_3BO_3	6.2mg

DL-tryptophan	2.0mg
water	100mL

Minimal mineral medium for *Pseudomonas putida*[4a]

Na_2HPO_4, heptahydrate	7.55g
KH_2PO_4	2.0g
$(NH_4)_2SO_4$	1.0g
trace mineral stock	4mL
camphor	2.5g/L
water	1L

LB medium (Luria-Bertani broth)

Bacto Tryptone	5.0g
Bacto Yeast Extract	2.5g
NaCl	5.0g
water	500mL

LB-Agar (for 20 plates)

LB medium	500mL
Bacto Agar	10.0g

LB/Amp medium

LB medium (autoclaved)	500mL
Ampicillin	1.0g in 10mL water, sterilized by filtration
	add 2ml for each 500ml medium

mixed when cooled to below 50°C under sterile conditions

LB/Amp-Agar (for 20 plates)

LB medium	500mL

Bacto Agar	10g
autoclaved	
Ampicillin	1.0g in 10mL water, sterilized by filtration

mixed when cooled to 50°C under sterile conditions

LB/Amp/X-Gal/IPTG plate

on each LB/Amp plate spread

X-Gal (20mg/mL in DMF)	40μL
IPTG (200mg/mL in water, filter sterilized)	4μL

Nutrient Broth

Bacto Peptone	2.5g
Bacto Yeast Extract	1.0g
Bacto Beef Extract	0.5g
NaCl	2.5g
pH	7.4±0.2
water	500mL

Nutrient agar

nutrient broth	500mL
Bacto agar	10.0g

SOC medium

The following solutions were prepared separately:

KCl	1.86g
water	100mL
$MgCl_2$	19.0g

water	90mL
sterilized by filtration	
glucose	18.0g
water	90mL
autoclaved	
Bacto Tryptone	10.0g
Bacto Yeast Extract	2.5g
NaCl	0.25g
water	450mL
KCl solution (0.25M)	5mL
pH	7.0
add water to	490mL
autoclaved	
$MgCl_2$ (2M, sterile)	2.5mL
glucose (1M, sterile)	10mL

added under sterile conditions

Isolation of plasmids

All centrifugations were performed at 4°C unless otherwise noted. Pellet re-suspension was done by vortexing for 0.5-1 minute. After addition of each solution, the mixture was gently swirled and/or inverted several times to mix the solutions. The ethanol used for precipitating and washing DNA was pre-chilled at -20°C. The following procedures were used to recover DNA from reaction mixtures unless otherwise noted: The ionic strength was adjusted to 0.2M by addition of 4M NaCl. Ethanol (95%, 2.5 times by volume) was added and mixed by gentle inversion. The mixture was stored at -20°C from 2 hours (small scale) to overnight (large scale). The DNA was recovered by centrifugation at 4°C. After washing with ethanol (75%), the DNA pellet was briefly lyophilized to remove last traces of ethanol and re-dissolved in TE buffer.

Midiprep of plasmid DNA from ATCC17453[9d] *Pseudomonas putida* (ATCC17453) was streaked on an LB-Agar plate and grown at room temperature until colonies were 1-2mm in size. One colony was used to inoculate 40mL mineral medium supplemented with camphor (2.5g/L) as the only carbon source. It was grown at 30°C, 200rpm for 2 days. The saturated culture was centrifuged at 10,000rpm for 15 minutes. The pellet was suspended in 10mL phosphate buffer and centrifuged again. The pellet was re-suspended in 5.5mL of sucrose-Tris buffer and lysozyme (100μL, 10mg/mL) was added. After incubating at 0°C for 5 minutes, EDTA (2mL, 0.25M) was added and the mixture was incubated at 0°C for another 5 minutes. SDS (4mL, 10%) was added and the mixture was subjected to a heat pulse by placing the tube in a 55°C heating block for 15

seconds. The centrifugation tube was removed and gently inverted 5 times to mix the solution. The heat pulse was repeated 7 times. The pH was adjusted with 2M NaOH to 12.1-12.3 (ca. 2mL added). Tris-HCl (3mL, 2M) was added to bring the pH to around 8. SDS (5.2mL, 10%) was added immediately followed by NaCl (6.3mL, 4M) and the mixture was gently inverted for 1 minute. The mixture was stored at 4°C overnight. The precipitate was removed by centrifugation, and 1/3 volume of PEG solution (42%) was added. The mixture was allowed to stand at 4°C for 6 hours and centrifuged at 6,000 rpm for 40 minutes. The pellet was washed once with 2mL 70% ethanol and re-dissolved in 1mL of TE buffer.

Largeprep of plasmid DNA[9d] *Pseudomonas putida* was streaked on an LB-agar plate and grown at room temperature until colonies were 1-2mm in size. One colony was used to inoculate 400mL mineral medium with camphor (2.5g/L) as the only carbon source. It was grown at 30°C, 200rpm until saturation ($OD_{600} = 4.2$). The culture was centrifuged at 3000rpm for 20 minutes. The pellet was washed once with 100mL of phosphate buffer and re-suspended in 50mL sucrose-Tris buffer. Lysozyme (10mg in 1mL TE) was added and the mixture was incubated at 0°C for 5 minutes. EDTA (20mL, 0.25M) was added and the mixture was incubated at 0°C for 5 minutes. SDS (20mL, 20%) was added and the mixture was subjected to 8 round of heat shock by placing the centrifugation tube in a 60°C water bath for 1 minute with gentle rotation followed by taking out the tube and gentle inversion for 8 seconds. NaOH (15mL, 2M) was added to bring the pH to 12, and the mixture was gently inverted for 3 minutes.

Tris-HCl (20mL, 2M) was added to bring the pH to 8, and SDS (26mL, 20%) was added immediately followed by NaCl (63mL. 4M). The mixture was inverted for 1 minute and stored at 4°C overnight. The precipitate was removed by centrifugation at 3000rpm for 40 minutes. The supernatant was filtered through 4 layers of KimWipe®, and 1/3 volume of PEG (42%) was added. The mixture was stored at 4°C overnight. The precipitate was collected by centrifugation at 4°C, 3,000rpm for 1 hour. The pellet was washed once with ethanol, re-dissolved in 4mL TE containing 80μg RNAse and stored at 4°C.

Purification of plasmid DNA by ultracentrifugation DNA solution from the large prep was diluted to 7mL with TE. 7.35g CsCl was added followed by EB (750μL, 10mg/mL), and stored in dark for 10 minutes at room temperature. The mixture was centrifuged at 3,500rpm for 10 minutes and the supernatant was transferred to a fresh tube. The density of the solution was adjusted to 1.55-1.57g/mL by dropwise addition of TE buffer. The solution was transferred to a 10mL sealable ultracentrifugation tube via a sterile syringe. The empty space was filled with mineral oil and the weight was adjusted to within 5mg difference from that of the balance tube. The centrifugation tube was heat sealed and ultracentrifuged at 45,000rpm for 36 hours at room temperature. The centrifugation tube was clamped vertically and was punctured with a needle near the top of the tube. The DNA solution was slowly withdrawn with a sterile syringe via a second, wide bore needle punctured through the tube directly under the DNA band. The solution was placed in a fresh centrifugation tube and repeatedly extracted with *n*-

butanol saturated with water until no further pink colour of EB was visible in organic extracts. The aqueous layer was transferred to a 3x5cm dialysis bag and dialyzed against 1L of TE buffer at 4°C. The TE buffer was replaced every 4 hours. After 3 changes, the content of the dialysis bag was transferred to a fresh centrifugation tube. NaCl solution (175μL, 4M) was added followed by ethanol (8.75mL). The mixture was stored at -20°C for 1 hour and centrifuged at 3,500rpm for 10 minutes. The pellet was washed once with ethanol (2mL), and re-dissolved in 3mL TE buffer. An aliquot of this solution (10μl) was diluted with distilled water to 100μL and transferred to a 1mm quartz cuvet and optical density (OD) at 268nm and 280nm were taken. OD_{260} = 0.1655, OD_{280} = 0.0864, OD_{260}/OD_{280} = 1.91. DNA concentration was determined to be 0.66mg/mL (determined from OD_{260}, 0.4mg/mL for each OD_{260} unit).

Control labeling reaction with Genius Labeling Kit Standard DNA sample (vial 2, 5μL) was heated at 95°C for 10 minutes and chilled to 0°C in an ice bath. Hexanucleotide mixture (vial 5, 2μL), dNTP labeling mixture (vial 6, 2μL), water (10μL) followed by Klenow fragment enzyme (vial 7, 1μL) were added and mixed by gentle stirring with the pipette tip. The mixture was incubated at 37°C for 60 minutes. The reaction was stopped by addition of EDTA (2μL, 0.2M) and the DNA was recovered by ethanol precipitation.

Southern Blot control reaction with Genius Kit[17] The product from the control labeling reaction and reference sample (vial 3, 5ng/μL) were used in this test. Serial dilutions

were made by mixing 1μL of more concentrated sample and 9μL of TE. Samples of 0.1x (500pg/μL), 0.01x (50pg/μL), and 0.001x (5pg/μL) dilution were prepared. Original sample, 0.1x and 0.01x dilutions were also used to prepare 1:1 dilutions which had DNA concentration of 2500pg/μL, 250pg/μL and 25pg/μL. A small piece of nitrocellulose membrane was soaked in water and transferred to 20x SSC. The membrane was dried between two pieces of filtering paper and was placed on a fresh piece of filtering paper. Serial dilution samples (1μl each) were spotted to the membrane by touching the pipette tip to the membrane and allowing the liquid to be drawn through the membrane. The membrane was air dried at room temperature and was further dried in a vacuum oven at 80°C, 0.1mmHg for 2 hours. The membrane was placed in a pouch and blocking solution was added (20mL for each 100cm^2 membrane), the pouch was sealed and was incubated at 68°C for 1 hour. The pouch was opened and the blocking solution was discarded. The membrane was washed twice in 2x SSC and 0.1% SDS (1mL 20x SSC, 0.1mL 10% SDS, 9mL water) for 5 minutes at room temperature, followed by two washings in 0.1x SSC and 0.1% SDS (50μL 20x SSC, 100μL 10% SDS, 10mL water) at 68°C for 15 minutes. When lower stringency was needed, the washing condition was changed to 1x SSC, 1% SDS, 55°C, 15 minutes, or 0.1x SSC, 0.1% SDS, 55°C, 15 minutes, or 1x SSC, 1% SDS, 37°C, 20 minutes, or 1x SSC, 1% SDS, room temperature, 30 minutes. The membrane was then washed in Tris-NaCl buffer at room temperature for 1 minute, followed by 30 minutes in the blocking solution. The membrane was washed briefly in Tris-NaCl, and was incubated in a solution of digoxygenin alkaline phosphatase (vial 8, 1μL) in Tris-NaCl (5mL) at room

temperature for 30 minutes. The solution was discarded and the membrane was washed twice with 100mL of Tris-NaCl buffer for 15 minutes at room temperature. The membrane was placed in a new pouch and a solution of NBT (vial 9, 45μL), X-phosphate (vial 10, 35μL), and TSM buffer (10mL). The pouch was sealed and placed in dark at room temperature for 1 day without any disturbance. The membrane was removed and rinsed with TE buffer, and the spots were recorded by photocopy or photographic methods when the membrane was still wet. From the colour of the spots the concentration of labeled DNA from the labeling reaction was determined to be 500pg/μL, which corresponded to a labeling efficiency of 10%. Sample concentration of 5pg/μL was detected in Southern Blot analysis using this protocol.

Digestion of *CAM* plasmid with restriction endonucleoases

Endonucleoases were purchased from New England Biolabs or Boehringer Mannheim Biochemicals. Boehringer Mannheim SuRE/Cut system was used as the digestion buffer. Distilled water was purified through Milli-Q system and autoclaved. Plasmid DNA (2μL) was added to a sterile 0.6ml Eppendorf tube containing distilled water (15μL), digestion buffer (2μL) and mixed by gentle stirring with the pipette tip. Endonucleoase (1μL, or 0.5μl each if two endonucleoases were used.) was added and mixed well by gentle stirring, and the mixture was incubated at 37°C for 4 hours. When a batch of digestions were to be conducted together, the reaction tubes were temporarily stored on ice until all the tubes were ready and were put into the incubator together. Agarose gel electrophoreses were conducted in 6x10cm polyacrylate trays. The ends of

the tray were sealed with two pieces of label tape and the comb was placed approximately 0.5cm from one end and 0.5-1mm from the bottom. 0.4g agarose was suspended in 40mL 0.5x diluted TBE buffer and heated in a microwave oven with occasional swirling until the agarose had dissolved. The solution was cooled to ca. 50°C, EB (0.5μL) was added and the solution was poured into the tray and allowed to set at room temperature for 2 hours. The comb was removed and the gel was installed in the electrophoresis apparatus. TBE buffer (0.5x) was used as the working electrolyte. The digestion mixture (3μL) was added to a 0.6mL Eppendorf tube containing 3μL distilled water and 2μL of loading buffer. The mixture was loaded to one lane in the gel and electrophoresis was conducted at 86V at room temperature. When higher resolution was needed, the gel was developed at 50V. When tracking dye bromophenol blue has reached the end of the gel, electrophoresis was stopped and the bands were visualized under UV light. Photographic method was used to record the gel.

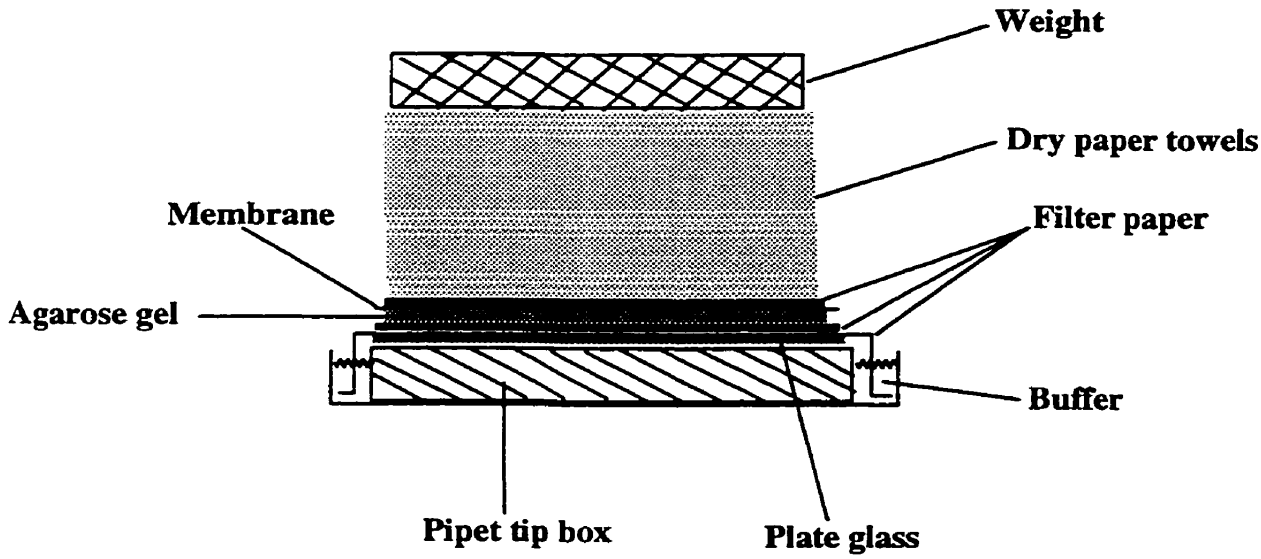

Figure 21: DNA transfer in Southern blot

Southern Blot of DNA fragments separated by electrophoresis The apparatus setup is illustrated in Figure 21. An empty pipette tip box was covered with a piece of plate glass to obtain a flat platform. The whole unit was wrapped in two layers of filter paper and placed in a shallow tray containing 20x SSC. Four more layers of filter paper were carefully placed on top of the platform. Agarose gel from electrophoresis was inspected under UV light and any unused area was trimmed off. The left-bottom corner of the gel was cut off as a marker for the orientation. The gel was soaked in denaturing solution (NaOH 0.5M, NaCl 1.5M) for 30 minutes at room temperature with occasional agitation. After rinsing with distilled water, the gel was soaked in neutralizing buffer (Tris-HCl 1M, NaCl 1.5M) for 30 minutes at room temperature. The gel was rinsed with distilled water and placed upside down (the opening of the loading well facing the filter paper) on the platform. A piece of nitrocellulose membrane was trimmed to leave a small rim (about 0.5-1mm) around the gel and wetted with 20x SSC. The membrane was placed on the gel, Four pieces of filter paper were trimmed to the same size of the gel and were wetted in 20x SSC and placed on the membrane. Care was taken to avoid air bubbles between any of the layers. Dry paper towels were cut to the same size as the filter paper and were placed on the filter paper. Some weight was applied on top of the paper towels. Wetted paper towels were replaced when necessary to ensure sufficient liquid flow through the membrane. The transfer was continued for at least 8 hours. The membrane was washed in 10x SSC to remove any gel debris, air dried between two pieces of filter paper and further dried at 80°C, 0.1mmHg to fix DNA fragments. Subsequent steps followed the protocol of the Southern Blot control test (page 28).

Labeling of *camR* binding region by 3′-tailing with terminal transferase[28] The DNA probes (2μl each, 100μg/ml in TE) corresponding to *camR* binding regions were placed in 0.6mL Eppendorf tubes containing 12μL water, and denatured at 95°C for 10 minutes. After chilling in an ice bath, TTB (10x, 2μL), $CoCl_2$ (10mM, 2μL), dig-dUTP (0.25mM, 1μL), and terminal transferase (1μL) were added. The mixture was incubated at 37°C for 15 minutes. The reactions were terminated by chilling on ice and addition of EDTA (0. 5M, 10μL). The resulting solutions were stored at -20°C.

Larger scale labeling reactions DNA probes (4μL each, 100μg/mL in TE) were placed in 0.6mL Eppendorf tubes and diluted with water (9μL). After denaturalization, TTB (10x, 2μL), $CoCl_2$(10mM, 2μL), dig-dUTP(0.25mM, 2μL), and terminal transferase(1μL) were added and the mixture was incubated at 37°C for 60 minutes. The reactions were terminated by chilling on ice and addition of EDTA (0.5M, 10μL). The resulting solutions were stored at -20°C. Serial dilutions and Southern Blot check were performed as described before (page 28). 1x, 0.1x, 0.05x, 0.01x, 0.005x, 0.001x solution were prepared. Labeled DNA sample (Genius Kit Vial 4, 50ng/μL) was used as a reference standard. By comparing the intensity of the final spots, the effective concentration of labeled DNA was determined to be ca. 50pg/μL, and the detection limit was 0.25pg/μL.

Preparation of vector for ligation Cloning vector pUC19 (0.67mg/mL, 14.9μL, 10μg) was diluted in 70μL water. Boehringer Mannheim buffer B (10μL) was added followed by *Bam*HI (5μL). The mixture was incubated at 37°C and monitored by periodically removing a small aliquot (1μl) and checking by electrophoresis. The digestion finished in 30 minutes. Linearized DNA was recovered by ethanol precipitation and re-dissolved in Tris-HCl (90μL, 10mM, pH 8.3). CIP buffer (10x, 10μL), and calf alkaline phosphatase (0.01x dilution in TE, 5μL) were added and the mixture was incubated at 37°C for 30 minutes. The reaction was terminated by the addition of EDTA (0.5M, 3μL) and heat deactivation at 65°C for 40 minutes. The mixture was extracted once with saturated phenol in chloroform (200μL), the aqueous layer was transferred to a fresh Eppendorf tube and the DNA was recovered by ethanol precipitation. The pellet was re-dissolved in TE (10μL) and stored at -20°C.

Preparation of inserts for ligation *Sau*3A I (1μL) was diluted with a mixture of water (7μL), BSA (1mg/mL, 1μL), and *Sau*3A I digestion buffer (NEB, 10x, 1μL). *CAM* plasmid DNA (2mg/mL, 2μL), *Sau*3A I digestion buffer (New England Biolabs, 10x, 2μL), BSA (1mg/mL, 2μL), water (13μL), and diluted enzyme (1μL) were mixed and incubated at 37°C. An aliquot of the reaction mixture (1μl) were withdrawn every 10 minutes and treated by addition of EDTA (0.2M, 1μL) and heat deactivation at 65°C for 20 minutes. The aliquots were checked by gel electrophoresis, which showed digestion of 30 minutes at 37°C with 0.1x diluted enzyme was suitable to give the majority of fragments between 1-3kb. Larger scale digestion was performed using 65μL water,

10μL DNA, 10μL BSA, 10μL digestion buffer, 5μL 0. 1x diluted *Sau*3A I at 37°C for 30 minutes. The reaction was terminated by addition of EDTA (0. 5M, 1μL) and heat deactivation at 65°C for 20 minutes. The DNA was recovered by ethanol precipitation and re-dissolved in 20μl TE.

General procedures for DNA purification by preparative agarose gel electrophoresis

The agarose gel used for purification of the ligate product was prepared from 1% agarose and 0.5x TBE containing 10μg/mL EB. The gel was prepared in the mid-tray of the instrument and the wide comb was used to increase the loading capacity of the gel. DNA samples suitable reference samples were loaded and developed as usual. Lower voltage was used to obtain better resolution. When the desired band had migrated more than 2/3 of the total gel length, the electrophoresis was stopped and the gel was examined under hand-held low power UV light. The gel was trimmed to leave as little as possible containing the desired band. The trimmed gel was transferred into a 1.6mL Eppendorf tube and frozen at -20°C for 1.5 hours. The sample was quickly thawed at 37°C and crushed with a clean spatula. The slurry was transferred to filter cups and centrifuged at 10,000rpm for 15 minutes at room temperature. The residue was re-soaked with 200μL TE buffer for 5 minutes and then re-centrifuged. DNA in the filtrate was recovered by ethanol precipitation.

General procedures for ligation of vector and insert The total amount of DNA in the mixture should be approximately 2μg. The amounts of vector (cut and dephosphorylated) and insert were determined by the following equations:

Let A = vector size (kb), B = insert size (kb)

then $\text{vector} = \frac{A}{A+B} \times 2\ (\mu g)$

$\text{insert} = \frac{B}{A+B} \times 2\ (\mu g)$

Appropriate amount of vector and insert solutions were placed in a 0.6mL Eppendorf tube, and enough water was added to make 18μL. A small sample (1μL) of this mixture was removed and kept in a fresh Eppendorf tube at 0°C as a reference. T4 ligase buffer (2μL) was added followed by T4 ligase (1μL). The reaction mixture was maintained at 16°C overnight. The reaction was checked by electrophoresis, the 1μL DNA sample set aside before ligation and the uncut vector were used as reference samples.

Ligation of pUC19 and *CAM* plasmid digest When calculating the amount of each component to be used in the ligation, the size of the insert (0.6-4kb) was taken as 0.6kb.

$$\text{pUC19 (3.2 kb)} = \frac{3.2}{3.2+0.6} \times 2\mu g = 1.7\mu g = 1.7\mu L\ (1mg/mL)$$

$$\textit{CAM}\ \text{digest (0.6 - 4 kb)} = \frac{0.6}{3.2+0.6} \times 2\mu g = 0.32\mu g = 3.2\mu L\ (100\mu g/mL)$$

The ligation mixture consisted of water (12μL), T4 ligase buffer (10x, 2μL), pUC19 (cut and dephosphorylated, 1mg/mL in TE, 3.4μL, excess 100%), *CAM* digest (0.6-4kb,

100μg/mL in TE, 3μL, in three 1μL portions in 1 hour intervals), and T4 ligase(1μL). The mixture was incubated in a 0.6ml Eppendorf tube at 16°C for 12 hours. The reaction was checked by electrophoresis using *CAM* digest, pUC19 (cut and dephosphorylated) and pUC19 (circular) as reference samples. There were new bands that corresponded to the correctly ligated products. The ligation mixture was stored at -20°C.

Preparation of acceptor cells for transformation *E. coli* strain *XL1Blue* was streaked on a nutrient-agar plate and incubated at 37°C until colonies were 1-2mm in size. One colony was used to inoculate 10mL LB medium and grown at 37°C, 200rpm overnight. 1mL of this culture was used to inoculate 100mL LB medium and grown at 37°C, 200rpm until OD_{600} was 0.5 (ca. 2 hours). The culture was centrifuged at 4,000rpm for 15 minutes at 4°C. The cell pellet was re-suspended in 100mL of ice cold water and centrifuged again at 4,000rpm for 15 minutes at 4°C. The washing was repeated once with 50mL of ice-cold water, and once with 2mL of ice-cold glycerol (10%). The cell pellet was finally suspended in 600μL ice-cold 10% glycerol and stored as 40μL aliquots in a -70°C freezer. Acceptor cells prepared in this way can be stored for extended period, and can be used directly in electroporation upon thawing.

Transformation of *E. coli* by electroporation Three vials of acceptor cells were thawed and kept on ice. Plasmid sample(2μL), pUC18(positive control, 1μL), and TE (negative control, 1μL) were added respectively. These mixtures were loaded into ice-cold

electroporation cells, and were electroporated at 2.5 KV. After the discharge, 1mL SOC medium was added quickly to each electroporation cell and the mixtures were incubated at 37°C for 1.5 hours. Diluted culture (200μL, 0.1x in TE) was spread on each LB/Amp/X-Gal/IPTG plate. One plate for each control and five plates for the transformation were used and incubated at 37°C for 24 hours. Transformants with appropriate inserts gave white colonies.

Miniprep with Wizard Kit[20] A single colony of *E. coli* was used to inoculate 10mL LB medium in a 25mL test tube and grown at 37°C, 200 rpm overnight. 1.5mL of the culture was centrifuged at 10,000rpm for 2 minutes at room temperature. The cell pellet was re-suspended in Wizard I (cell resuspension buffer, 200μL), then Wizard II (cell lysing solution, 200μL) was added and mixed by gentle inversion until the mixture became clear and viscous. Wizard III (neutralizing solution, 200μL) was added and mixed by gentle inversion. The precipitated debris was removed by centrifugation for 5 minutes at 10,000rpm at room temperature. A filter cup was attached to a 3mL disposable syringe with the plunger removed. The supernatant containing the plasmid was transferred to the syringe barrel, 1mL DNA resin was added and mixed by gentle tapping and rotation for 1 minute to allow complete absorption of the plasmid. The liquid was pushed through with the plunger and was discarded. The filter cup was detached from the syringe and the plunger was removed. The filter cup was re-attached and 2mL of Wizard IV (washing buffer) was added and was pushed through by the plunger. The filter cup containing the DNA resin was removed and attached to a 1.5mL

Eppendorf tube. The assembly was centrifuged at 10,000rpm for 2 minutes in order to remove any liquid. The plasmid was released from the resin by re-hydrolyzing with 50μL of water for 1 minute and centrifuged into a fresh Eppendorf tube. The resulting DNA solution was checked by electrophoresis and stored at -20°C.

Miniprep with Quantum Kit[21] A single colony of *E. coli* was used to inoculate 8mL LB/Amp medium in a 25mL test tube and grown at 37°C, 200rpm for 20h. The culture was centrifuged at 1,000rpm for 20 minutes. The cell pellet was transferred to a 1.5mL Eppendorf tube and centrifuged again at 10,000rpm for 1minute. The cell pellet was suspended in Quantum I (cell resuspension buffer, 200μL), then Quantum II (cell lysing solution, 200μl) was added quickly and mixed by repeated inversion until the mixture became clear and very viscous. Quantum III (neutralizing solution, 200μL) was added and mixed by brief but swift shaking. The mixture was allowed to settle for 2 minutes and centrifuged at 10,000rpm for 5 minutes. The supernatant containing the plasmid was transferred to a filter cup installed on a 1.5mL Eppendorf tube. Quantum IV (Quantum Miniprep Matrix, 200μl) was added and waited for 2 minutes with occasional tapping to allow complete absorption of the plasmid. The assembly was centrifuged at 10,000rpm for 1 minute and the filtrate was discarded. Quantum V (washing buffer, 500μL) was added and the assembly was tapped vigorously to re-suspend the matrix. The assembly was centrifuged at 10,000rpm for 1 minute and the washing was repeated once. The filter cup was transferred to a new Eppendorf tube, any liquid on the outer wall was wiped off. The lid was left open for 2 minutes to let last trace of ethanol evaporate. The DNA was released by rehydrating the resin with 50μL water. DNA solution was recovered by

centrifuging at 10,000rpm for 2 minutes. The resulting solution was checked by gel electrophoresis and was stored at -20°C.

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Chapter Eight: Conclusions

This thesis work has extended the repertoire of oxidation reactions catalyzed by the designer yeast overexpressing cyclohexanone monooxygenase from soil bacterium *Acinetobacter* sp. NCIB 9871.

The oxidations of prochiral 4-substituted cyclohexanones indicated that compounds with short, *non-oxygenated* substituents in the 4-position are preferred substrates for CHMO, which was in agreement with the early report by Taschner.* Both nonpolar (alkyl and allyl) and polar (halo) groups were acceptable and gave good reactivities and enantioselectivities. On the other hand, *oxygenated* substituents were less successful. Compounds with short oxygenated groups (-OH, -OMe) were oxidized with lowered enantioselectivities, and those with longer substituents (-OEt, -O-allyl) failed to react. The longest chain allowed in the 4-position was found to be about 3-4 atoms. These results were rationalized using various models proposed for CHMO active site.

Both 2- and 3-alkylated cyclopentanones have been successfully oxidized. 2-Alkyl-cyclopentanones were oxidized with complete kinetic resolution when the substituent chains were four carbons and longer. 3-Substituted cyclopentanones were oxidized with low to moderate enantioselectivities, but the regioselectivity became complete (>99%) when the chain length reached 4 carbons.

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* See Ref. 18 in Chapter One.

Several 2-substituted cyclopentanones bearing functionalized side chains were evaluated as substrates for the oxidizing yeast. It was found that compounds with unprotected alcohol and carboxylic acid side chains were poor substrates because of the low reactivity and low enantioselectivity in the oxidations. Compounds with etherial side chains were better substrates, but the selectivities were still lower than those of simple alkyl substituted cyclopentanones with similar chain lengths. An unfavourable hydrogen bond within the active site of CHMO has been proposed to explain the lower reactivity and lower selectivity in these reactions.

A study of the bio-oxidation of sulfides, dithiolanes and dithianes revealed the possible complexity of live-cell biotransformations. The enantio- and diastereoselectivity of these reactions were found to be highly dependent on the structure of the substrates, the bioreagent used, and the reaction conditions. In cases when the oxidation catalyzed by bioengineered yeast and *E.coli* gave comparable results to those obtained by isolated CHMO or the parent soil bacterium *Acinetobacter* NCIB 9871, the experimental simplicity of yeast-mediated reactions makes it an attractive alternative in organic synthesis. The oxidations of 2,2-disubstituted dithianes bearing one ester chain and one ether chain were found to be particularly enantio- and diastereoselective, which is valuable both in the terms of preparing the chiral sulfoxides and in understanding the active site of the enzyme.

In order to further develop the yeast reagent and to extend the idea of "designer yeast" to other useful biotransformations, the cloning of diketocamphane monooxygenase from *Pseudomonas putida* ATCC17453 was initiated during an exchange program at the University of Florida. Two different approaches, one being the "*camR* hypothesis" and the other being the shotgun method, were used. A DNA library of over 600 fragments from the *CAM* plasmid has been constructed, and the subsequent cloning is an on-going project at the University of Florida.

To demonstrate the utility of the oxidizing yeast reagent in the synthesis of pharmaceutically important natural products, a short route for mevinolin analogue synthesis was designed using the designer yeast to introduce the chirality in the final product. Different approaches were tested to synthesize a key intermediate. Although this project was unfinished due to the limited time frame of this thesis work, the short synthetic approach demonstrated the potential of using the designer yeast in organic synthesis.

Appendix One: Protocols Used in Yeast- and *E. coli*-Mediated Oxidations

Protocols used in yeast-mediated oxidations

Transformation of baker's yeast

Yeast strain *Saccharomyces cerevisiae* 15C was streaked on a URA(+) plate and incubated at 30°C until the colonies were 1-2mm in size. One colony was used to inoculate 100mL YPD in a 250mL Erlenmeyer flask and incubated on an orbital shaker at 30°C, 200rpm until OD_{600} had reached 2.0. An aliquot of 1.5mL was placed in a sterile Eppendorf tube and centrifuged at 5000rpm for 3 minutes. The cell pellet was washed with TE (0.5mL) and centrifuged again.* The washing was repeated once with TE/LiOAc (0.5mL) and the yeast cells were resuspended in 0.5mL TE/LiOAc. An aliquot of 0.1mL was placed in a fresh sterile Eppendorf tube and calf thymus DNA (2.15mg/mL, 4.6μL), pKR001 plasmid DNA (837μg/mL, 2μL) and PEG/TE/LiOAc (0.7mL) were added and well mixed. The mixture was incubated at 30°C for 1 hour without shaking followed by a heat pulse by immersing the Eppendorf tube in a 41°C circulating water bath for 25 minutes. After cooling to room temperature, the content of the tube was spread on URA(-)

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*From this step on all mixing was done by gentle inversion and stirring with the pipet tip.

plates at 0.1mL/plate and incubated at 30°C until the colonies were 1-2mm in size.

One colony was used to inoculate 20mL YPD in a 125mL Erlenmeyer flask and incubated at 30°C, 250rpm overnight. At that time the culture should have reached OD_{600} between 4-6.* The cells were harvested by centrifugation at 3000 x g, 0°C for 5 minutes. The cell pellet was washed twice with TE and resuspended in YPD containing 15% glycerol at 50mg/mL. The mixture was divided into 0.5mL aliquots and stored in a -80°C freezer.

Propagation and maintenance of yeast reagent

The yeast was streaked directly from the frozen stock† on a URA(-) plate and incubated at 30°C until the colonies were 1-2mm in size. Colonies from this plate were used for the propagation and preparation of the yeast reagent. The plate could be sealed with Parafilm® and temporarily stored‡ in a refrigerator at 4°C. The same protocol as described in the previous section was used for making yeast stocks when necessary.

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* OD_{600} was measured in 1:10 diluted sample using distilled water as the reference, and the reading was multiplied by 10.

† Yeast stock should be kept at low temperature at all times. Vials that have been warmed beyond -40°C or thawed should be discarded.

‡ Colonies that have been on URA(-) plates for more than one week are known to start losing their activity.

The propagation of the carrier yeast strain 15C without the plasmid was identical except that URA(+) plates were used.

Protocols for preparation of yeast for reactions

The growth of yeast can be divided into 4 phases (Figure 22).* The cell density and activity both increase during the log phase, and reach the maximum in the stationary phase. When the cells were incubated for an extended period, loss of viable cells and activity will slowly occur (death phase). The preferred period to perform the reaction was found to be the mid-log phase and the stationary phase. When the cells had reached the death phase, the reaction would slow down and a much larger amount of metabolites would form.

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* Adapted from the on-line lecture notes by McKenna, S., Mondal, D., Long Island University, Brooklyn.
http://www.bklyndev.liunet.edu/FacultyProjects/WebClass/micro-web/html-files/ChapterB-1.html

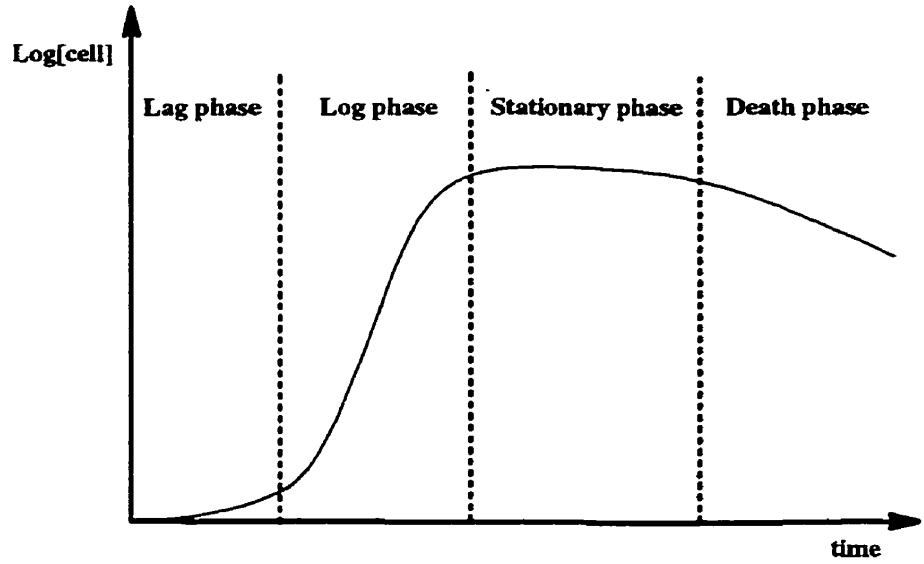

Figure 22: Qualitative diagram of cell growth

Successful yeast propagation protocols should be able to synchronize the cell growth, minimize any possible mutations, maximize the mass yield from a given volume of culture, and maximize the yeast activity.

The original protocol (**method A**) was developed during the study of yeast-mediated oxidations of cyclohexanone substrates by Dr. Stewart's group at the University of Florida. Yeast from frozen stock was streaked on a URA(-) plate and incubated at 30°C until the colonies were 1-2mm in size. YPD (100mL) in a 250mL baffled Erlenmeyer flask was inoculated with a single yeast colony. In order to increase the amount of yeast cells, several flasks could be prepared at the same time, each inoculated with a separate colony. The flasks were agitated on an orbital shaker at 30°C, 200rpm for 20-28

hours until the OD_{600} has reached 6-8.* The cultures were combined and the cells were harvested by centrifugation at 3000 x *g* for 10 minutes at 0°C. The yeast pellet was washed three times with TE. After the final spin the yeast cells were suspended in 15% glycerol in TE at 0.2g/mL wet mass, divided into 1mL aliquots and frozen in a -80°C freezer.

This protocol generally produced yeast with consistent but moderate activities; several aspects in this method could be improved. First of all, the mass yield from a single colony was low, since each colony could be used to inoculate only 100mL of medium, which will produce approximately 0.5g wet mass of yeast cells at OD_{600} 6-8.† Secondly, the cell growth was not synchronized; this was because the original cell growth in a colony was not even (Figure 23).

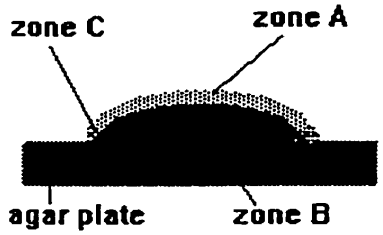

Figure 23: Uneven cell growth on an agar plate

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* Cultures that had $OD_{600} < 6$ after 28h were discarded

† It is inappropriate to inoculate more medium with a single colony, because the time required for the culture to reach the desired OD will significantly increase if the culture volume were too high. An extended incubation period increases the possibility of mutation and loss of activity.

The cells in **zone A** have enough air supply, but the nutrient supply is limited because it has to pass through layers of cells underneath, and the cell growth is slower; cells in **zone B** have sufficient nutrient supply from the agar plate, but air supply is limited and the cells have to adapt to the relatively anaerobic growth conditions; only cells near the rim of the colony (**zone C**) which have both sufficient nutrient supply and air supply will therefore have the most vigorous growth. When cells in different growth conditions are used to inoculate the growth medium, the resulting culture also contain cells in different growth conditions and the cell activity is not maximized.

An alternative method (**Method B**) was developed to prepare larger amounts of yeast from a single colony. In this protocol, 10mL YPD in a 50mL Erlenmeyer flask was inoculated with a single yeast colony and agitated on an orbital shaker at 30°C, 250rpm overnight. This culture was used to inoculate YPD (1:100 ratio by volume) in a large baffled Erlenmeyer flask. Cell growth, harvest and storage followed the protocol described in **Method A**. In this method, the first overnight culture served to bring all viable cells to the stationary phase, and the cell growth was started in a synchronized fashion when diluted with more YPD. This method consistently gave large quantities of highly active yeast, and was used in preparing yeast-reagent for reactions.

It was found empirically that yeast cells that had been briefly exposed to their preferred substrates showed increased activities towards other substrates. A second modification (**Method C**) was based on the above observation. A single colony of yeast was used to inoculate 200mL YPD in a 500mL baffled Erlenmeyer flask and agitated on an orbital shaker at 30°C, 250rpm. When the OD_{600} of the growing yeast had reached 4.0, usually within 24h, the cells were separated by centrifugation at 3000 x g for 10 minutes at 0°C and the yeast pellet was transferred to a 500mL baffled Erlenmeyer flask containing 200mL YPG. Cyclohexanone (20μL) was added and the reaction was allowed to proceed for 3-4 hours until OD_{600} was 6-8. The yeast was harvested and stored as described in **Method A**. This method produced yeast with the highest activity, but the variations between batches seemed to be larger, and an increased proportion of metabolites was generated during the reactions. Therefore, this method was used only for reactions that were otherwise too slow.

General procedure for yeast-mediated oxidations

The substrate was added to 100mL YPG in a 250mL baffled Erlenmeyer flask at a concentration of 10mM. The frozen yeast was thawed and added to the reaction flask at a final concentration of 2mg/mL, and the flask was agitated at 30°C, 250rpm. The reaction was monitored by periodically

withdrawing 100μL of the medium and extracting each sample with an equal volume of EtOAc or CH_2Cl_2 by vigorously vortexing for 30 seconds, followed by centrifugation at 7500rpm for 1 minute. The organic layer was withdrawn and analyzed by GC, HPLC, or TLC. Ethyl benzoate or diethyl 1,2-dibenzoate (50 ppm) was used as an internal standard for the GC analyses.

When the reaction had finished, the culture was centrifuged. The yeast pellet was re-suspended in 50mL of distilled water and extracted twice with ethyl acetate (100mL). The aqueous layer was saturated with NaCl and extracted with 200mL of ethyl acetate in 3 or 4 portions.* Combined extracts were washed once with brine and dried over anhydrous Na_2SO_4 or $MgSO_4$. Continuous liquid-liquid extraction with CH_2Cl_2 overnight could be used and this generally gave better results. After removing the solvent on a rotary evaporator, the residue was purified by flash chromatography on silica gel.

Strategies for optimization of reaction conditions

If substrate solubility or toxicity was a problem, it might be advantageous to include a stoichiometric amount of cyclodextrin (α-, β- or γ-, depending

---

* It is not advisable to directly extract the culture without first removing the cells because of severe emulsion problems.

on the substrate) in order to form an inclusion complex that would increase the solubility and reduce the toxicity of the substrate. In this case, the substrate and cyclodextrin were shaken in YPG until a uniform dispersion was achieved before the yeast was added. Because of the formation of inclusion complexes, it was necessary to use longer extraction times and a larger amount of solvent in the workup in order to achieve yields comparable to those obtained in the reactions run without cyclodextrins. Reactions with cyclodextrin were not always advantageous. It had been observed that in some cases the inclusion complex precipitated and the results were even worse. Lowering the substrate concentration or adding the substrate in smaller portions provided better solutions in these cases.

The cell density exerted a profound influence on the reactivity. High activity was observed during the log phase and stationary phase of the yeast. When the cell density had reached saturation the activity leveled off and eventually started to fall. At very high cell densities, yeast-reduction could become the predominant reaction. On the other hand, too low initial cell density was not suitable since the yeast would lose activity after a prolonged incubation period.

It was observed that aeration was beneficial to the reactions. To ensure enough oxygen in the system, the culture medium was kept at less than 1/3

of the total volume of the flask. The reaction flasks were covered with permeable materials such as 4-6 layers of KimWipe® instead of the more commonly used aluminum foil to allow the exchange of air. The Erlenmeyer flasks used in the reactions were baffled and the orbital shaker was set to a slightly higher speed to help the aeration of the culture. However, even with these modifications, large scale reactions (in excess of 1L) still suffered from diminished reactivity, presumably due to lower efficiency of agitation and starting material dispersion. For larger scale reactions a fermenter would be necessary.

Protocols for *E. coli*-mediated reactions

Propagation of *E. coli* strains

The *E. coli* strain BL21(DE3)(pMM04) was streaked from a frozen stock on LB-Ampicillin plates and incubated at 37°C until colonies were 1-2mm in size. One colony was used to inoculate 10mL of an LB-Ampicillin medium in a 50mL Erlenmeyer flask and incubated at 37°C, 250rpm overnight. Sterile glycerol (15% v/v) was added and the mixture was divided into 0.5mL aliquots and stored in a -80°C freezer. The carrier strain BL21(DE3) was propagated using the same protocol except that no ampicillin was used in the plates and medium.

General procedure for *E. coli*-mediated reactions

A saturated culture of *E. coli* was prepared as above. This culture was used at a 1:100 ratio to inoculate an LB-Ampicillin medium supplemented with 10% glucose in a baffled Erlenmeyer flask. The culture was incubated at 30°C, 250rpm until OD_{600} was approximately 1. IPTG stock solution was added (0.1μL per mL of medium) followed by the substrate. If cyclodextrin was necessary to alleviate the solubility problem, it was introduced at this stage. The culture was agitated at room temperature at 250rpm and monitored by GC, HPLC or TLC until the reaction was finished.

The culture was saturated with NaCl and extracted with ethyl acetate. Combined extracts were washed once with brine and dried with anhydrous Na_2SO_4 or $MgSO_4$. The solvent was removed on a rotary evaporator and the residue was purified by flash chromatography.

Appendix Two: General Experimental Conditions

General instrumentation

NMR spectra were obtained on Varian Unity 400, Brucker AMX 400 or Varian XL-200 instruments. All spectra were recorded in $CDCl_3$ solutions unless otherwise specified. Calibration of the spectra was performed by referencing to the solvent trace (δ=7.24ppm for ^1H spectra, and δ=77.0ppm for ^{13}C spectra). In cases when the solvent signal overlapped other peaks, the TMS peak in the solvent was used as the internal standard (d=0.0ppm for both ^1H and ^{13}C spectra). IR spectra were recorded from thin films on a Nicolet 520 FT-IR spectrophotometer or a Perkin Elmer 297 instrument. Optical rotations were measured on a Perkin Elmer 241 polarimeter operating at ambient temperature. All measurements were performed in $CDCl_3$ solutions unless otherwise specified. Packed column gas chromatography was performed on a Shimadzu GC-9A gas chromatograph employing a customer packed column (1/8" x 1m, 5% OV-101 on 100/120 Supelcoport®, Supelco Ltd.). Capillary gas chromatography was performed on a Hewlett Packard 5890 instrument employing a 0.54μm x 1.0mm x 15m DB-1301 column (J&W), and a Shimadzu GC-9A employing a 0.32μm x 0.25mm x 30m β-Dex 225 column (Supelco). All the GC instruments used flame ionization detectors and helium as the carrier gas. HPLC was performed on a Beckman System Gold personal chromatograph using an Econosphere 5μm x 4.6mm x 250mm Silica-C18 column (Alltech) coupled with a 4.6mm x 150mm Chiralcel OD-H column (Daicel Chemical

Industries Ltd.) operated at room temperature. Mixtures of hexanes and 2-propanol were used as the mobile phase. Distillations of products were carried out using a short path microdistillation apparatus or a Kugelrohr apparatus. Melting points were recorded from a Fisher/Johns apparatus without correction. Thin layer chromatography was performed on pre-coated silica gel 60 plates (Whatmann). Flash chromatography was performed on silica gel (200-425 mesh, Fisher Scientific or SiliCycle).

A Puffer Hubbard upright freezer operating at -80°C was used to store the yeast and *E. coli* strains. Cell culturing and biooxidations were performed on an Innova 4000 orbital shaker (New Brunswick Scientific). A Vortex Genie-2 vortexer (VWR) and a Biofuge 13 microcentrifuge (Baxter Canlab) were used in the monitoring of bioreactions. A CT 4 20 centrifuge (Jouan) employing 50mL tubes or 500mL buckets was used to remove cells after reactions. An Accumet 25 pH meter (Fisher Scientific) was used in preparing the media and buffers for biotransformations. Optical density was measured on a Spectronic 20D spectrophotometer (Milton Roy Co.)

Data analysis and plotting were performed with CA-Cricket Graph III (v.1.01, Computer Associates International, Inc.) and Curve Fit (v0.7e, Kevin Raner) software packages. Several computer programs were developed to perform internal standard calculations used in GC analysis (Appendix IV).

Descriptive conventions

The following convention was followed in describing the spectroscopic data:
IR: w = weak, m = medium, s = strong, vs = very strong, br = broad;
NMR: s = singlet, d = doublet, t = triplet, q = quintet, m = multiplet, br = broad.

Treatment of chemicals and solvents

$MgSO_4$ and Na_2SO_4 were dried in a 500°C furnace for 12 hours and cooled in a desiccator when received and stored in the original bottles. K_2CO_3 was dried in an 80°C oven and cooled to room temperature in a desiccator prior to use. Methylene chloride was dried over anhydrous K_2CO_3, fractionally distilled and stored over 3Å molecular sieves. Ether and THF were refluxed with and distilled from Na-K alloy in the presence of benzophenone in an argon atmosphere. Methanol and ethanol were dried by reacting with magnesium and distilled from the mixture. *t*-Butanol was dried by refluxing with sodium metal and distilled from the mixture in an nitrogen atmosphere. Acetone was dried with anhydrous $CaSO_4$ and distilled from $KMnO_4$. Ethyl acetate and hexane were purified by fractional distillation. Other solvents and reagents were obtained from commercial suppliers (Sigma-Aldrich, Lancaster Synthesis, and Fisher Scientific) and used as received unless otherwise indicated.

Miscellaneous

Unless otherwise noted, when an organic solvent or extract was dried with an appropriate drying agent, the drying agent was removed by filtration before subsequent distillation or rotary evaporation steps.

By default, when GC analysis was used to monitor a yeast or *E. coli*-mediated reaction, the internal standard method described in Appendix IV was used to normalize the reported "GC yield"; when GC was used to monitor a chemical transformation or to determine the purity of a product, direct instrument reading was used. No calibration curve was used in the analyses due to the semi-qualitative nature of this thesis work.

Analysis conditions for GC and HPLC

Table 25: Typical operating parameters for GC HP-5890

Chromatograph:			
Carrier gas:	helium 60ml/min., 100:1 splitting ratio.		
Oven temperature:	100°C, 4 minutes, ramp up at 10°C/min. to 180°C, 10 minute		
Injector:	250°C		
Detector:	300°C		
Range:	2		
Attenuation:	0		
Recorder:			
ATTEN	2	CHT SP	0.4
AR REJ	1000	THRSH	0
PK WD	0.10		

Table 26: Typical operating parameters for GC Shimadzu GC-9A

Packed column GC analysis			
Chromatograph:			
Carrier gas:	helium 50ml/min.		
Oven:	100°C, 2 minutes, ramp up at 6°C/min. to 180°C, 15 minutes.		
Injector and detector:	225°C.		
Data range:	10^2		
Recorder:			
WIDTH	10	SLOPE	15
DRIFT	0	MIN.AREA	100
T.DBL	0	STOP.TM	32
ATTEN	2	SPEED	2
METHOD$	441	FORMAT$	0
SPL.WT	100	IS.WT	1
Capillary column GC analysis			
Chromatograph:			
Carrier gas:	helium 100ml/min., 100:1 splitting ratio.		
Oven temperature:	100°C, 3 minutes, ramp up at 1°C/min. to 150°C, 1 minute, then ramp up at 10°C/min. to 180°C, 15 minutes.		
Injector and detector:	225°C.		
Data range:	10^1		
Recorder:			
WIDTH	8	SLOPE	50
DRIFT	0	MIN.AREA	50
T.DBL	0	STOP.TM	82
ATTEN	2	SPEED	2
METHOD$	441	FORMAT$	0
SPL.WT	100	IS.WT	1

Table 27: Typical operating parameters for HPLC

Mobile phase:	9:1 hexane and 2-propanol, 1mL/min.
UV Detector:	254nm for general analysis, 274nm for sulfoxides
Peak Threshold:	0.003AU

Appendix Three: Recipes

Dipping solutions for TLC visualization*

PMA solution

Phosphomolybdic acid	20g
Ceric sulphate	2.5g
H_2SO_4 (5%)	500mL

$KMnO_4$ solution

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

Vanillin solution

Vanillin	5g
MeOH	100mL
H_2O	200mL
H_2SO_4	0.5mL

---

*(a) Touchstone, J.C., *Practice of Thin Layer Chromatography*, 3rd. Ed., John Wiley and Sons, Inc., NY, 1992; (b) Casay, et. al., *Advanced Practical Organic Chemistry*, page 114.

Media and buffers used in biotransformations

URA(-) plates

Yeast nitrogen base without amino acids (DIFCO 0919-15)	0.67%	3.35g
Dextrose* (Fisher D15-3)	2%	10g
Bacto Agar (DIFCO 0145-17-0)	2%	10g
L-Triptophan (1g/100mL stock)	20mg/L	1mL
L-Histidine (1g/100mL stock)	20mg/L	1mL
L-Leucine (1g/100mL stock)	30mg/L	1.5mL

All ingredients except dextrose were suspended in 400mL water and autoclaved at 120°C for 20 minutes. The solution was sufficient for 20 plates.

* Autoclaved separately in 100mL water and mixed well when cooled.

The same recipe was used for **URA(+)** plates with the addition of 20mg uracil.

YPD

Yeast extract (DIFCO 0127-17-9)	5g
Bacto Peptone (DIFCO 0118-17-0)	10g
water	450mL
Dextrose	10g
water	50mL

Autoclaved separately and mixed when cooled.

The same recipe was used for **YPG** medium, with galactose substituted for dextrose.

TE

Tris, free base	1.21g
EDTA, disodium salt	0.37g
water	1L
pH (adjusted with 1:1 HCl and 50% NaOH)	7.5

Autoclaved at 120°C for 20 minutes

LiOAc/TE

LiOAc	1.65g
TE	250mL
pH	7.5

Autoclaved at 120°C for 20 minutes

LiOAc/PEG/TE

PEG 3000 (poly ethylene glycol)	17.5g
LiOAc/TE	50mL
pH	7.5

Autoclaved at 120°C for 20 minutes

Glycerol

Autoclaved in neat form and mixed with YPD or TE at 15% (v/v) when cooled.

Appendix Four: BASIC Program for Internal Standard Analysis

Computer program for non-chiral analysis

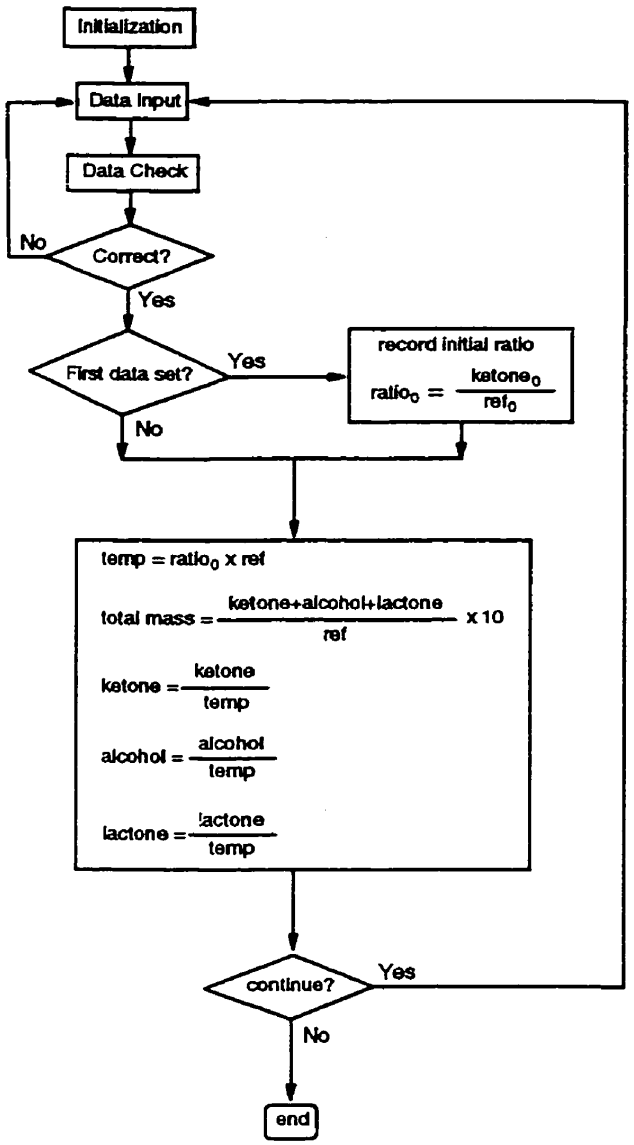


Figure 24: Flow chart for non-chiral column GC analysis

The program for interpreting the results from non-chiral GC analysis was based on the flow chart illustrated in Figure 24. This program was coded for Microsoft® MS-DOS QBASIC.

```
ref0 = -1
PRINT "Internal Standard method for OV101"
60 PRINT : PRINT
65 BEEP
70 PRINT "Input Data for OV101: "
80 INPUT "ketone="; ketone
90 INPUT "alcohol="; alcohol
100 IF ref0 = -1 THEN
      INPUT "reference="; ref0
      ref = ref0
      ratio0 = ketone / ref
   ELSE
      INPUT "reference= "; ref
   END IF
110 INPUT "metabolite="; metab
120 INPUT "lactone="; lactone
123 PRINT "ketone="; ketone, "alcohol="; alcohol, "reference="; ref, "metabolite="; metab,
        "lactone="; lactone
126 INPUT "Are DATA all right?"; a$
127 IF a$ = "n" OR a$ = "N" GOTO 70
```

```
128 CLS
130 temp = ratio0 * ref
140 IF ref <> 0 THEN PRINT "total ratio: "; INT((ketone + alcohol + lactone) / ref * 100 + .5) /
        10
150 PRINT "ketone="; INT(ketone / temp * 1000 + .5) / 10; "%"
160 PRINT "alcohol="; INT((alcohol / temp * 1000 + .5)) / 10; "%"
170 PRINT "lactone="; INT(lactone / temp * 1000 + .5) / 10; "%"
175 PRINT
180 INPUT "Go on (Q = stop)"; a$
190 IF a$ <> "q" AND a$ <> "Q" GOTO 60
210 END
```

Computer program for chiral analysis

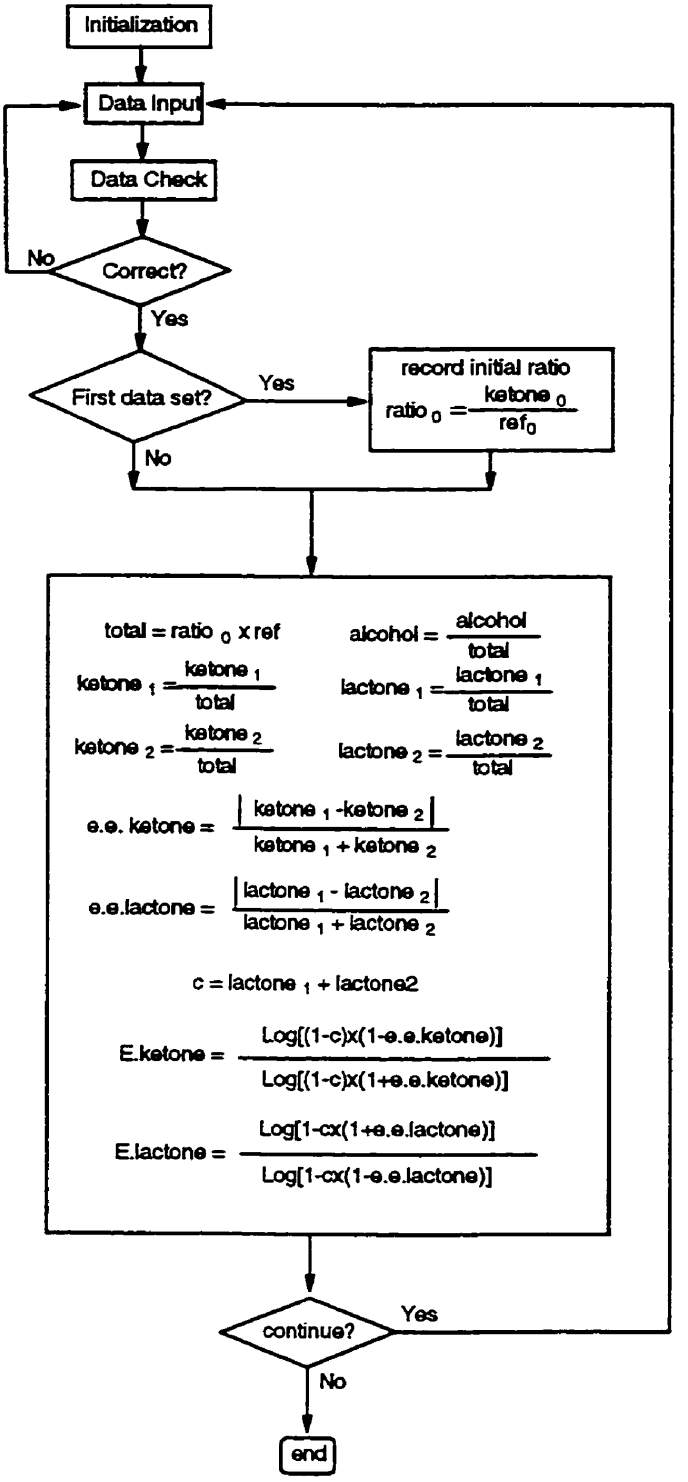


Figure 25: Flow chart for chiral GC analysis

The flow chart for chiral GC analysis is illustrated in Figure 25. The following is the computer program:

```
ketone1 = 0
ketone2 = 0
alcohol = 0
lactone1 = 0
lactone2 = 0
ref = -1
 CLS
PRINT "Internal Standard method calculator for chiral columns"
60 PRINT : PRINT : PRINT "choose what items will be used"
PRINT "when prompted, enter 0 for items that are not used"
PRINT "for items that are used, enter its number in ascending order"
PRINT : PRINT
INPUT "reference    ", fref
INPUT "ketone1      ", fket1
INPUT "ketone2      ", fket2
INPUT "alcohol      ", falc
INPUT "metabolite   ", fmet
INPUT "lactone1     ", flac1
INPUT "lactone2     ", flac2
CLS : PRINT "the following items will be used:": PRINT : PRINT
FOR i = 1 TO 7
```

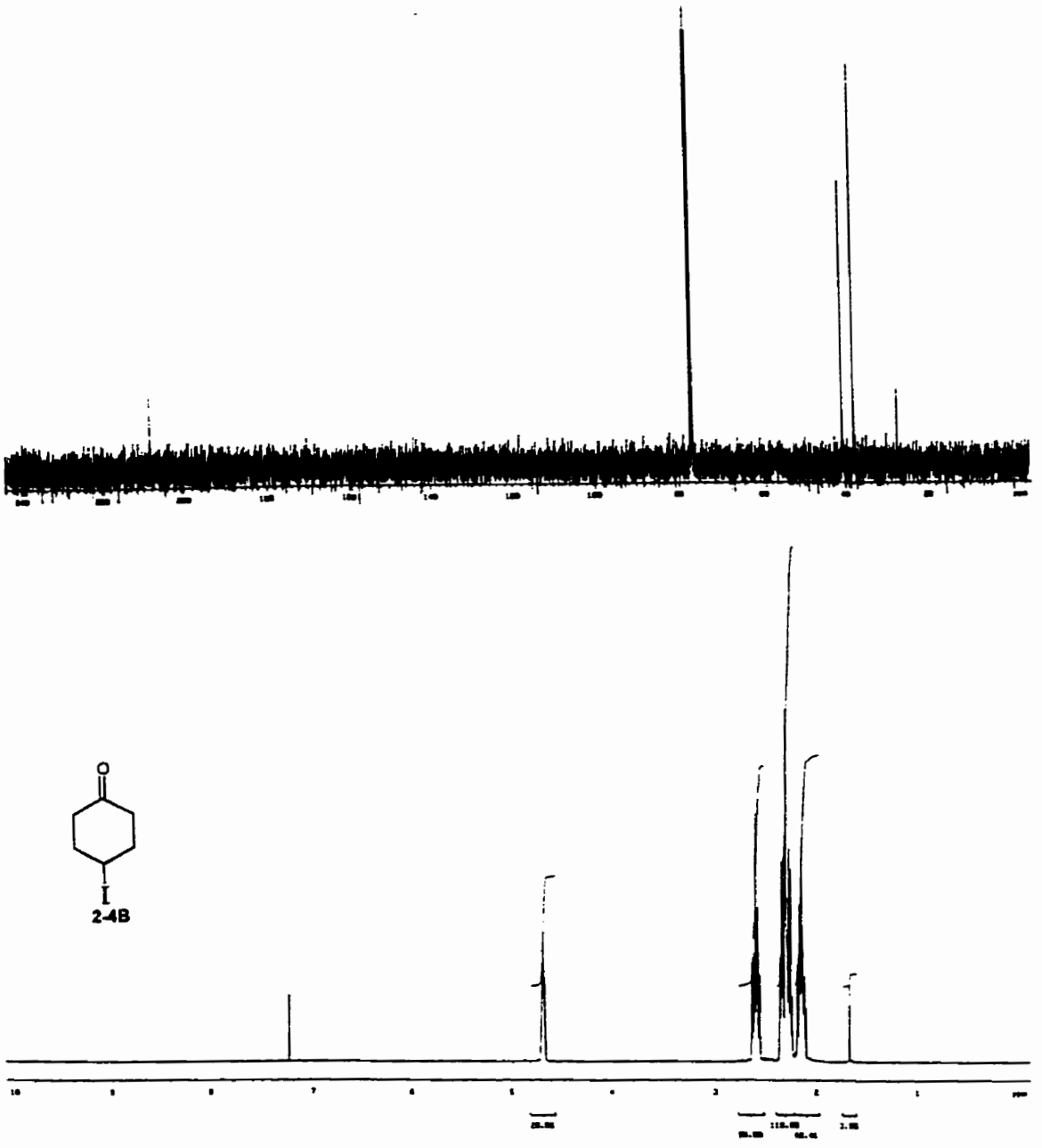
```
IF fref = i THEN PRINT "Reference"
IF fket1 = i THEN PRINT "ketone1"
IF fket2 = i THEN PRINT "ketone2"
IF falc = i THEN PRINT "alcohol"
IF fmet = i THEN PRINT "metabolite"
IF flac1 = i THEN PRINT "lactone1"
IF flac2 = i THEN PRINT "lactone2"
NEXT i
PRINT : PRINT : INPUT "everything all right     ", a$
IF a$ = "n" OR a$ = "N" THEN GOTO 60
CLS
BEEP
70 CLS
PRINT "Input Data: "
PRINT : PRINT
FOR i = 1 TO 7
IF fket1 = i THEN INPUT "ketone1="; ketone1
IF fket2 = i THEN INPUT "ketone2="; ketone2
IF falc = i THEN INPUT "alcohol="; alcohol
IF fref = i THEN
   IF ref = -1 THEN
        INPUT "reference="; ref0
     ELSE
        INPUT "reference="; ref
```

```
      END IF
    END IF
  IF fmet = i THEN INPUT "metabolite="; metab
  IF flac1 = i THEN INPUT "lactone1="; lactone1
  IF flac2 = i THEN INPUT "lactone2="; lactone2
  NEXT i
  IF ref = -1 THEN
        ref = ref0
        ratio0 = (ketone1 + ketone2 + alcohol + lactone1 + lactone2) / ref
    END IF
  sum = ketone1 + ketone2 + alcohol + ref + metab + lactone1 + lactone2
  IF sum > 100 THEN CLS : PRINT "Data Error:  total exceeded 100%": GOTO 70
  CLS : PRINT "Please check the data": PRINT : PRINT
  FOR i = 1 TO 7
  IF fket1 = i THEN PRINT "ketone1="; ketone1
  IF fket2 = i THEN PRINT "ketone2="; ketone2
  IF falc = i THEN PRINT "alcohol="; alcohol
  IF fref = i THEN PRINT "reference="; ref
  IF fmet = i THEN PRINT "metabolite="; metab
  IF flac1 = i THEN PRINT "lactone1="; lactone1
  IF flac2 = i THEN PRINT "lactone2="; lactone2
  NEXT i
INPUT "Are DATA all right?"; a$
IF a$ = "n" OR a$ = "N" THEN CLS : GOTO 70
```

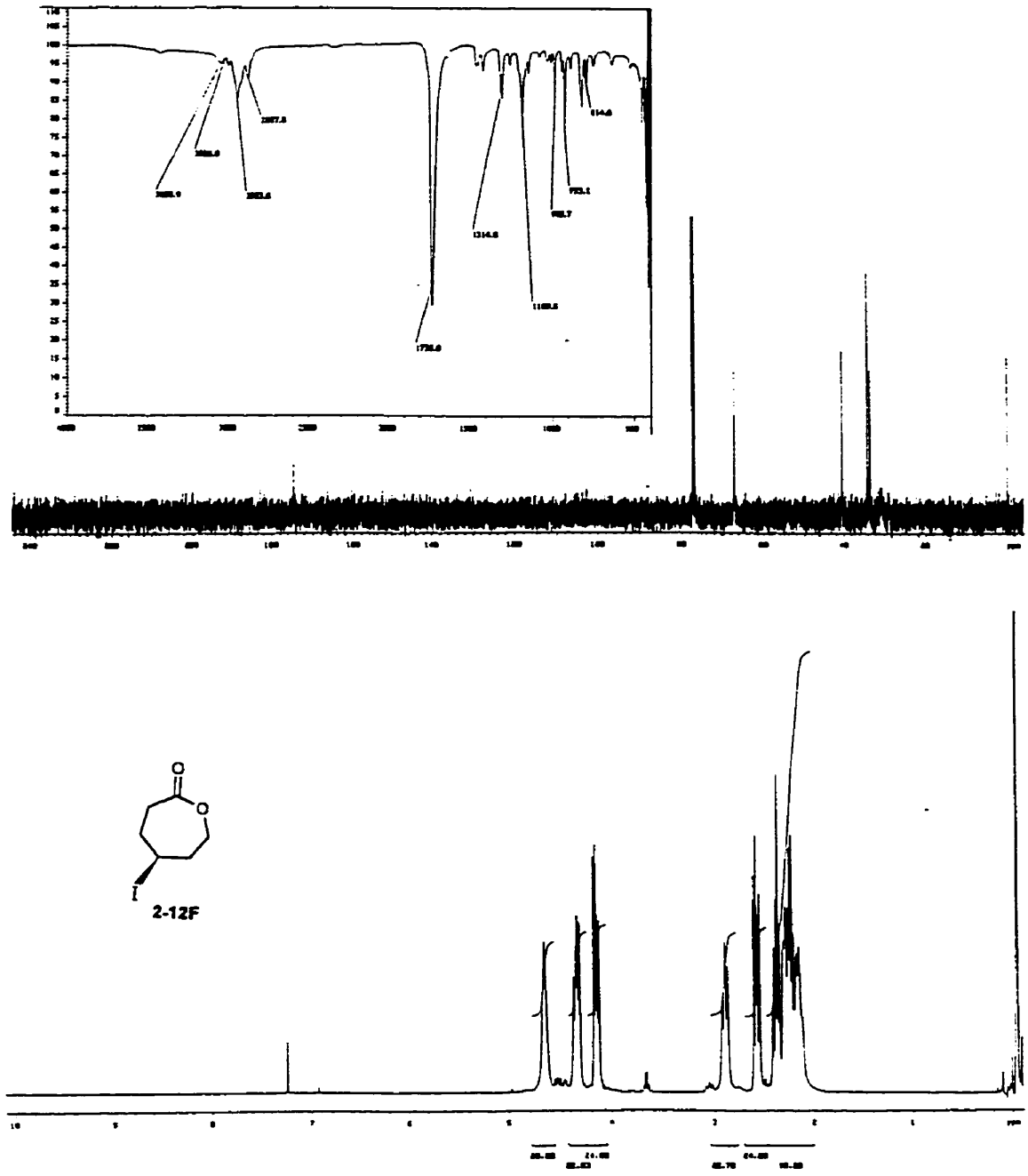
```
REM convert to fractional ratio
 total = ratio0 * ref
 sum = sum - ref - metab
 ketone1 = ketone1 / total
 ketone2 = ketone2 / total
 alcohol = alcohol / total
 lactone1 = lactone1 / total
 lactone2 = lactone2 / total
 c = lactone1 + lactone2
 ketone = ketone1 + ketone2
  IF c <> 0 AND flac1 <> 0 AND flac2 <> 0 THEN eelactone = ABS(lactone1 - lactone2) /
     (lactone1 + lactone2)
  IF ketone <> 0 AND fket1 <> 0 AND fket2 <> 0 THEN eeketone = ABS(ketone1 - ketone2) /
     (ketone1 + ketone2)
  IF c <> 0 AND ketone <> 0 AND fket1 <> 0 AND fket2 <> 0 THEN eketone = ABS(LOG((1
     - c) * (1 - eeketone)) / LOG((1 - c) * (1 + eeketone))) ELSE eketone = 0
   IF c <> 0 AND flac1 <> 0 AND eelactone <> 1 AND flac2 <> 0 THEN elactone =
     ABS(LOG(1 - c * (1 + eelactone)) / LOG(1 - c * (1 - eelactone))) ELSE elactone = 0
CLS
IF ref <> 0 AND fref <> 0 THEN PRINT "total ratio: "; INT(sum / ref * 100 + .5) / 10
IF fket1 <> 0 THEN PRINT "ketone1="; INT(ketone1 * 1000 + .5) / 10; "%"
IF fket2 <> 0 THEN PRINT "ketone2="; INT(ketone2 * 1000 + .5) / 10; "%"
IF fket1 <> 0 AND fket2 <> 0 THEN PRINT "e.e. of ketone="; INT(eeketone * 1000 + .5) / 10;
     "%"
```

```
IF eketone <> 0 THEN PRINT "E value (based on ketone) is "; INT(eketone * 10 + .5) / 10
IF falc <> 0 THEN PRINT "alcohol="; INT(alcohol * 10 + .5) / 10; "%"
IF fmet <> 0 AND ref <> 0 THEN PRINT "metabolite/ref="; INT(metab / ref * 1000 + .5) / 10;
    "%"
PRINT "lactone1="; INT(lactone1 * 1000 + .5) / 10; "%"
IF flac2 <> 0 THEN PRINT "lactone2="; INT(lactone2 * 1000 + .5) / 10; "%"
PRINT "fractional conversion="; INT(c * 1000 + .5) / 10; "%"
IF flac1 <> 0 AND flac2 <> 0 THEN PRINT "e.e. of lactone="; INT(eelactone * 1000 + .5)/10;
    "%"
IF elactone <> 0 THEN PRINT "E value (based on lactone) is "; INT(elactone * 10 + .5) / 10
PRINT
INPUT "q: stop; c: configure; <ENTER>: go on"; a$
IF a$ = "q" OR a$ = "Q" THEN GOTO 200
IF a$ = "c" OR a$ = "C" THEN GOTO 60
GOTO 70
200  END
```

Appendix Five: Selected Spectra of Representative Compounds

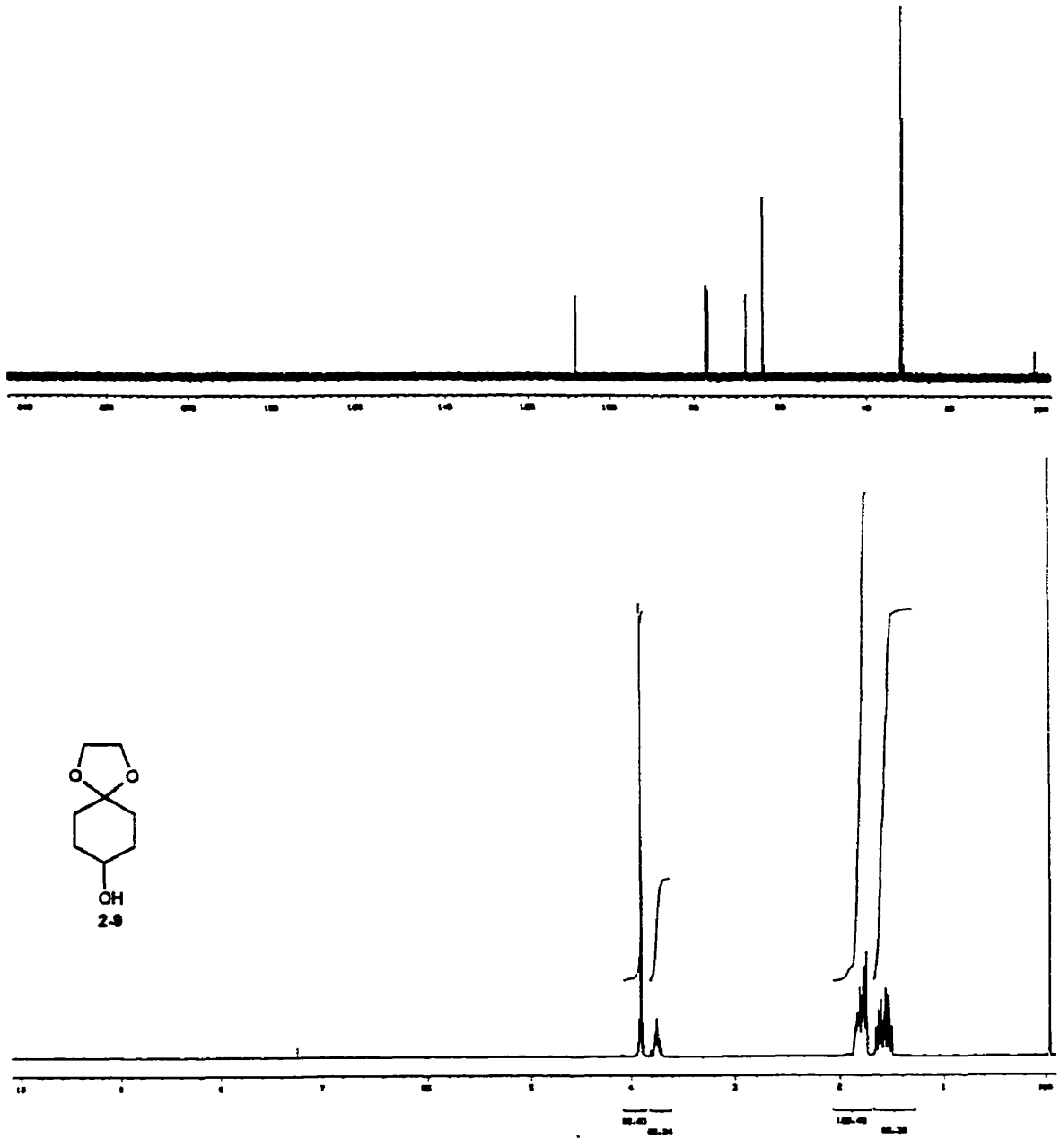


4-Iodocyclohexanone **2-4B**

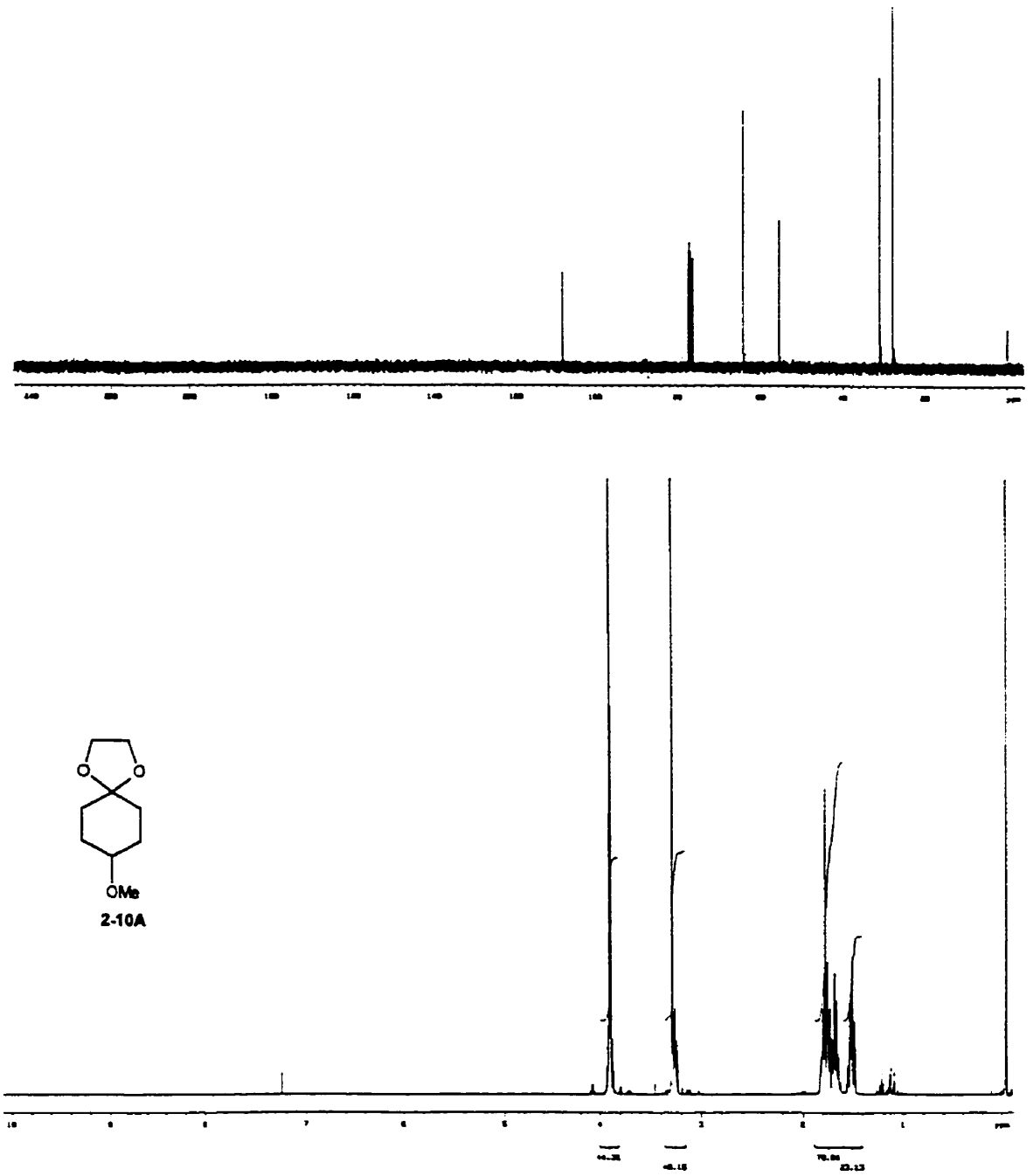


2-12F

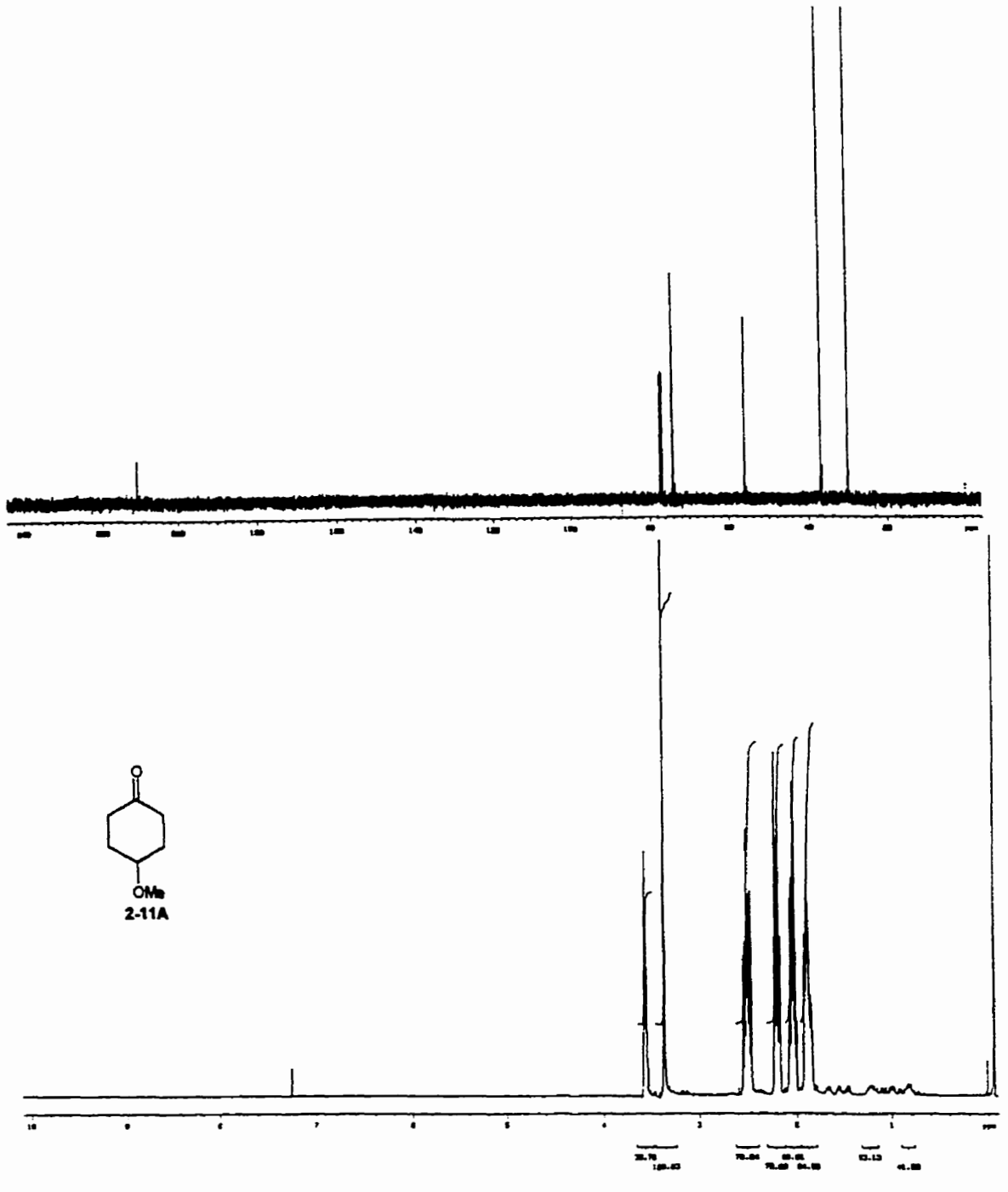
5-Iodooxepan-2-one **2-12F**



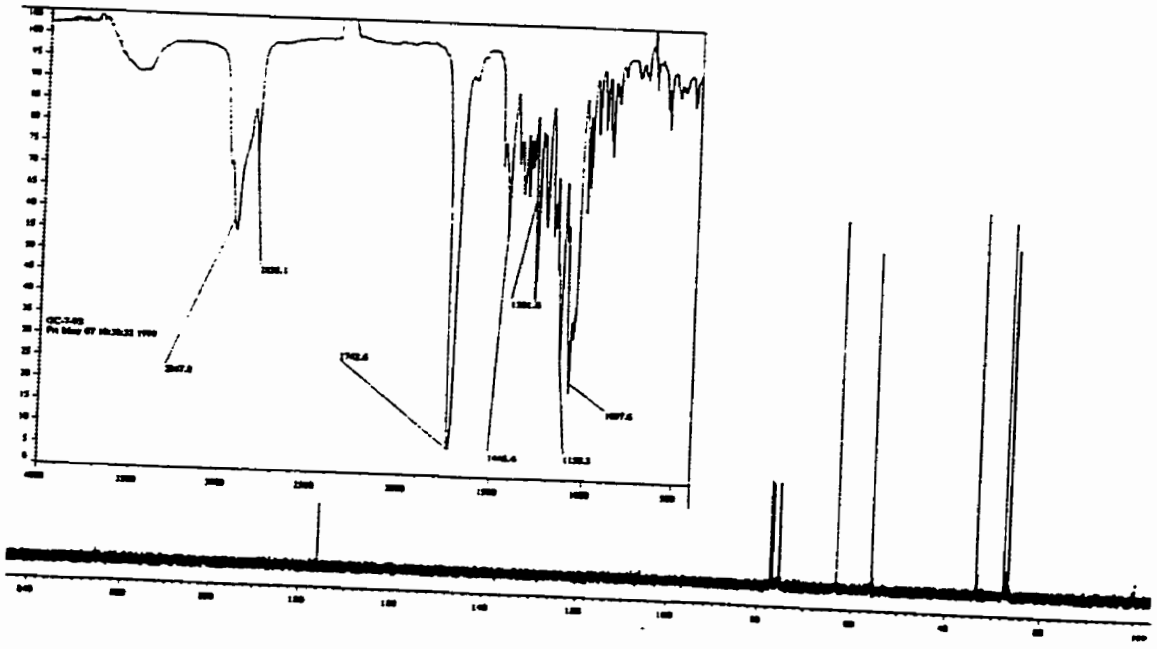
4-Hydroxycyclohexanone ethyleneglycol ketal 2-9

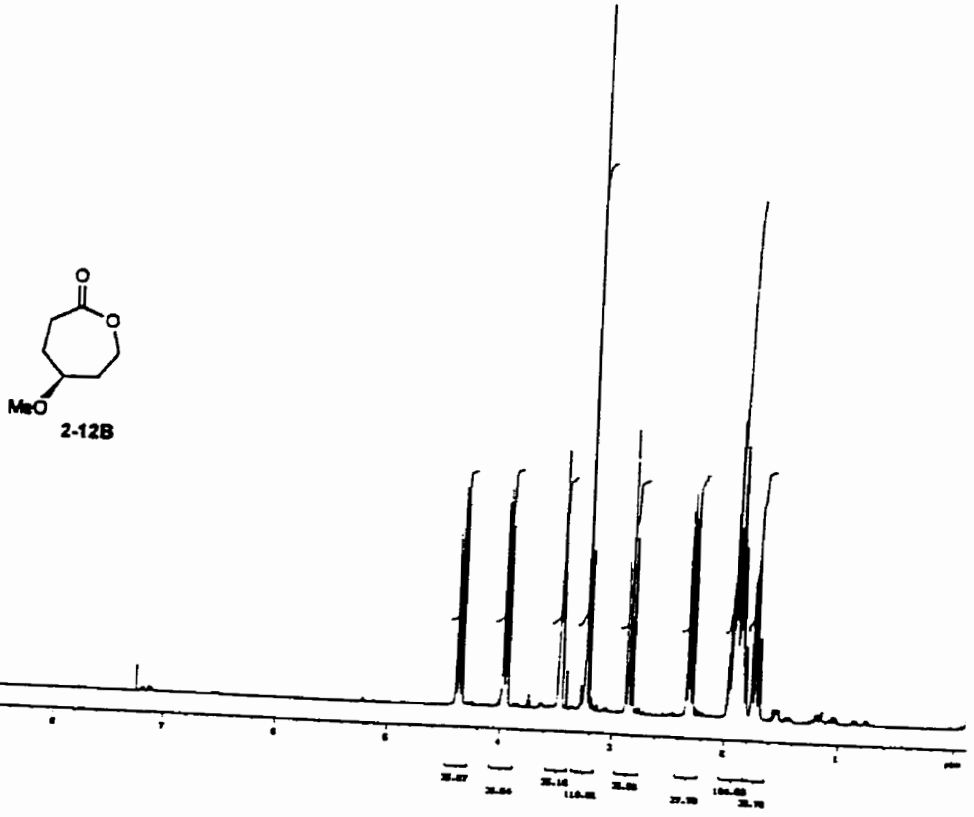


4-Methoxycyclohexanone ethyleneglycol ketal **2-10A**



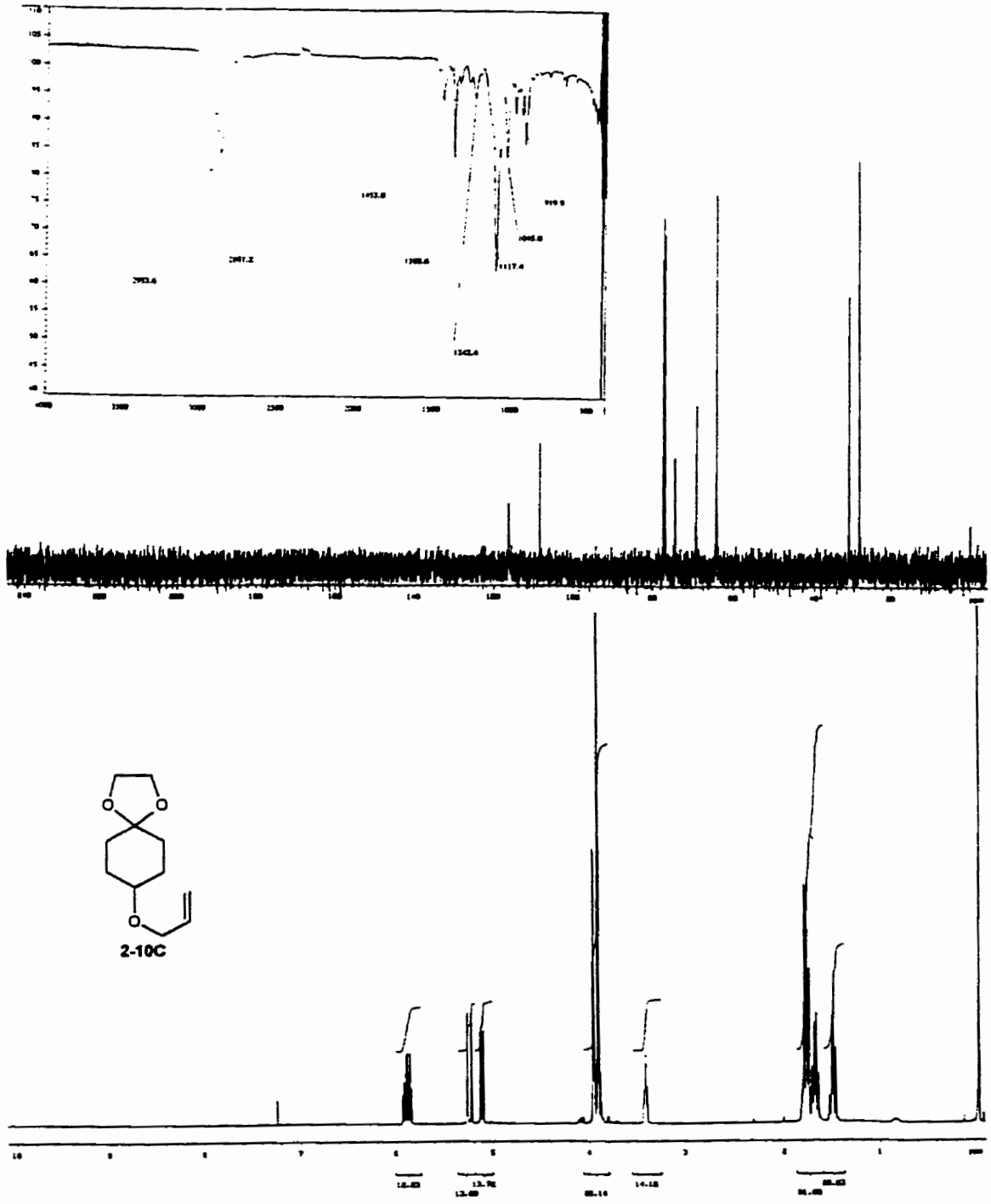
4-Methoxycyclohexanone 2-11A



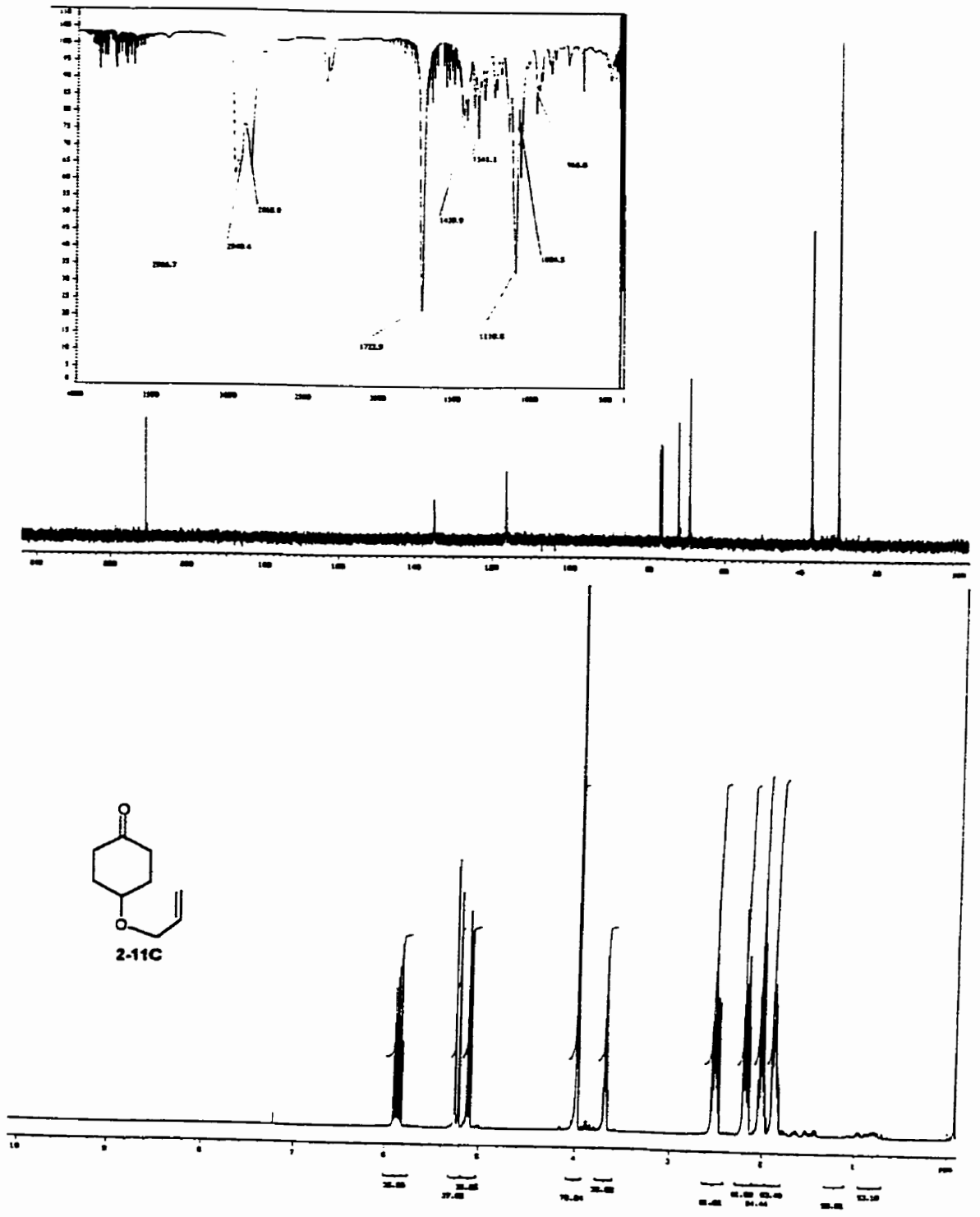


2-12B

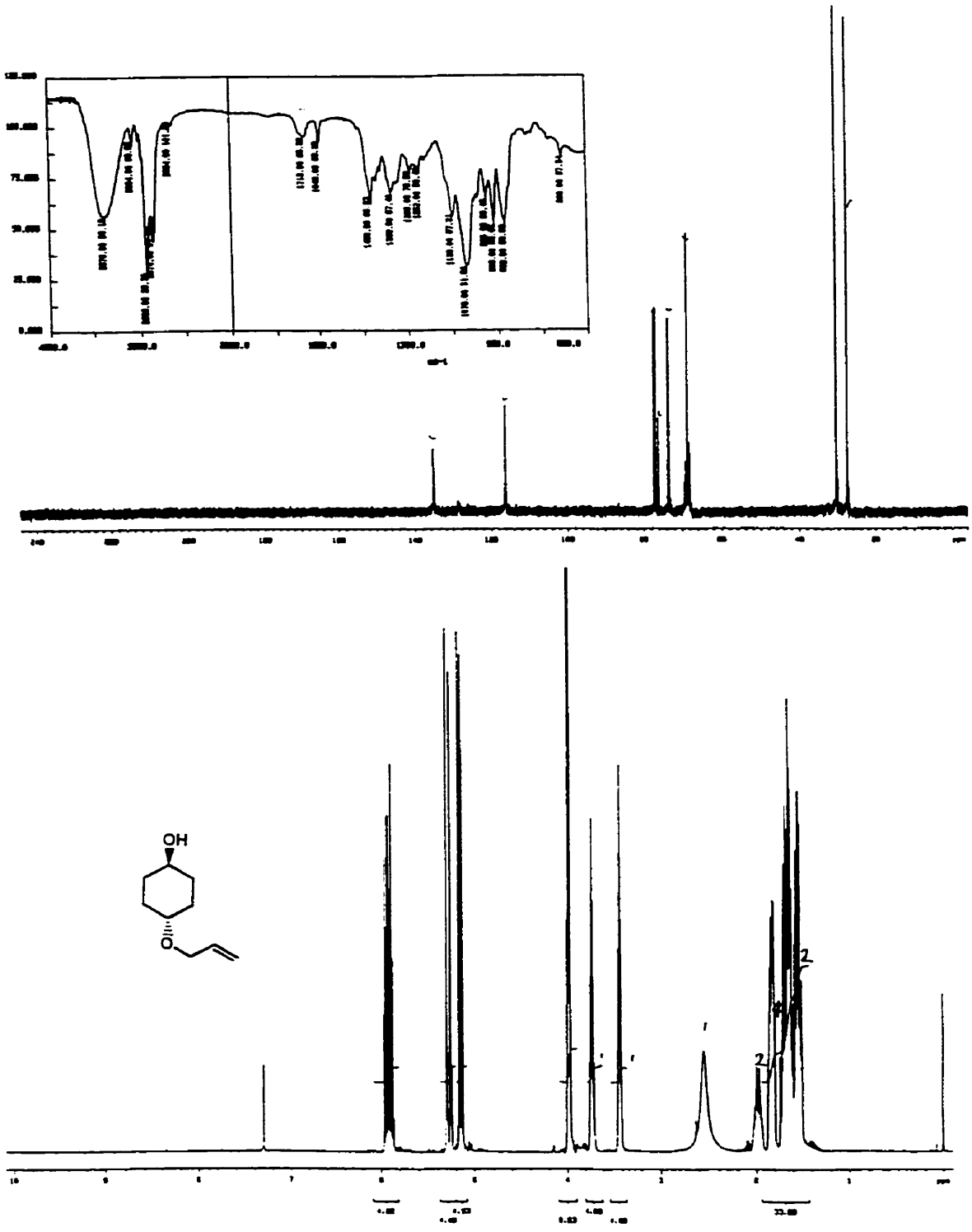
5-Methoxyoxepan-2-one **2-12B**



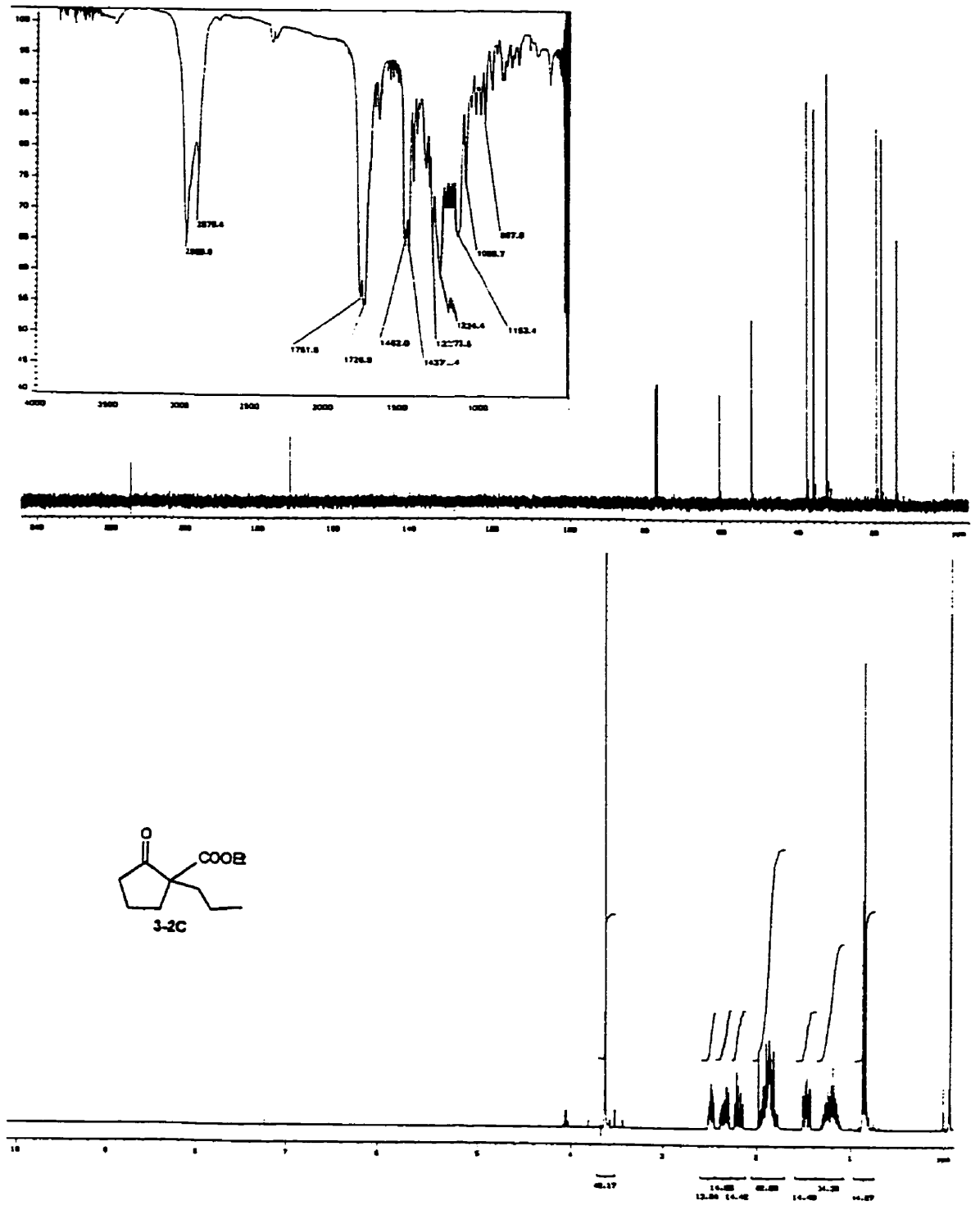
4-Allyloxycyclohexanone ethyleneglycol ketal **2-10C**



4-Allyloxycyclohexanone 2-11C

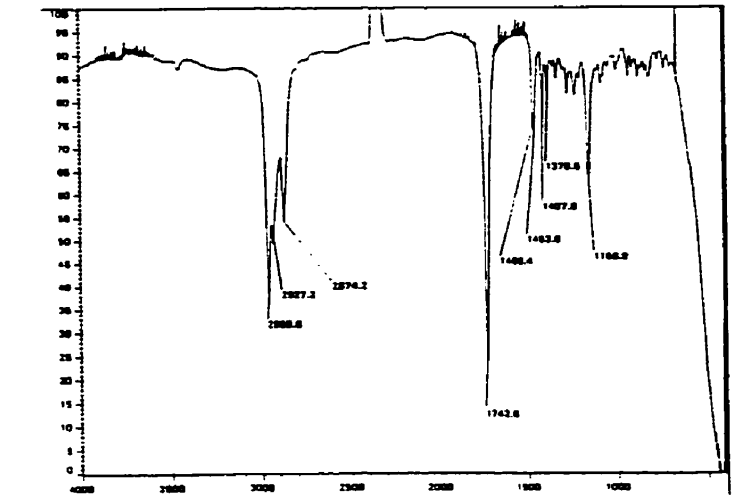


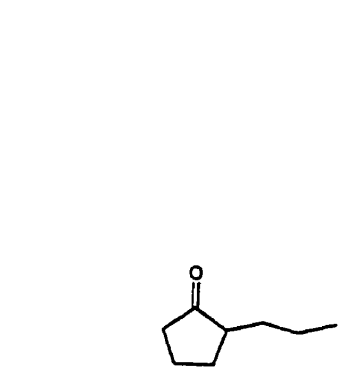
trans-4-Allyloxycyclohexanol



3-2C

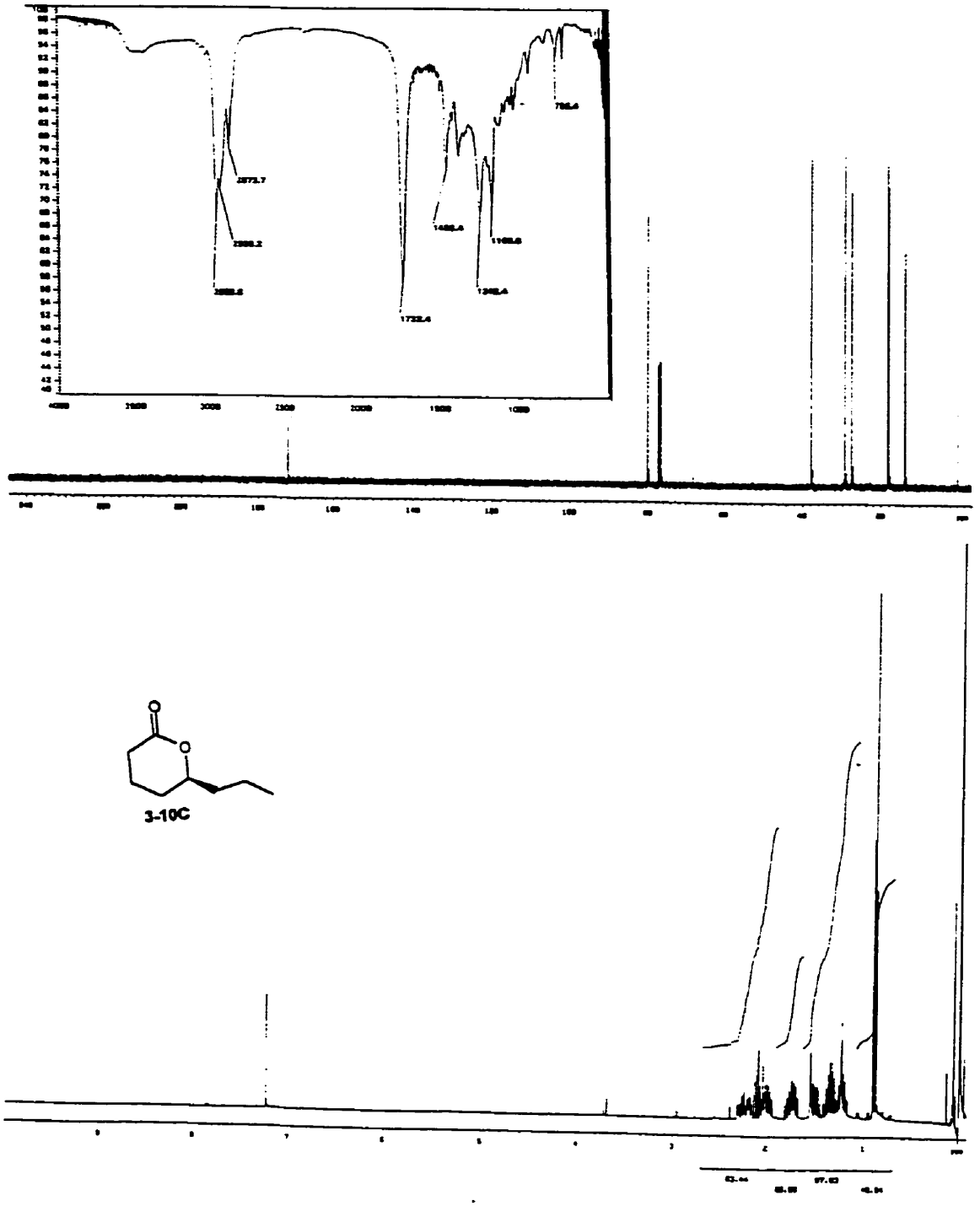
Ethyl-2-propylcyclo-pentanone-2-carboxylate **3-2C**





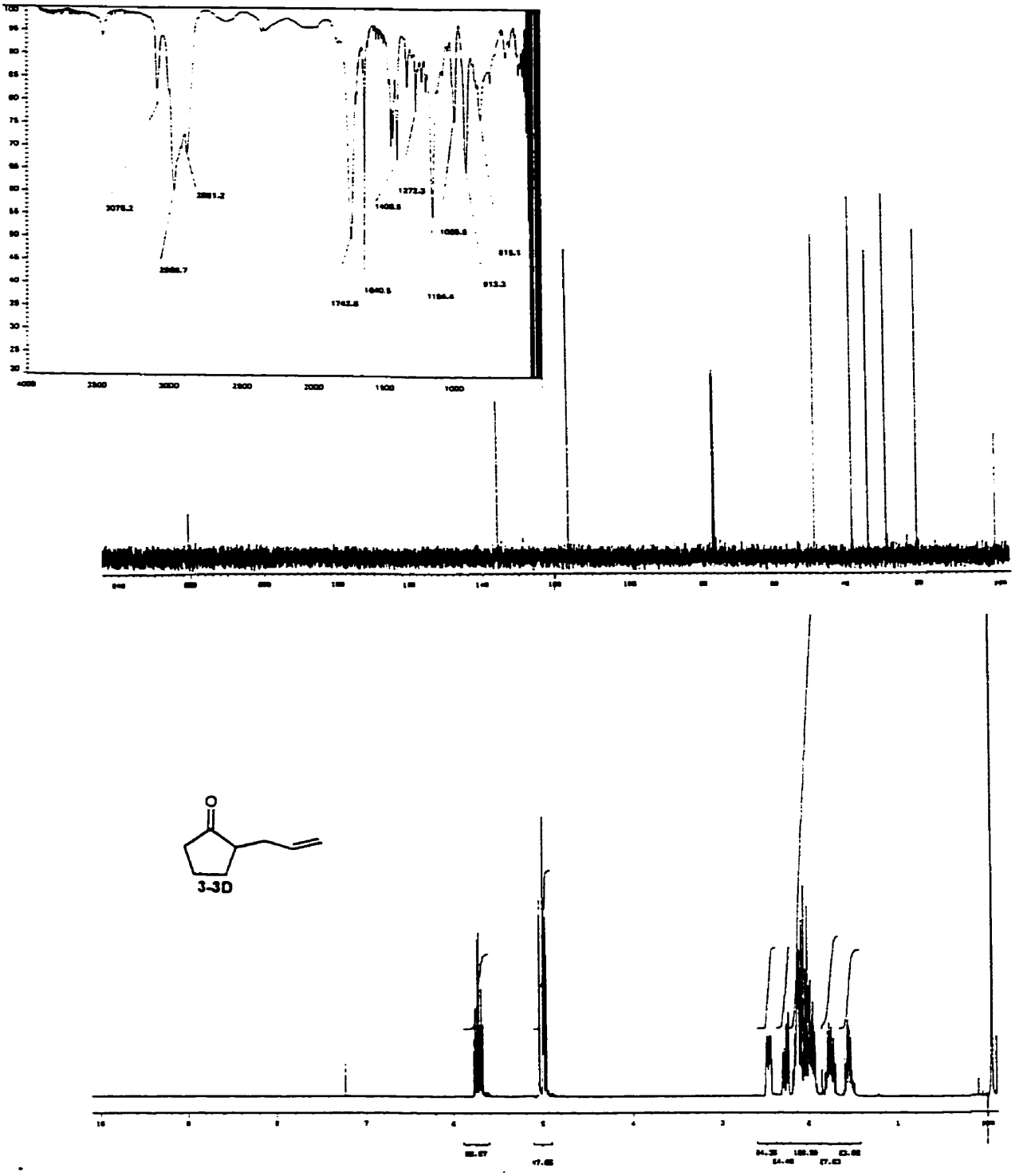
3-3C

2-Propylcyclopentanone **3-3C**

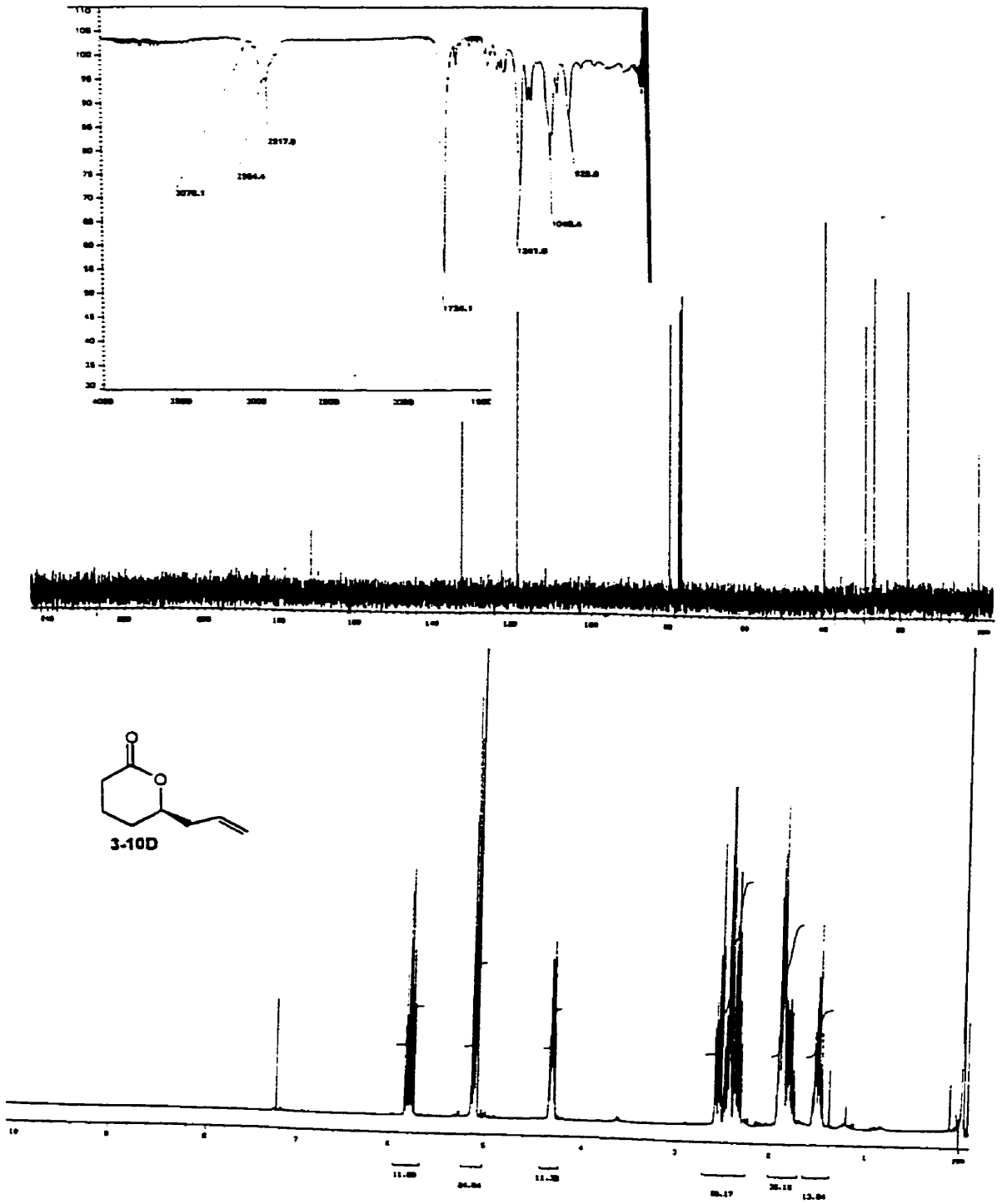


3-10C

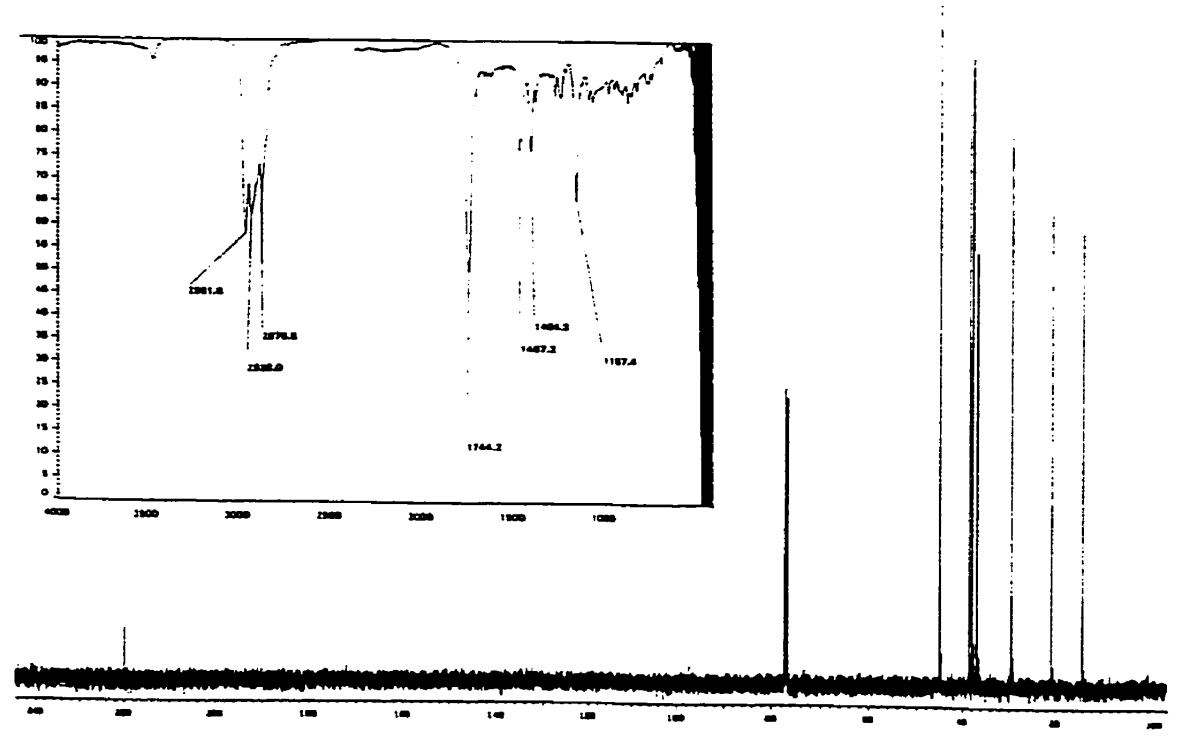
6-Propyltetrahydropyran-2-one **3-10C**

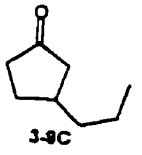


2-Allylcyclopentanone **3-3D**



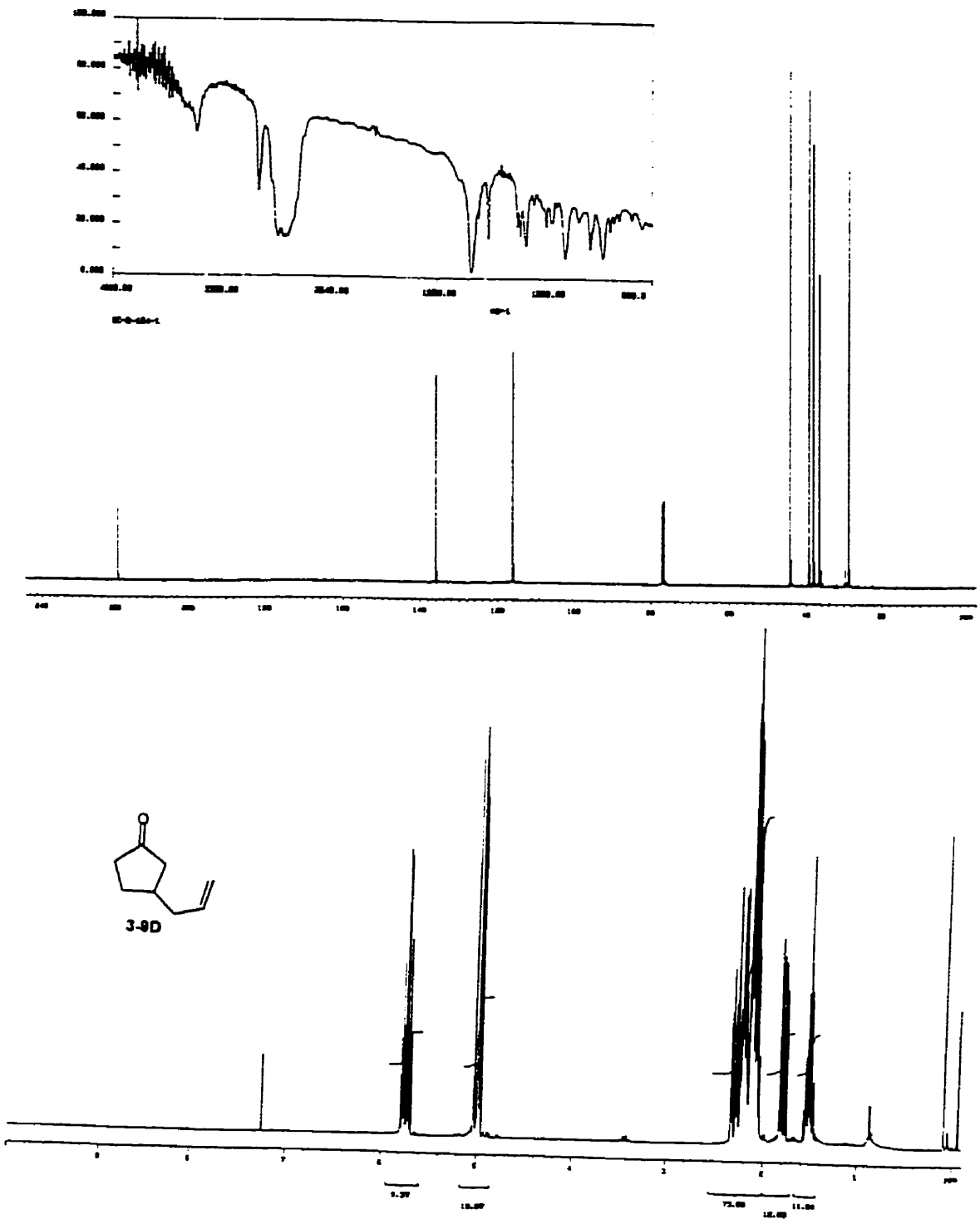
6-Allyltetrahydropyran-2-one **3-10D**



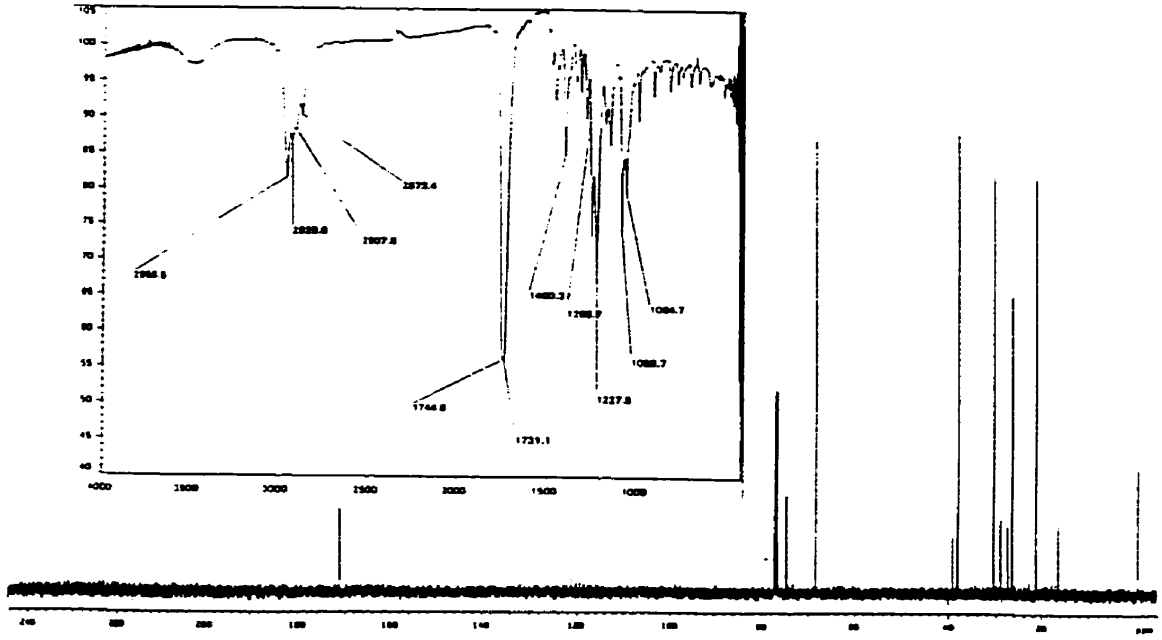


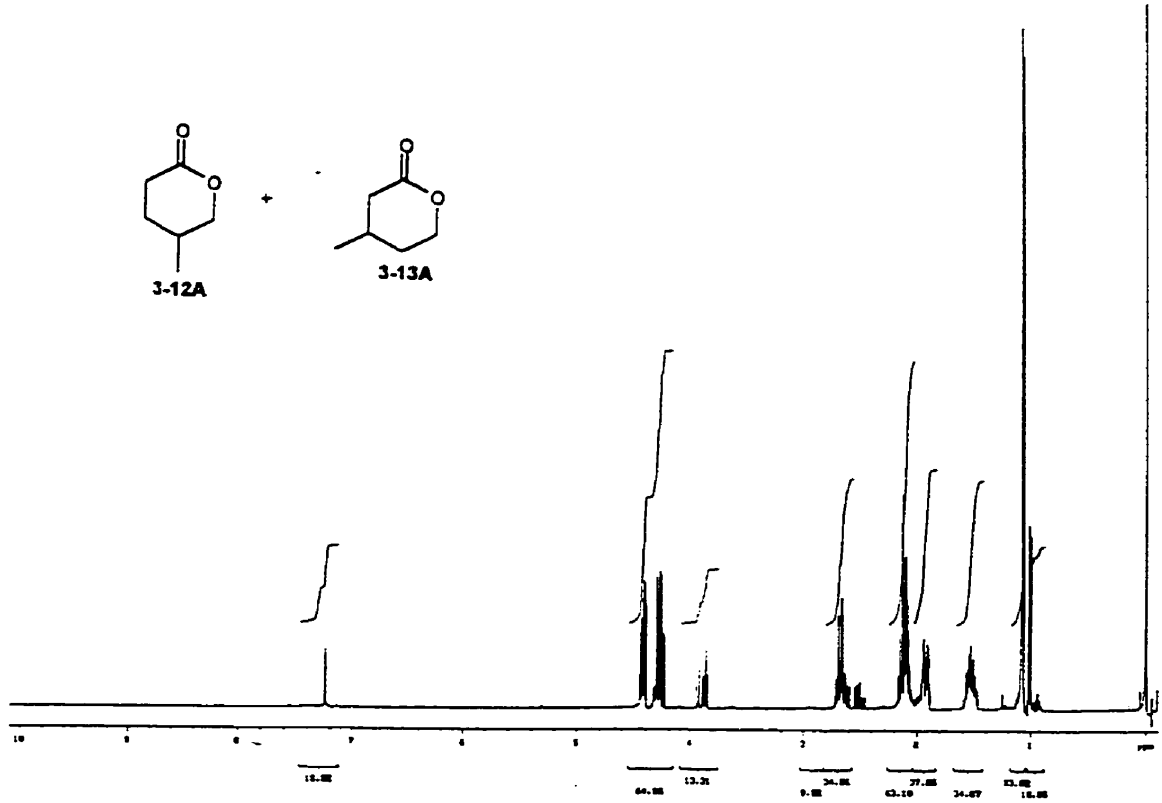
3-9C

3-Propylcyclopentanone **3-9C**

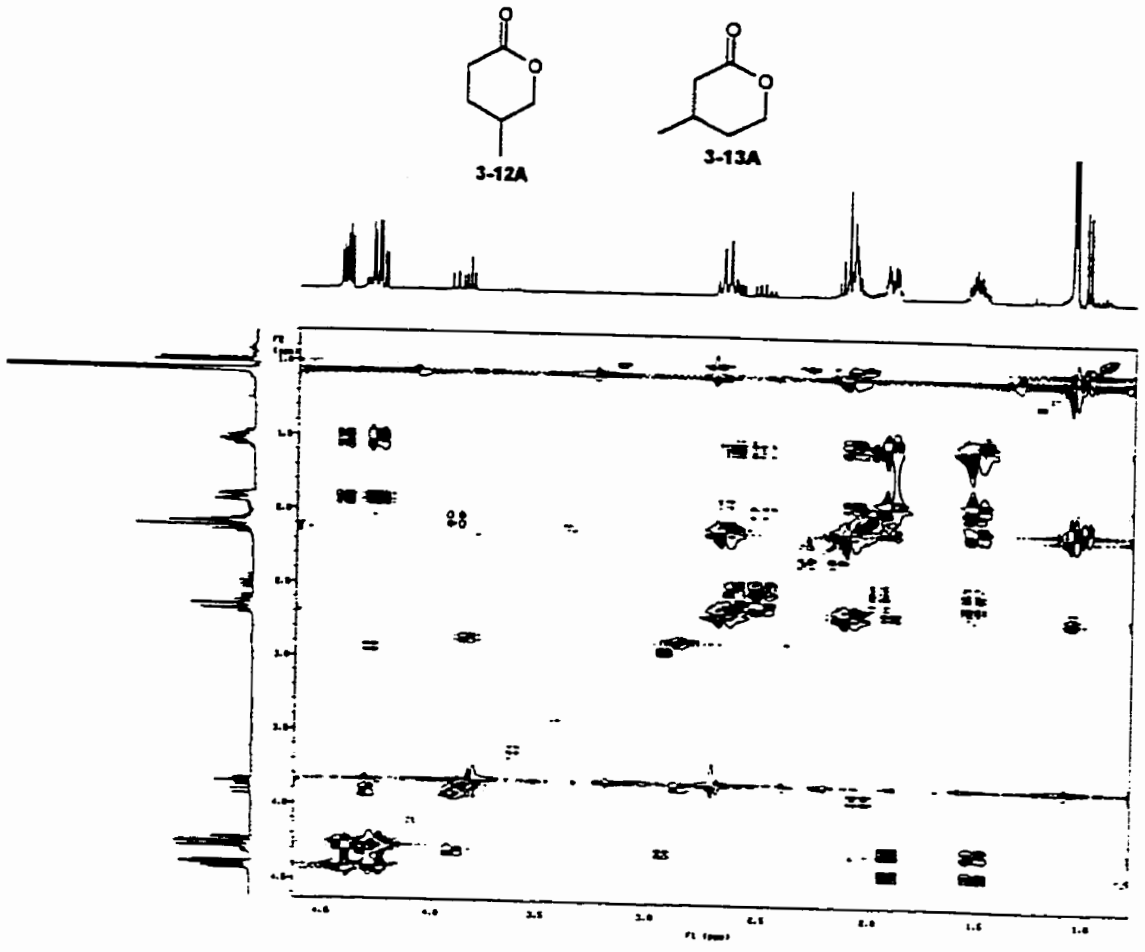


3-Allylcyclopentanone **3-9D**

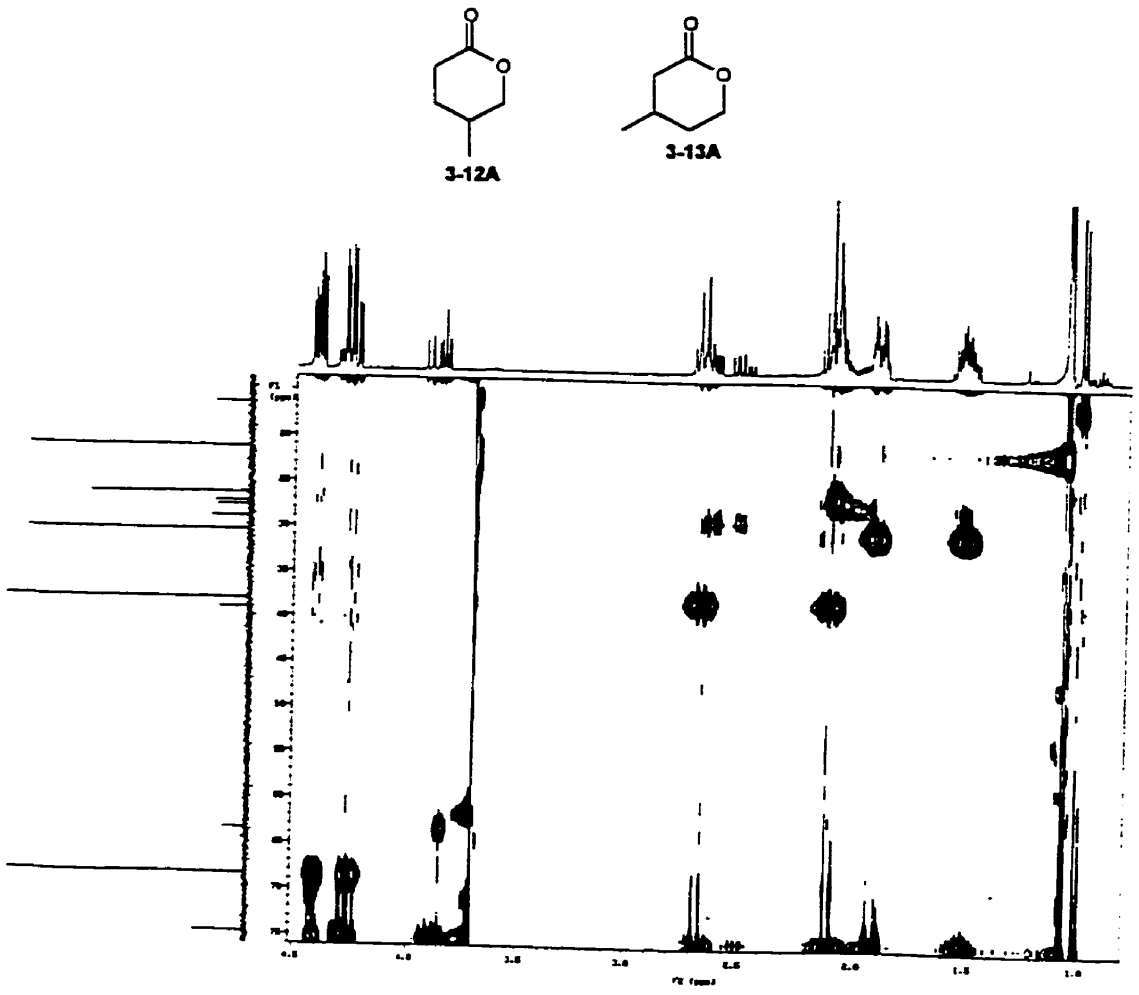




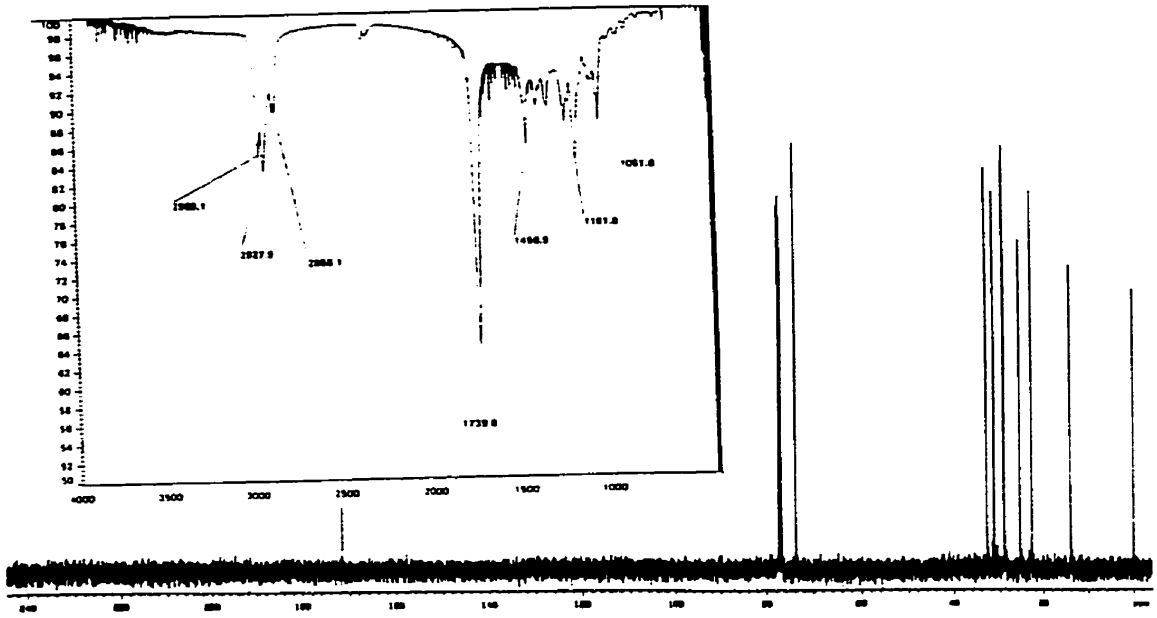
4- and 5-Methyltetrahydropyran-2-one **3-13A** and **3-12A**

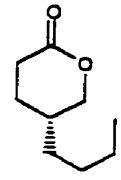


COSY experiment for **3-12A** and **3-13A**



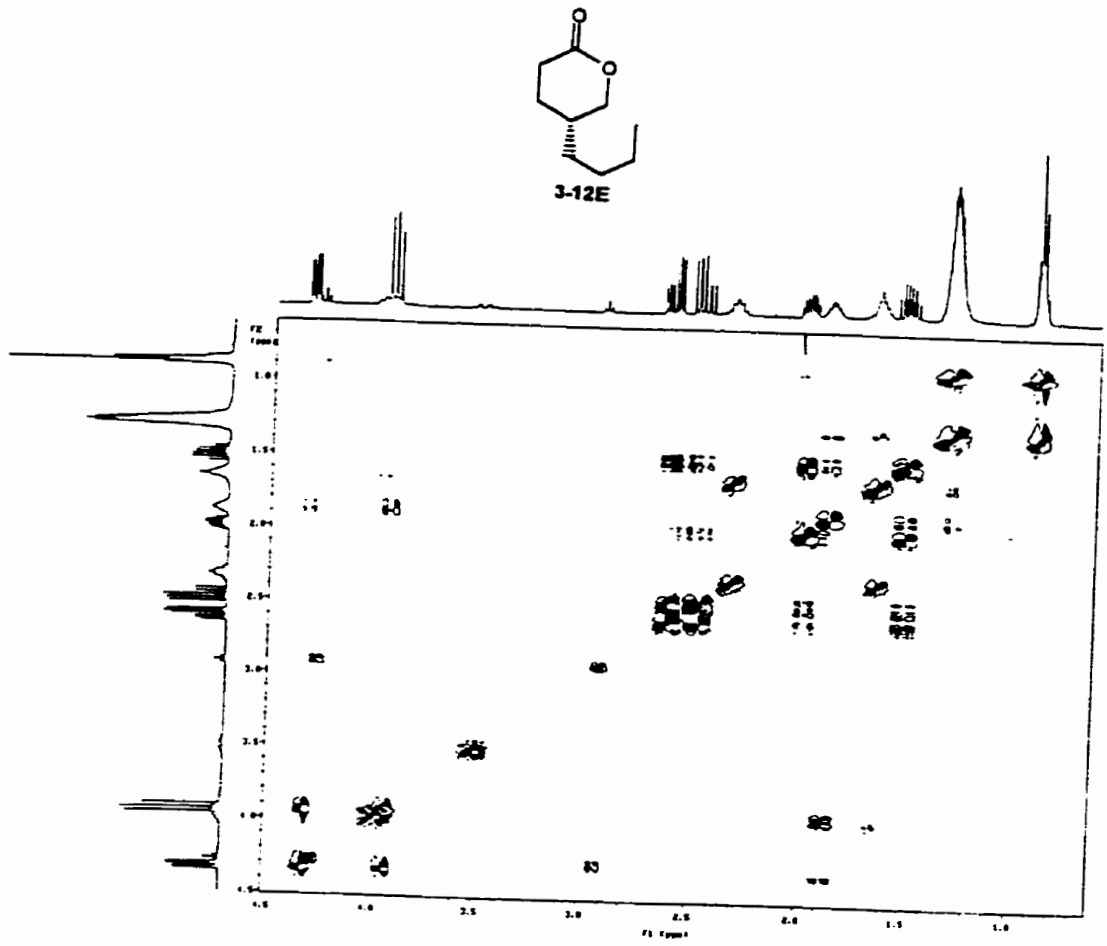
HMQC experiment for **3-12A** and **3-13A**



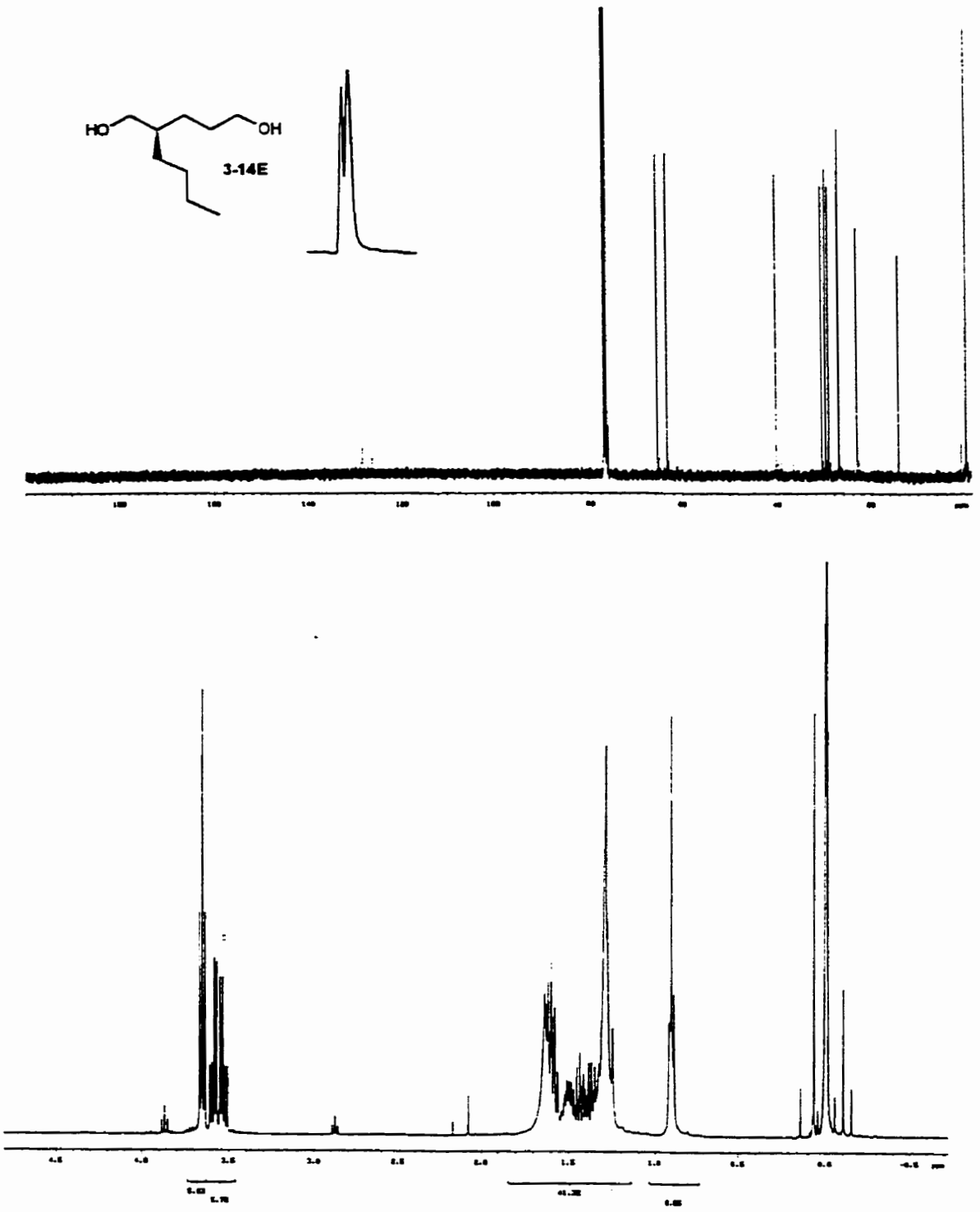


3-12E

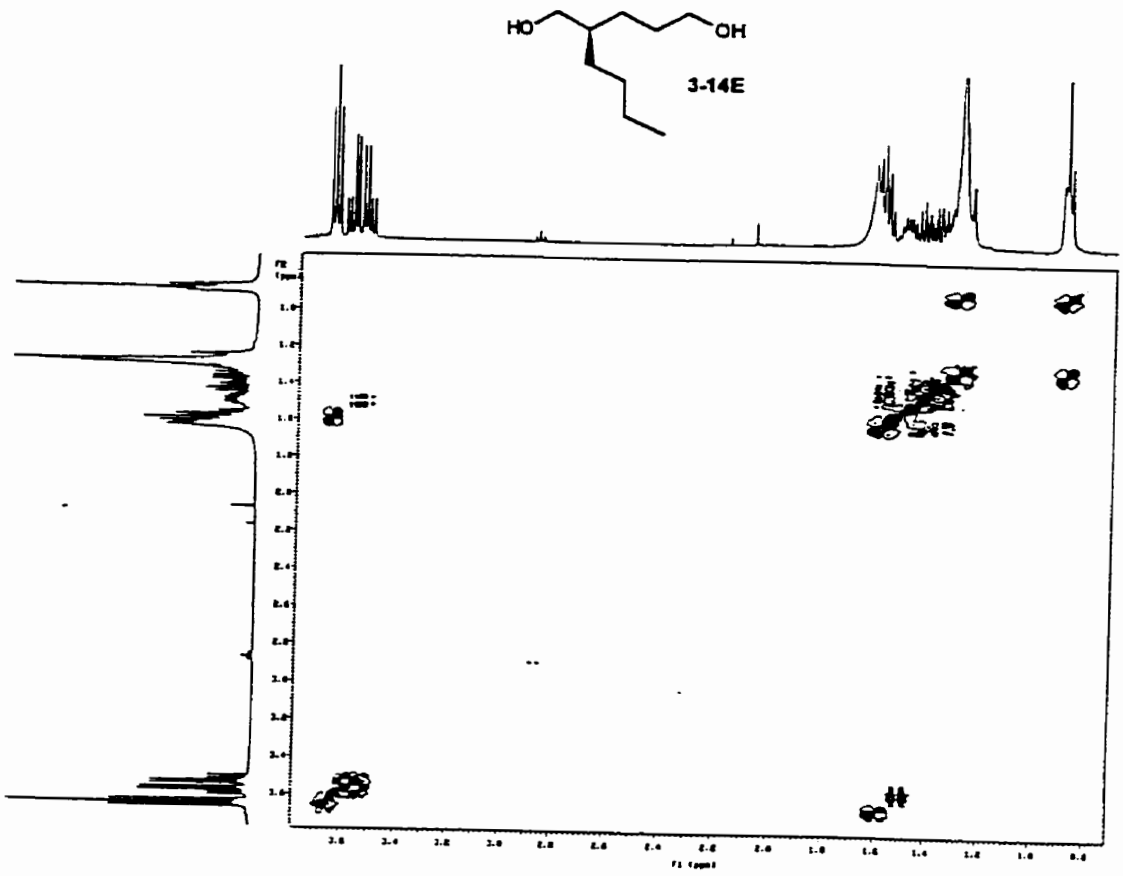
5-Butyltetrahydropyran-2-one 3-12E



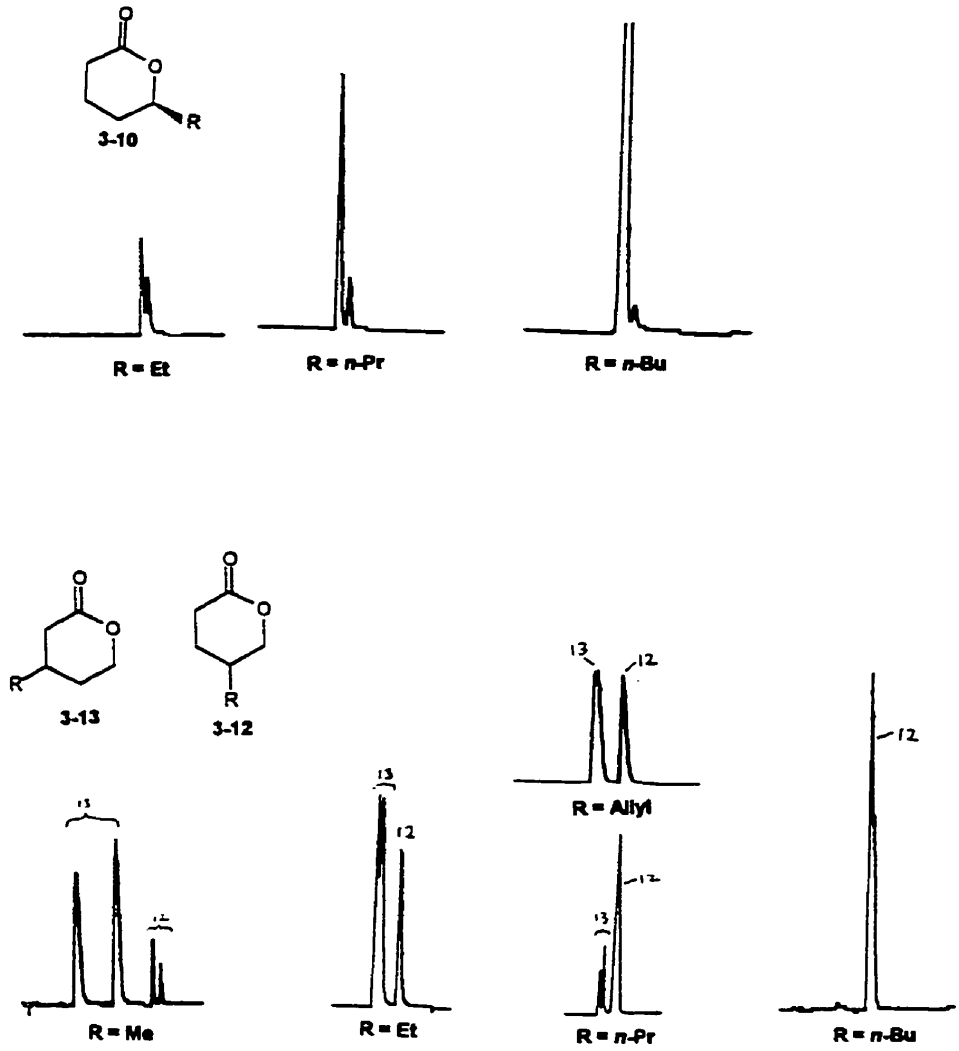
HMQC experiment for **3-12E**



GC and NMR of diol **3-14E** resulted from $LiAlH_4$ reduction of **3-12E**

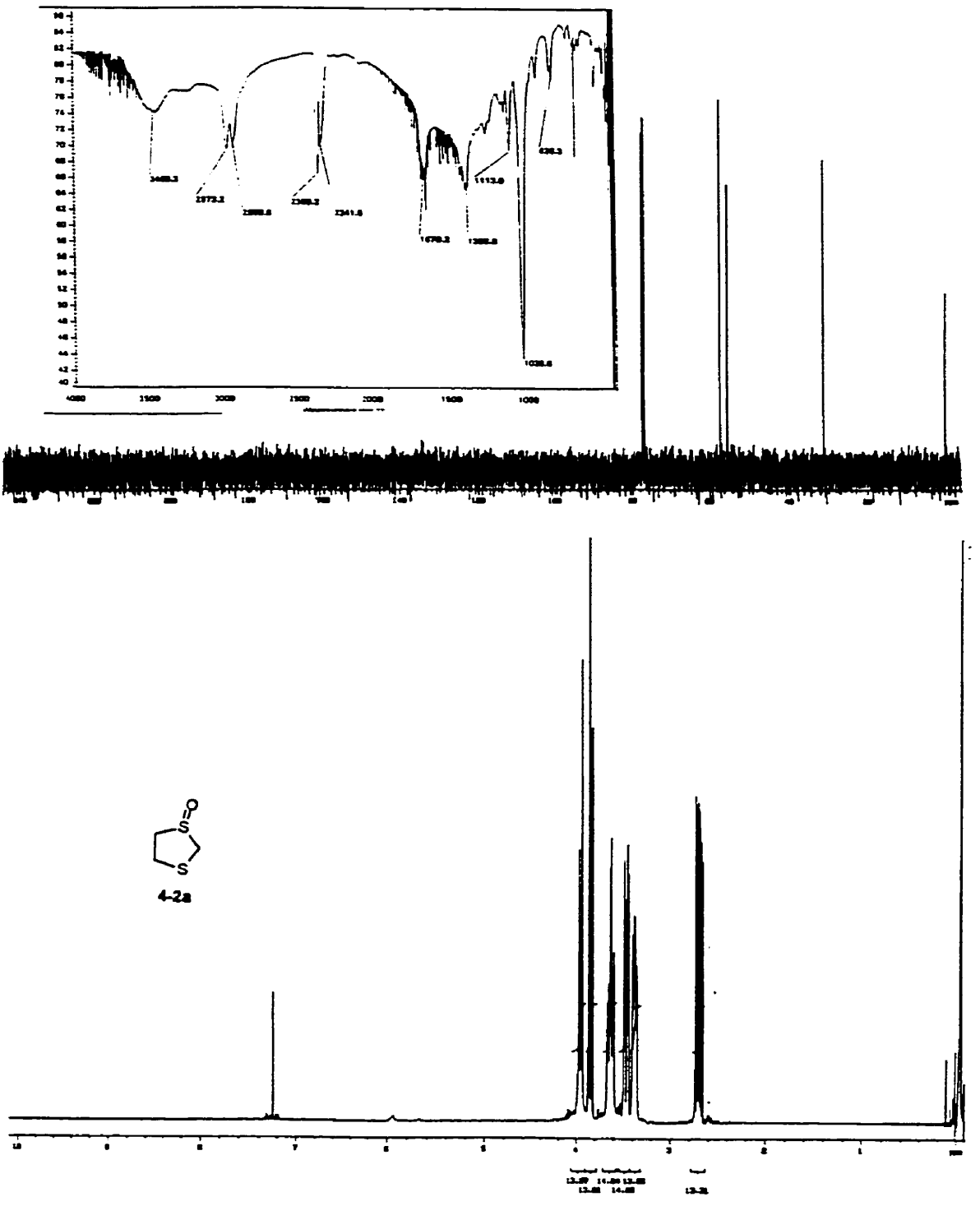


COSY experiment for **3-14E**

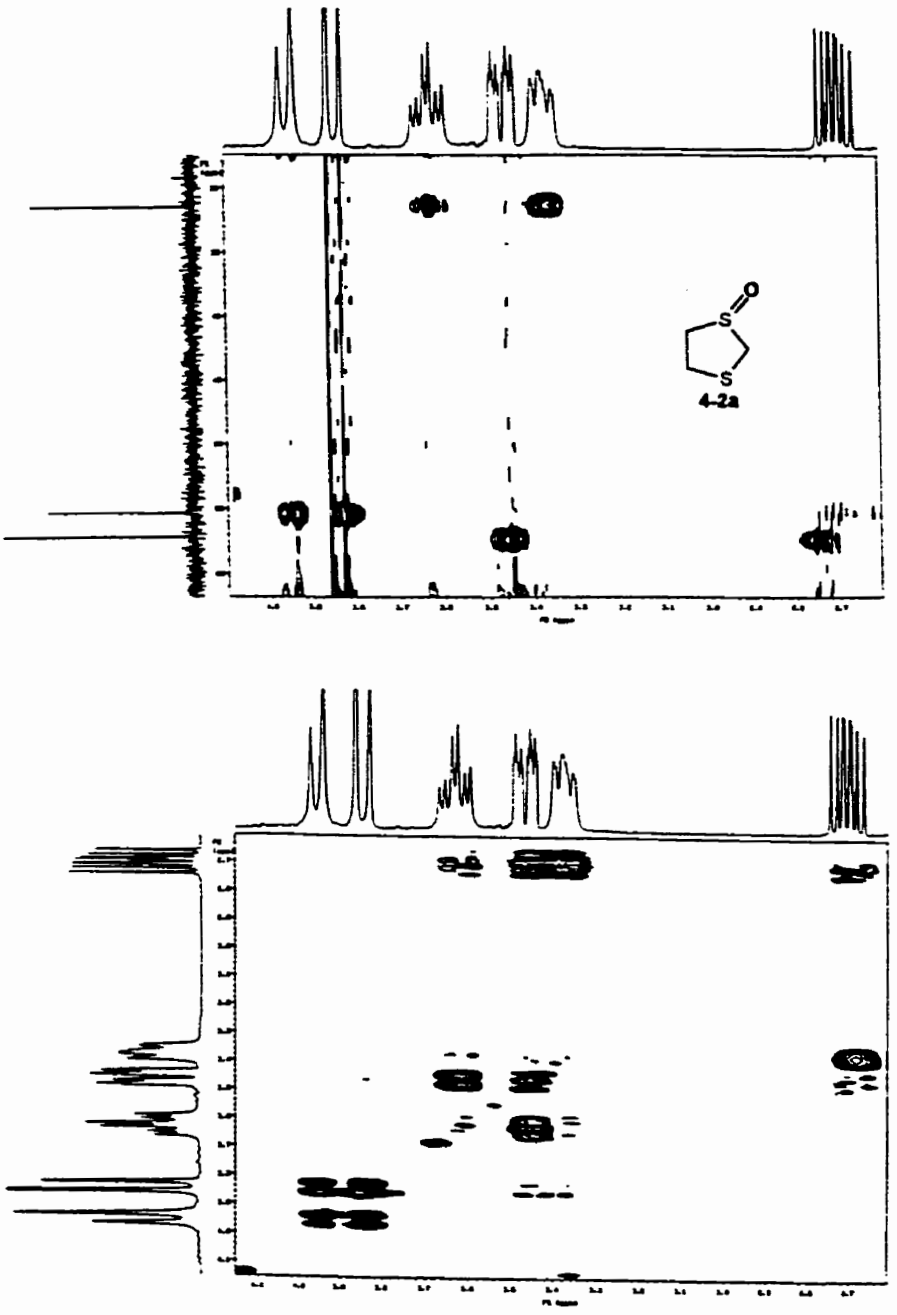


GC traces for Baeyer-Villiger products of 2- and 3-alkylcyclopentanones

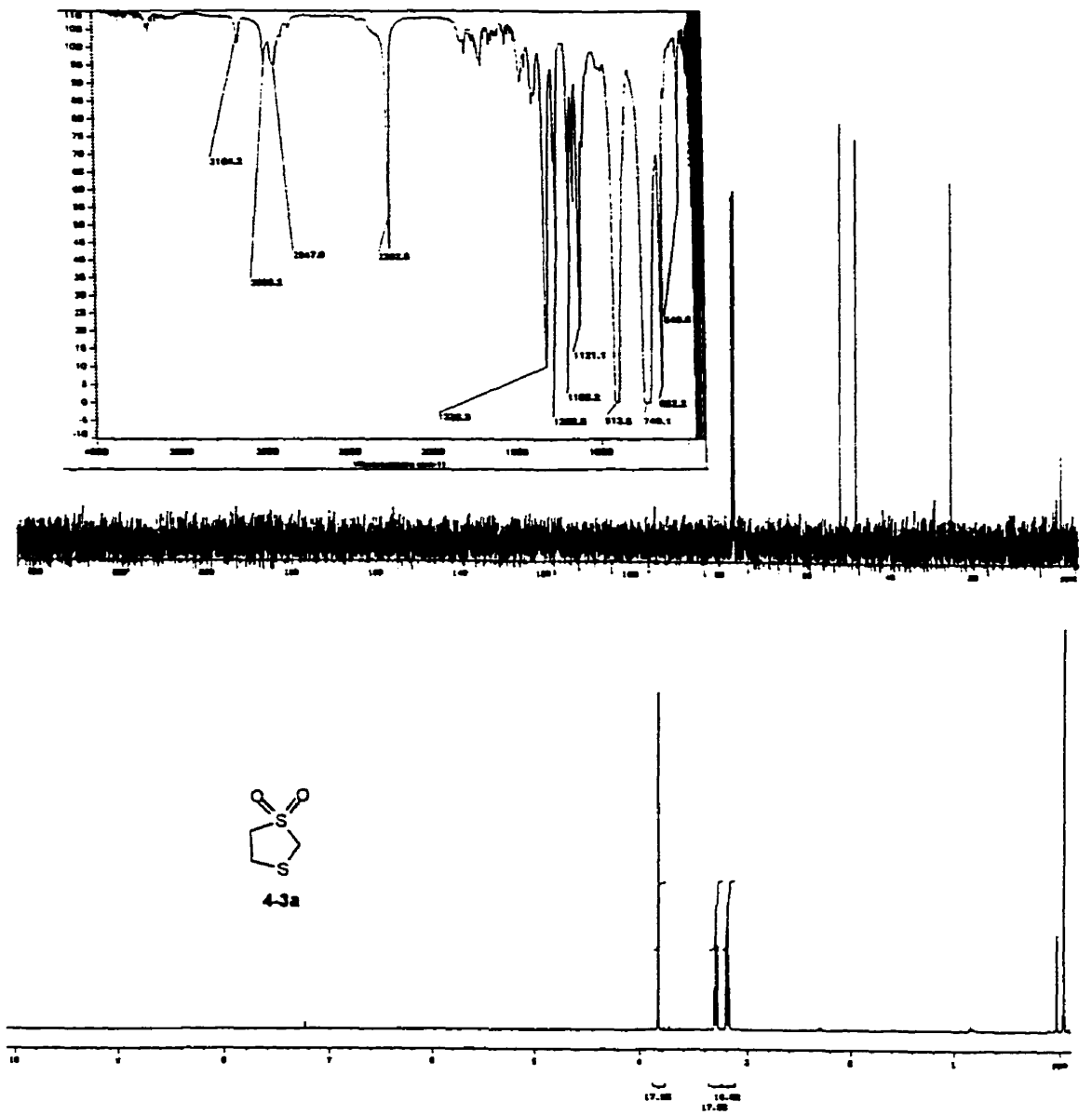
For 2-alkylcyclopentanones, the enantioselectivity increases as the chain gets longer; for 3-alkylcyclopentanones, the enantioselectivity is generally low, and there is a reversal in the regiopreference as the chain gets longer.



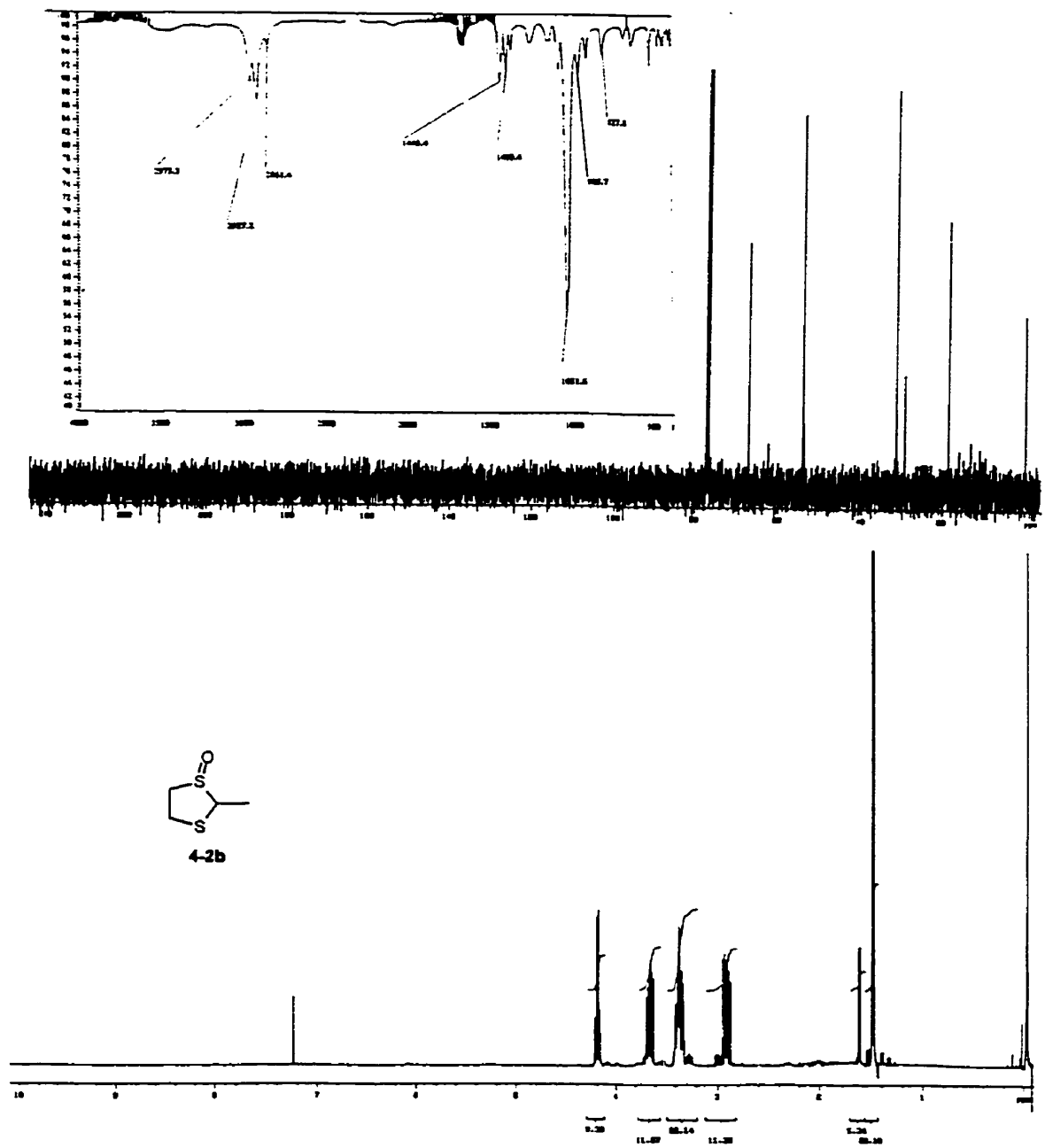
1,3-Dithiolane-1-oxide **4-2a**



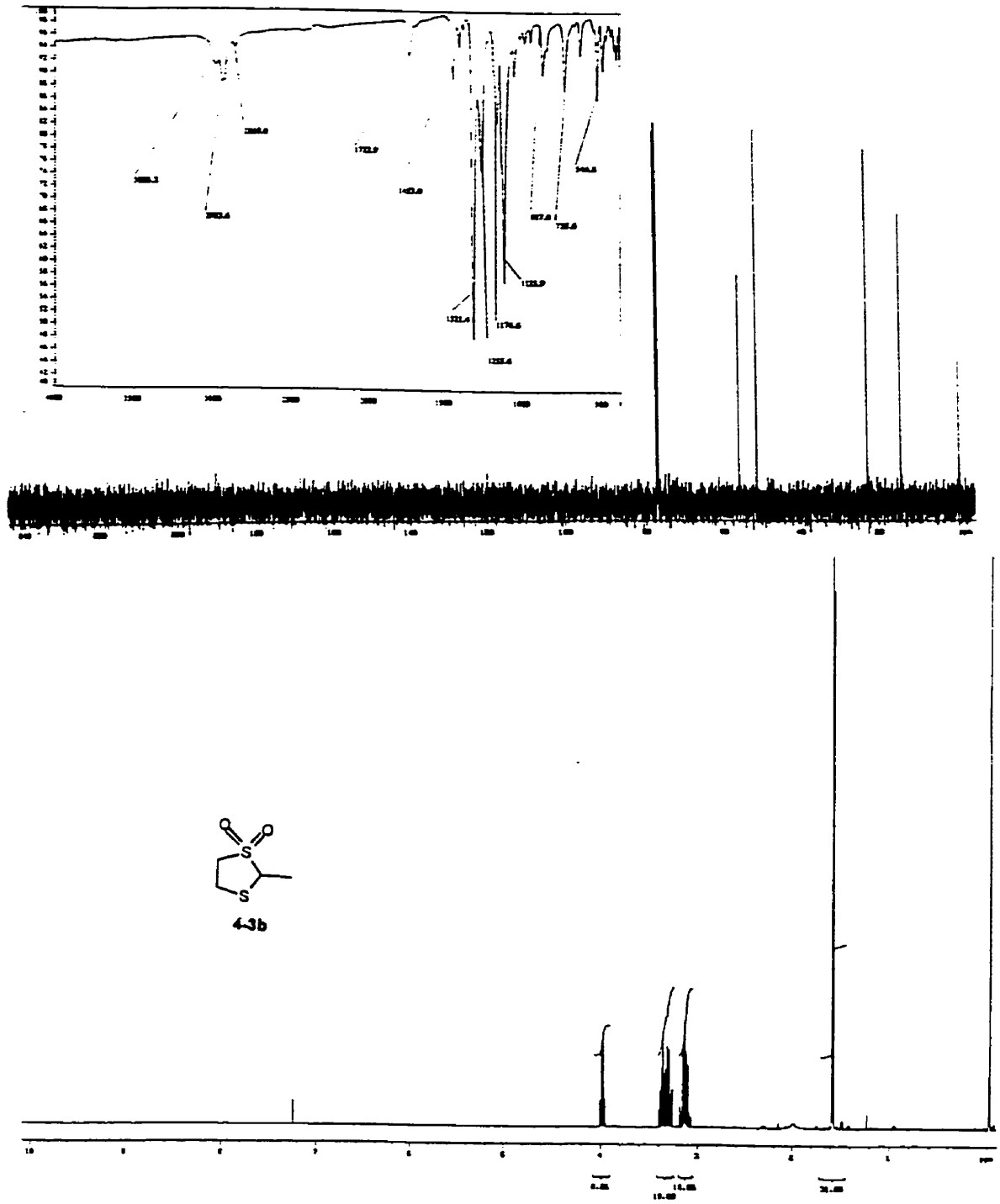
HMQC and COSY experiments for **4-2a**



1,3-Dithiolane-1-sulfone **4-3a**

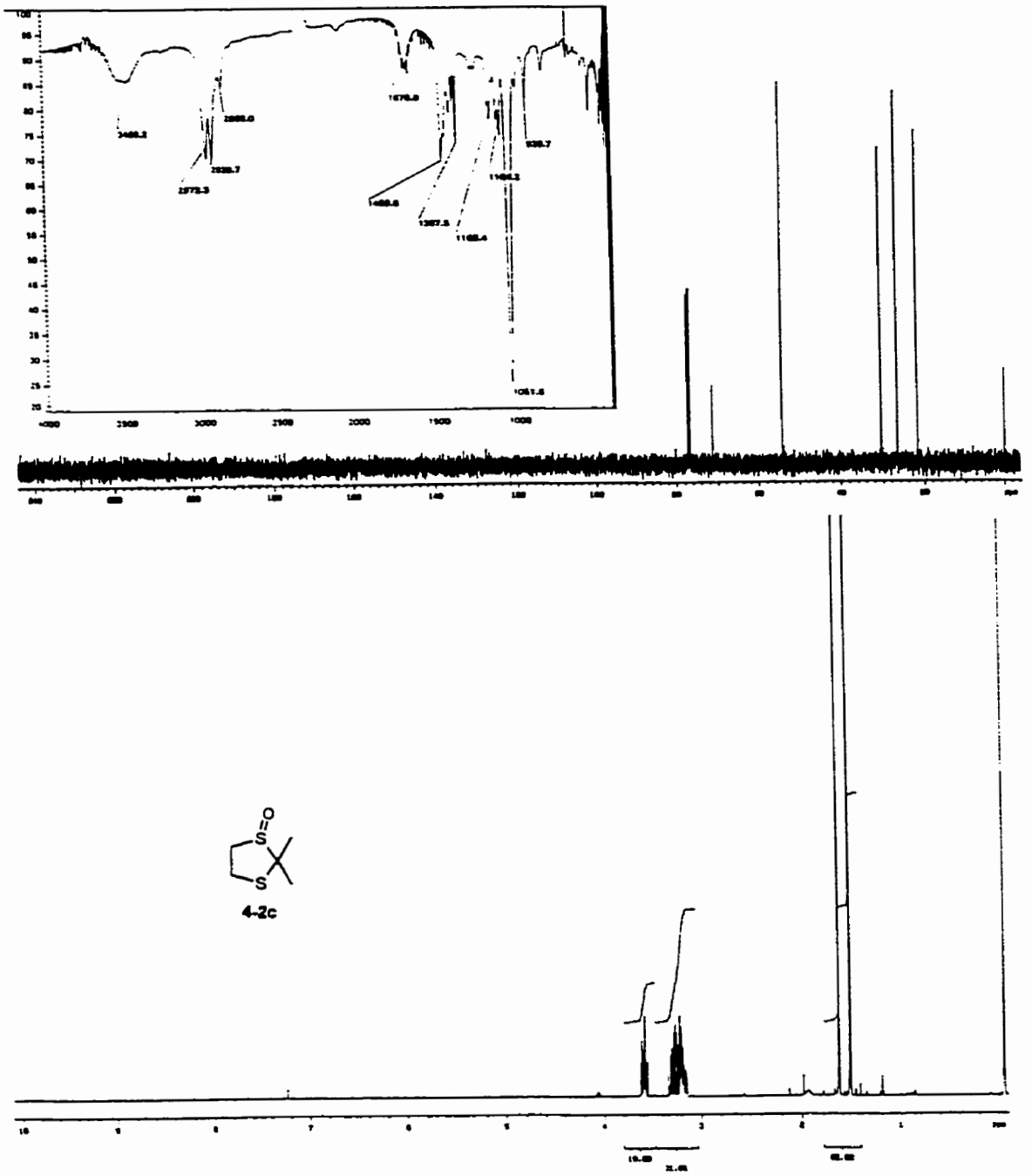


2-Methyl-1,3-dithiolane-1-sulfoxide **4-2b** (5:1 *trans:cis*)

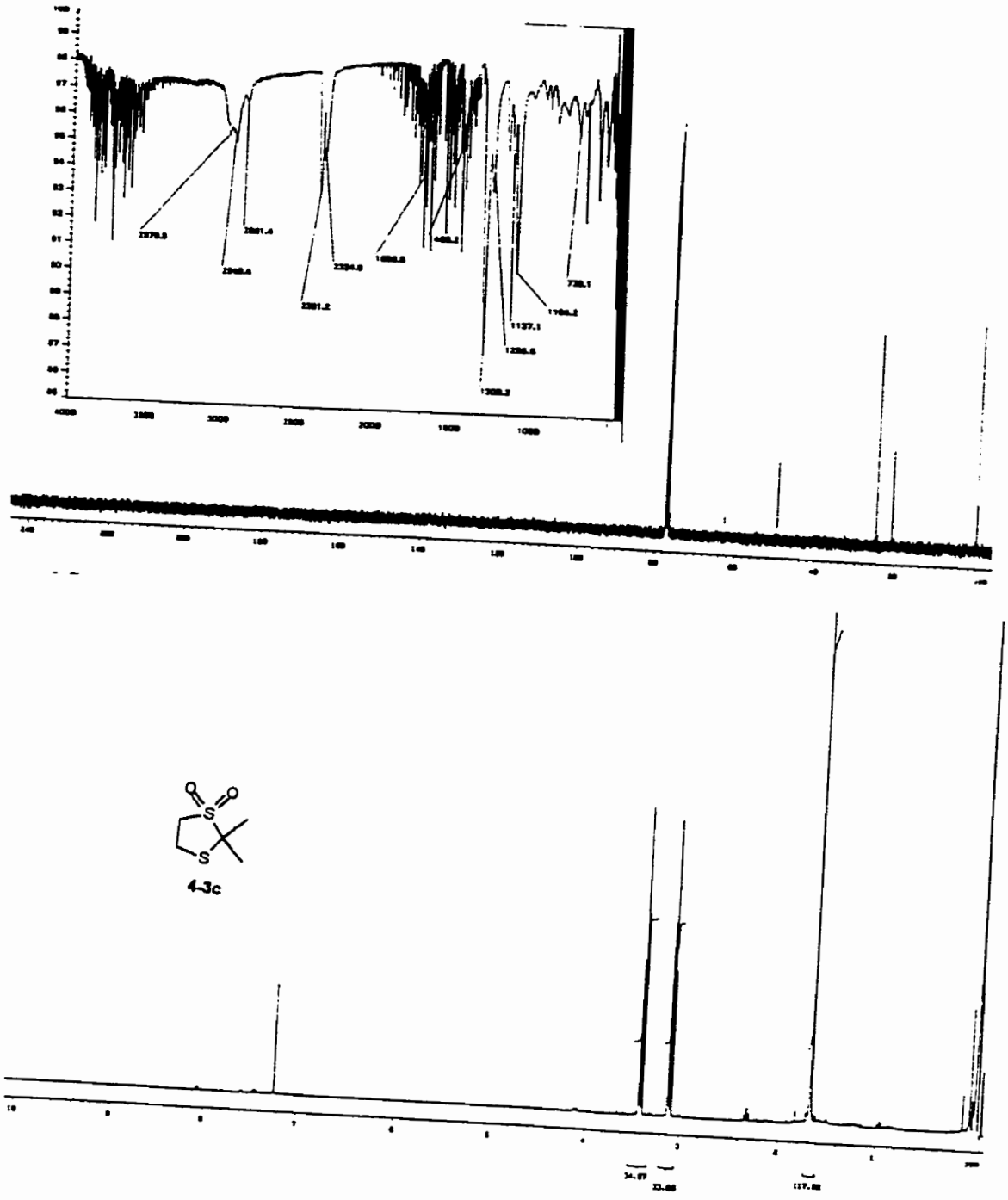


4-3b

2-Methyl-1,3-dithiolane-1-sulfone **4-3b**

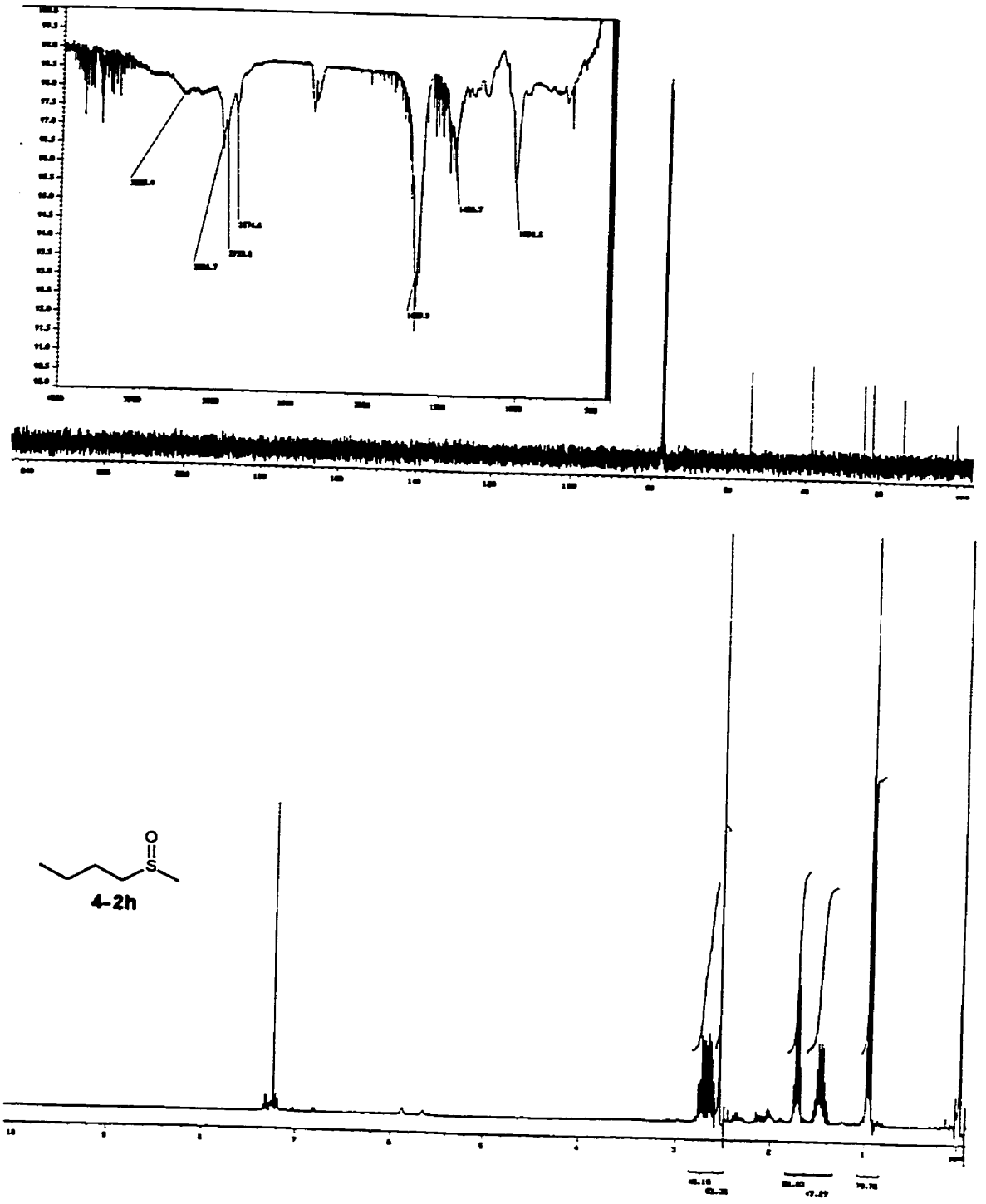


2,2-Dimethyl-1,3-dithiolane-1-sulfoxide **4-2c**

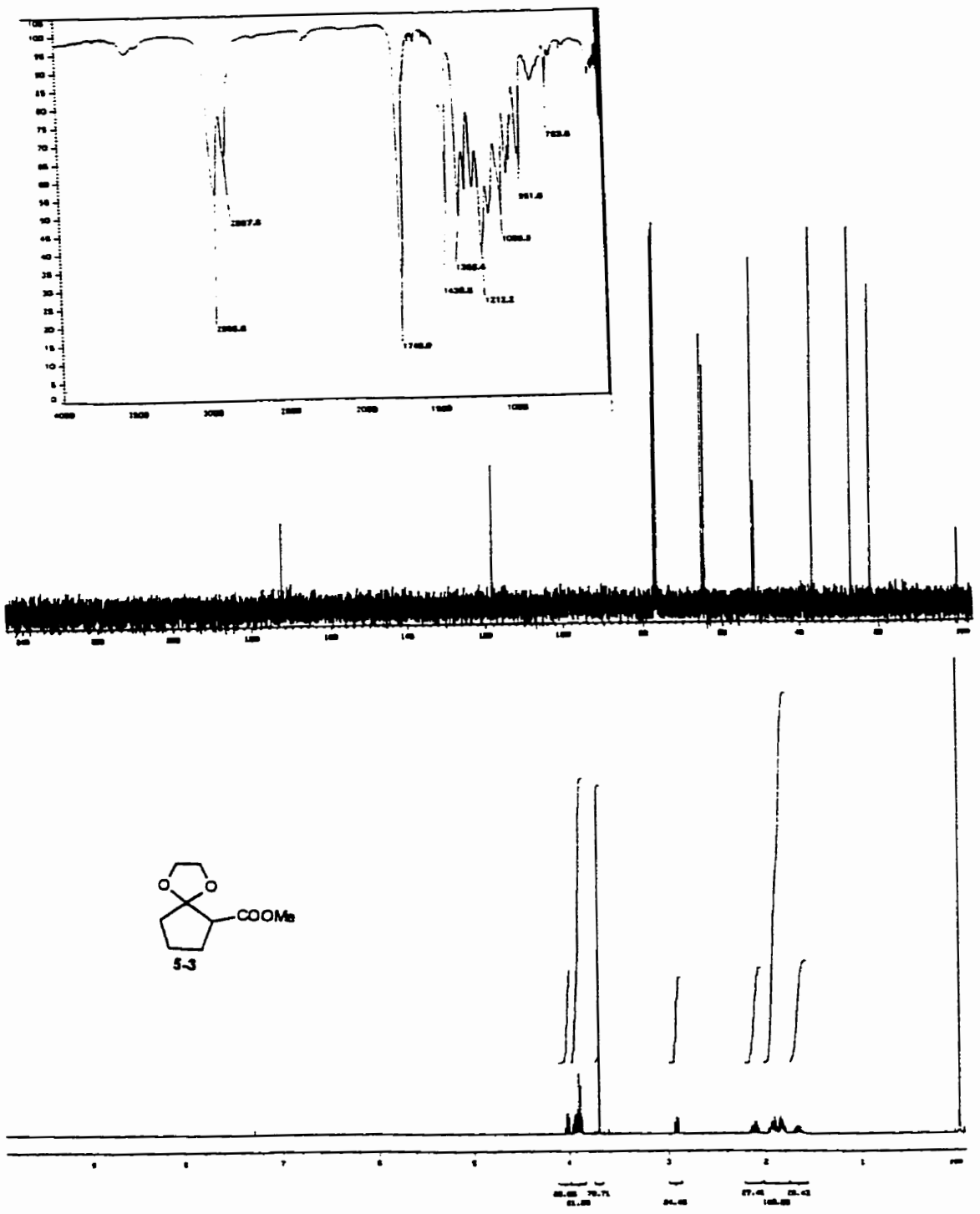


4-3c

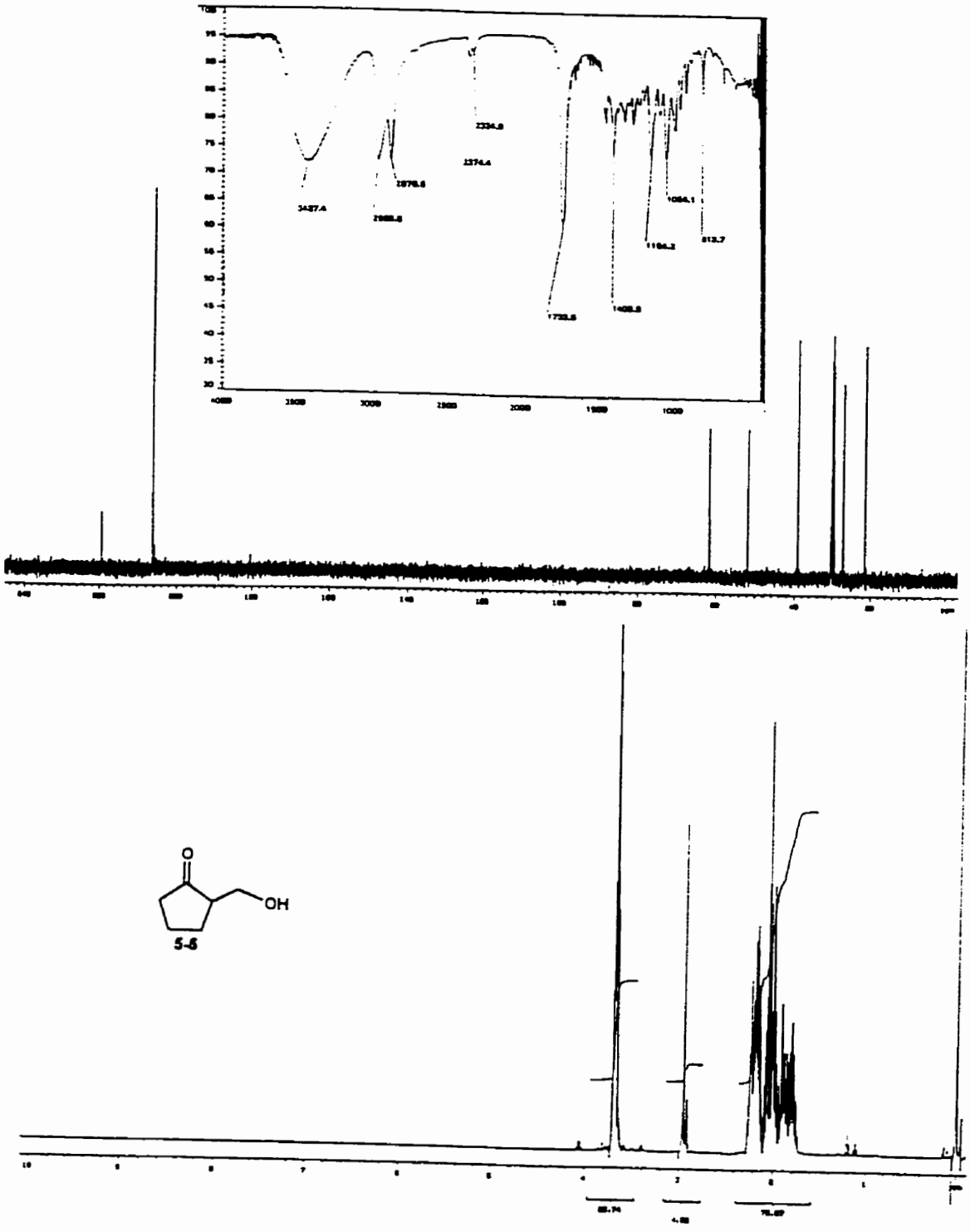
2,2-Dimethyl-1,3-dithiolane-1-sulfone **4-3c**



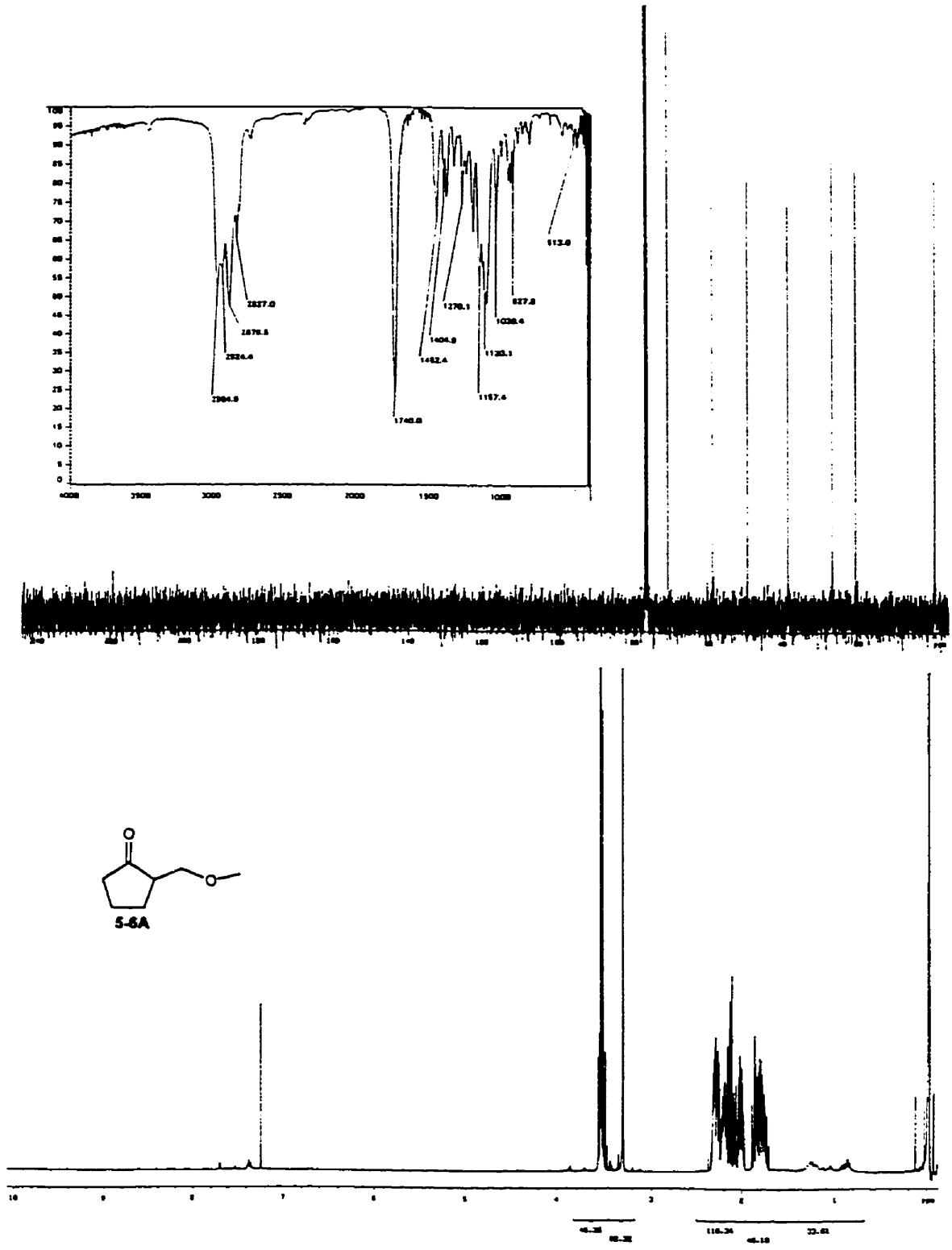
Butylmethylsulfoxide **4-2h**



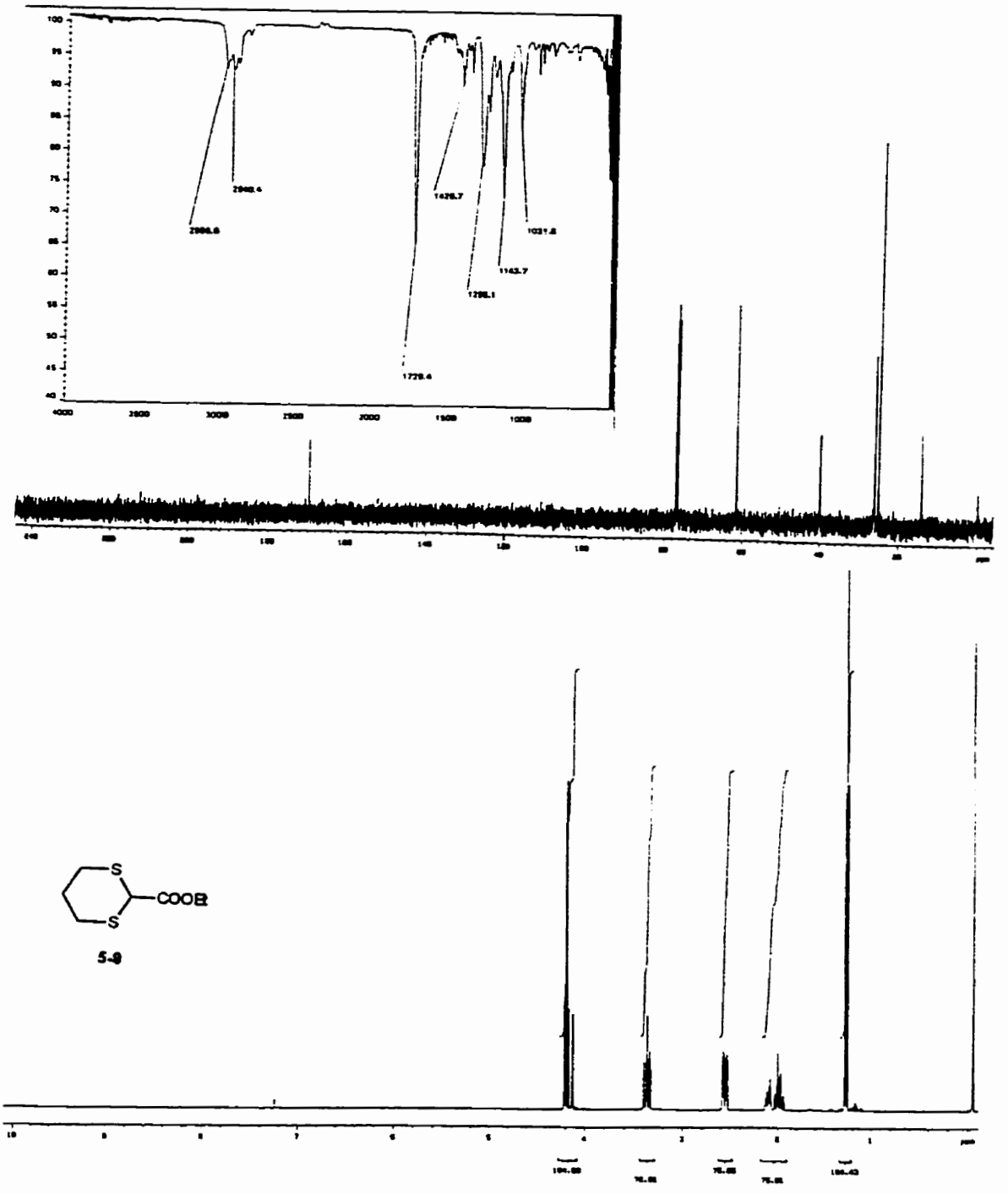
1,4-Dioxa-spiro[4.4]nonane-6-carboxylic acid ethyl ester 5-3



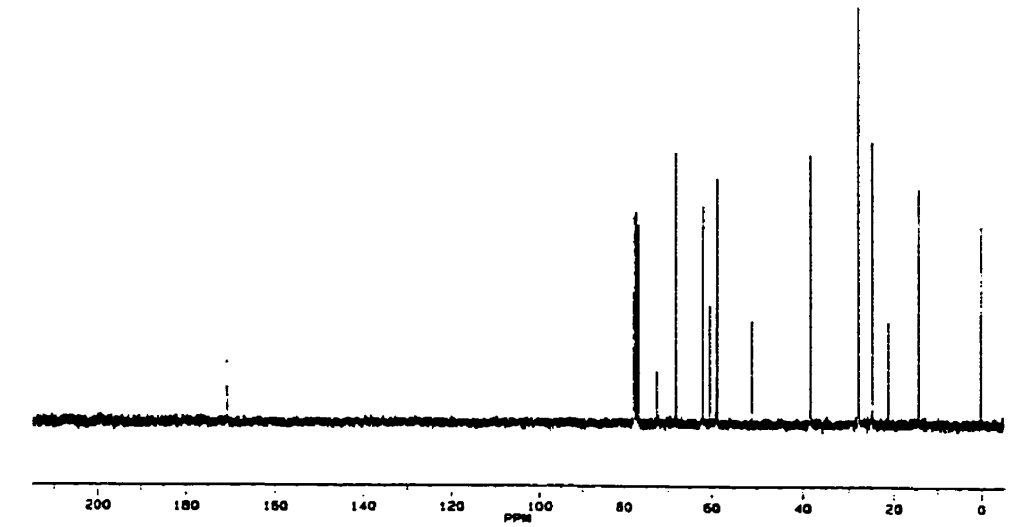
2-Hydroxymethylcyclopentanone 5-5

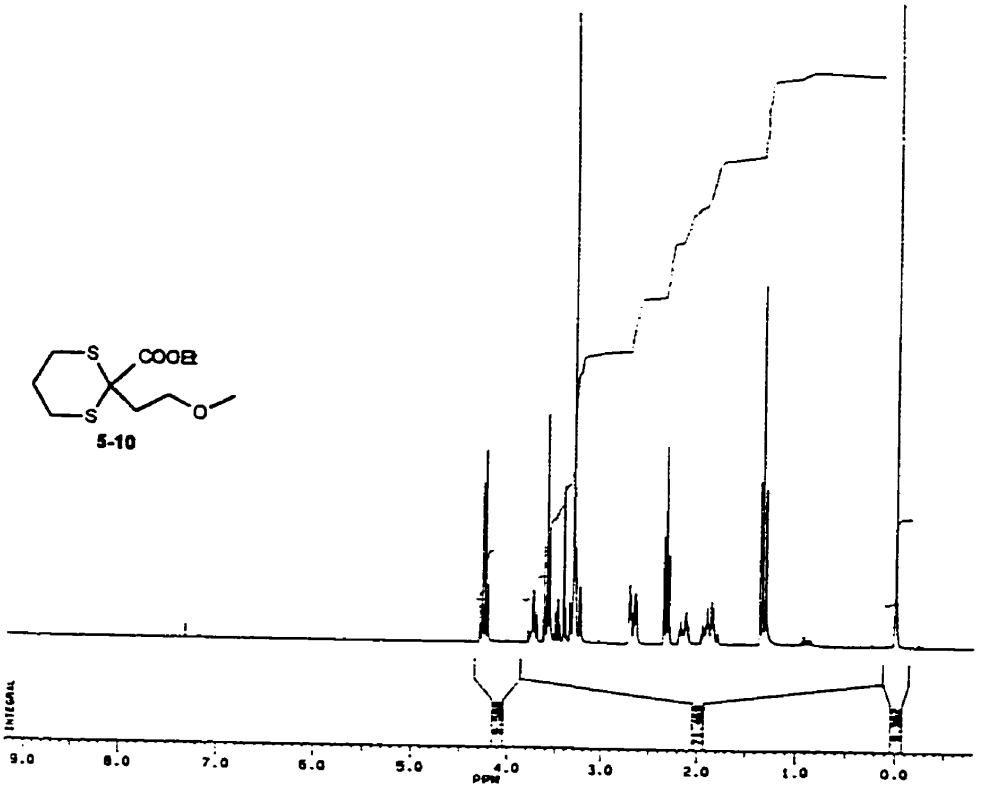


2-Methoxymethylcyclopentanone **5-6A**

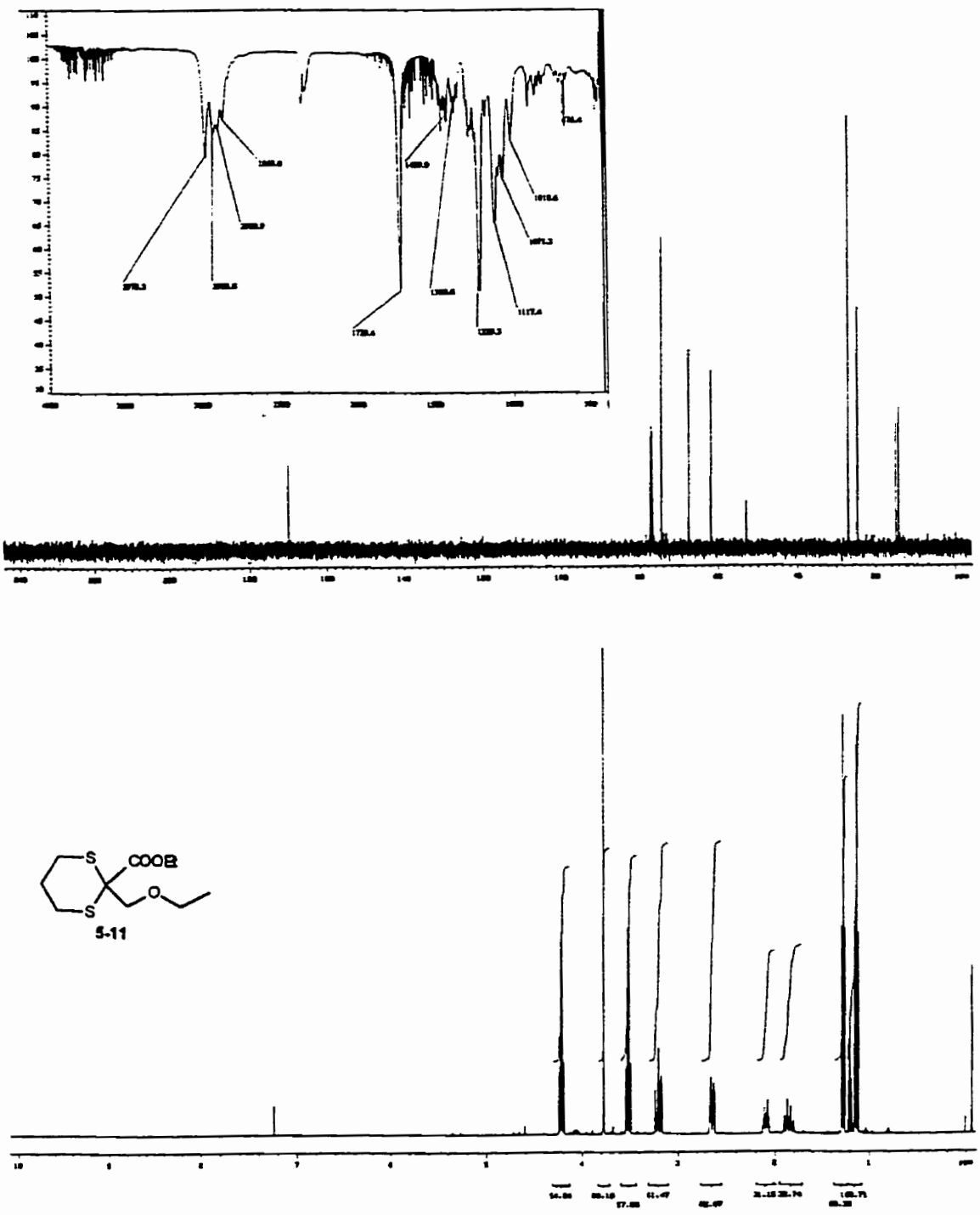


1,3-Dithiane-2-carboxylic acid ethyl ester 5-9

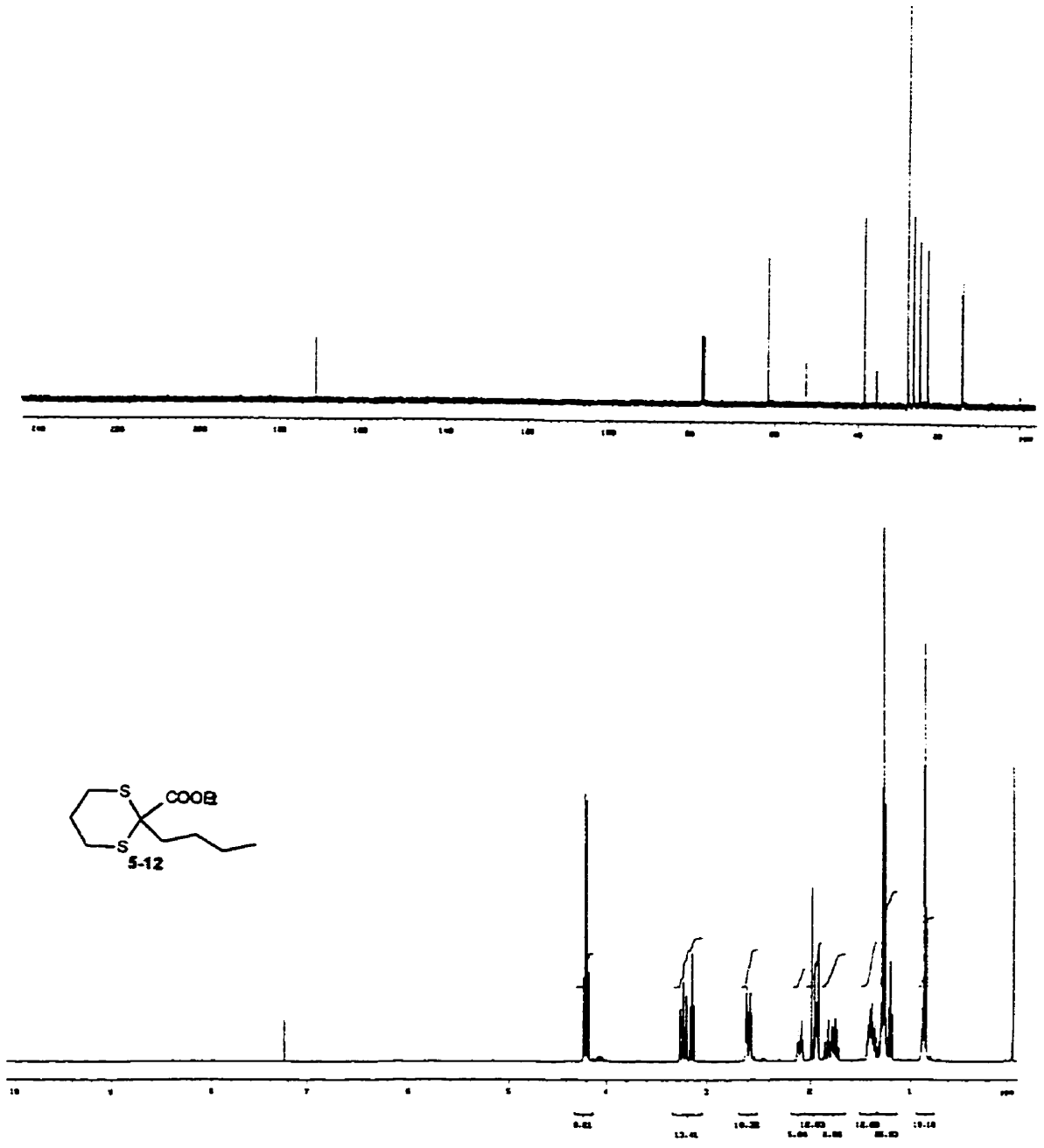




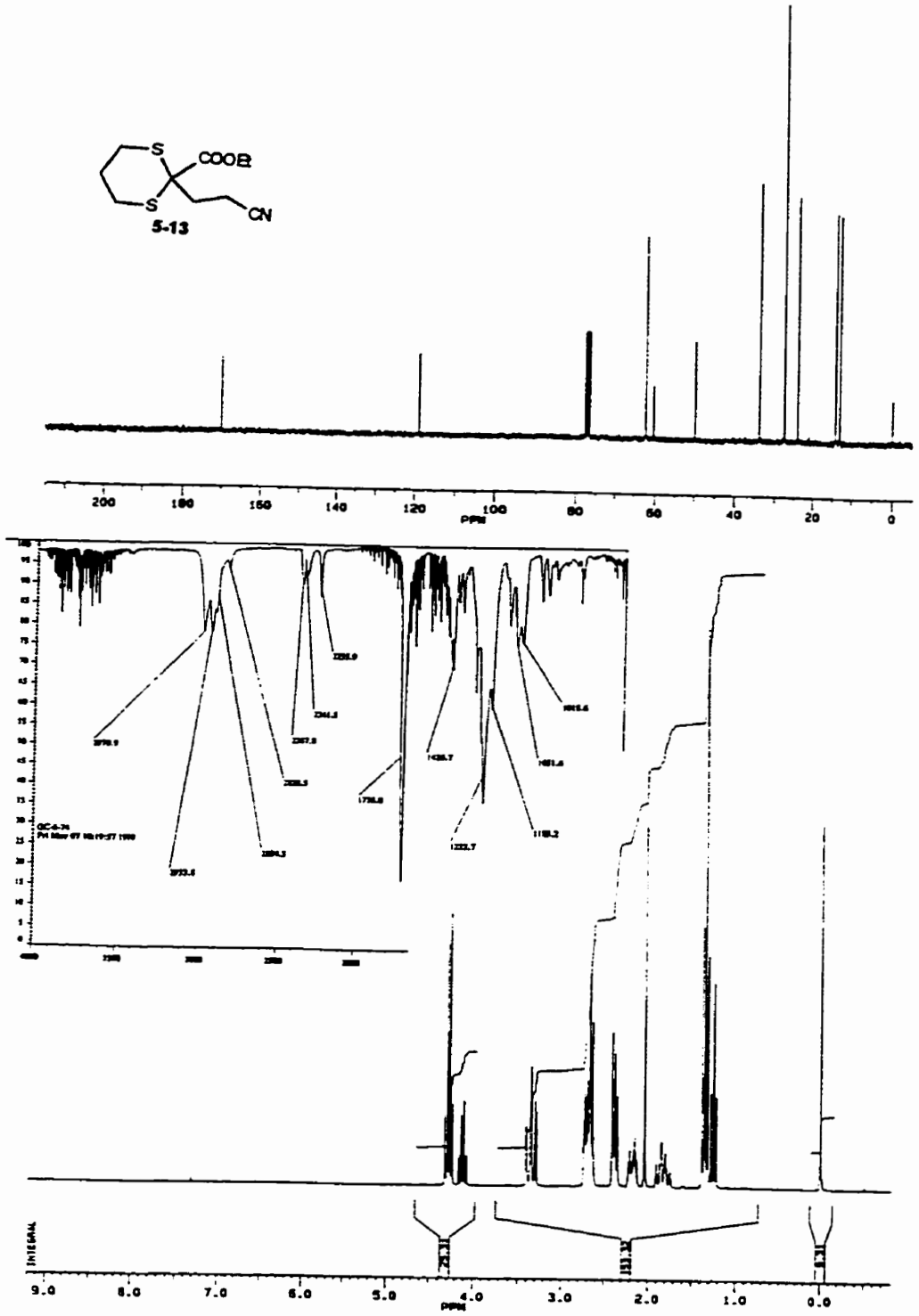
2-Methoxyethyl-1,3-dithiane-2-carboxylic acid ethyl ester **5-10**



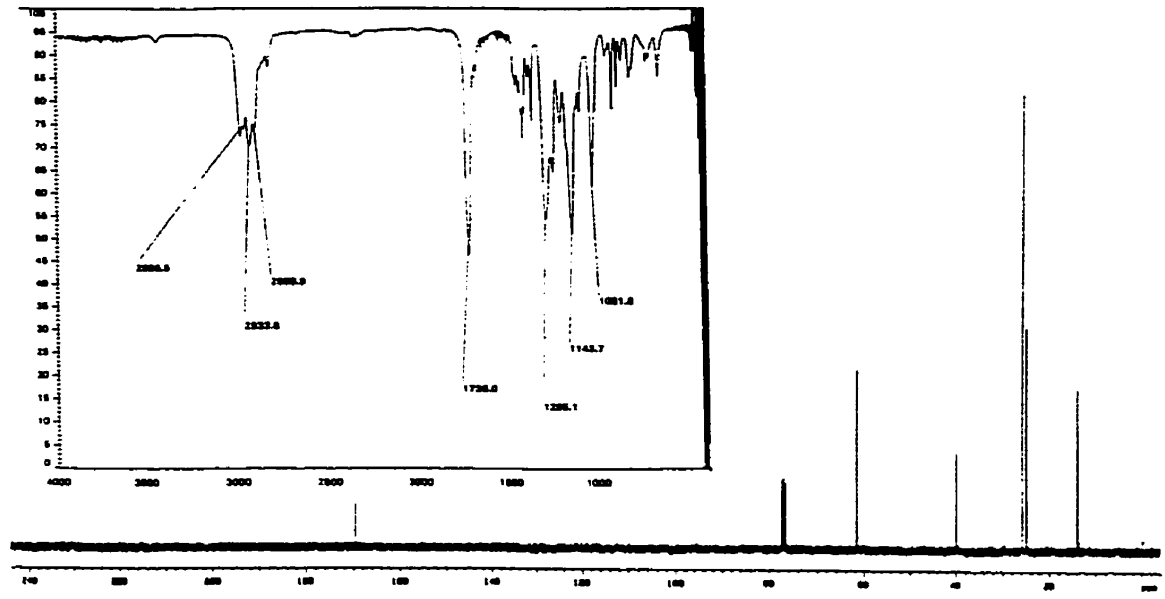
2-Ethoxymethyl-1,3-dithiane-2-carboxylic acid ethyl ester 5-11

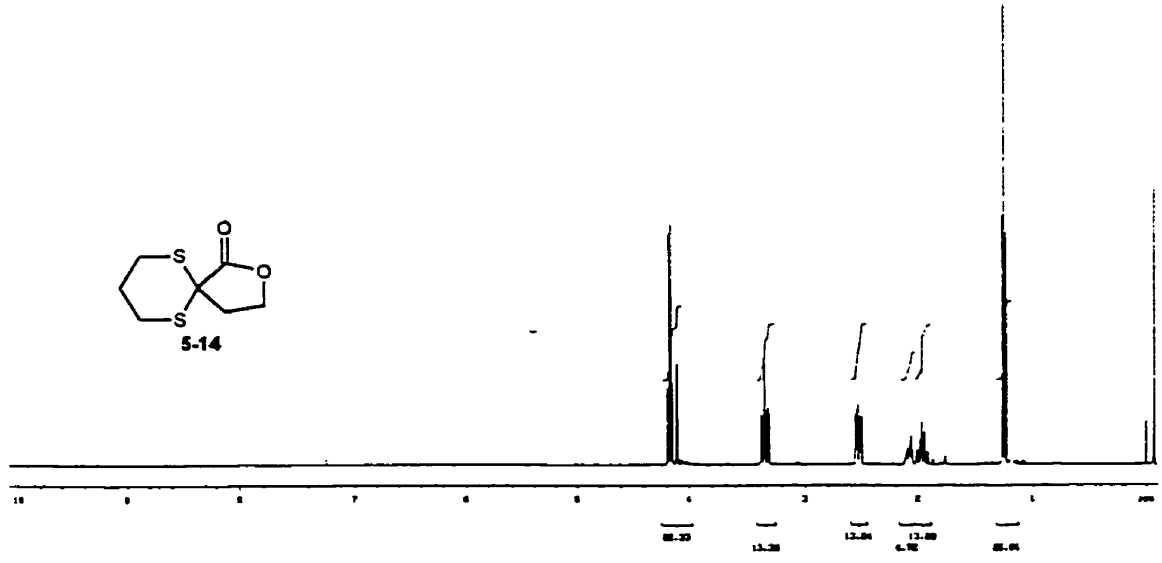


2-Butyl-1,3-dithiane-2-carboxylic acid ethyl ester 5-12

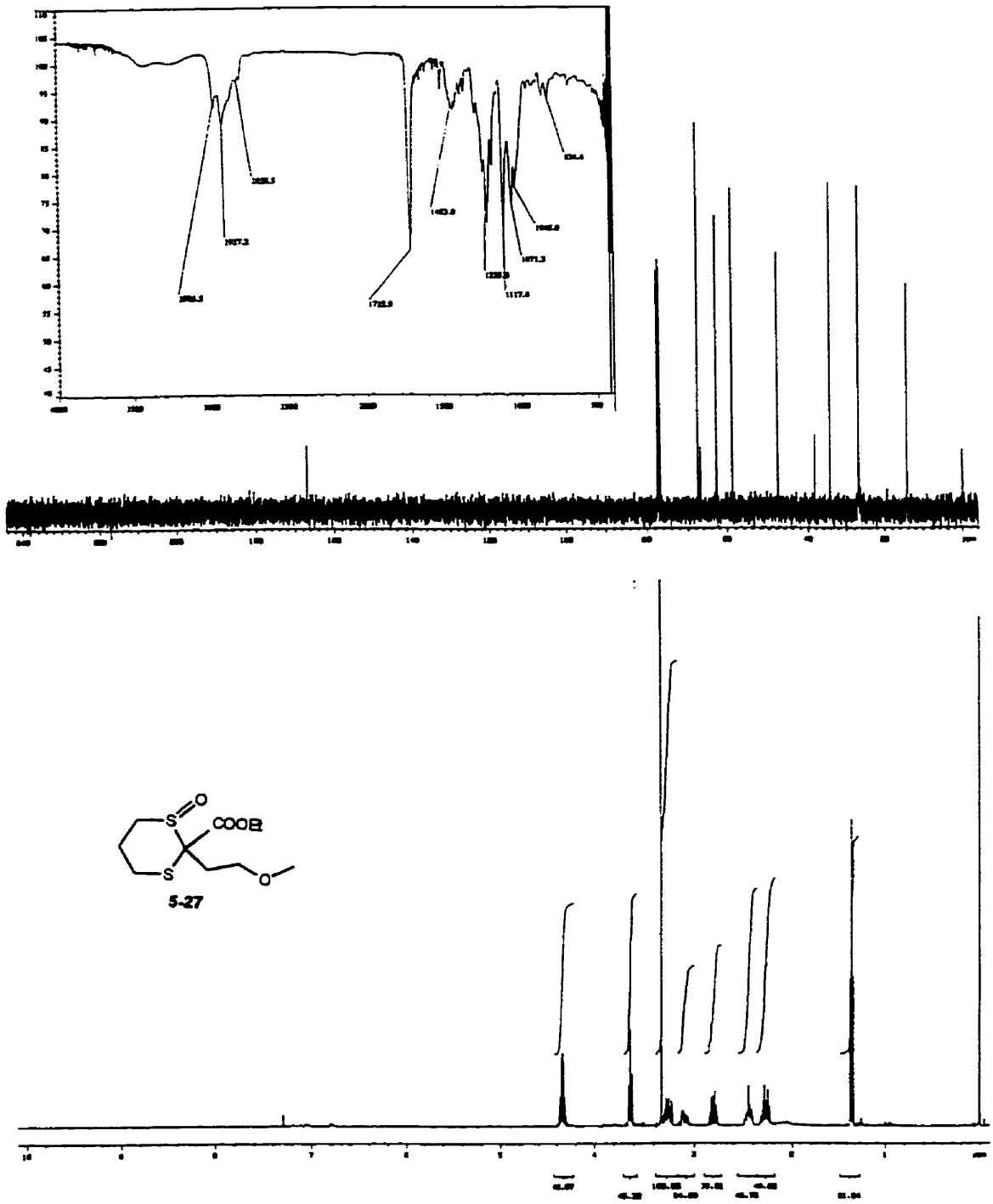


2-(2'-Cyanoethyl)-1,3-dithiane-2-carboxylic acid ethyl ester **5-13**

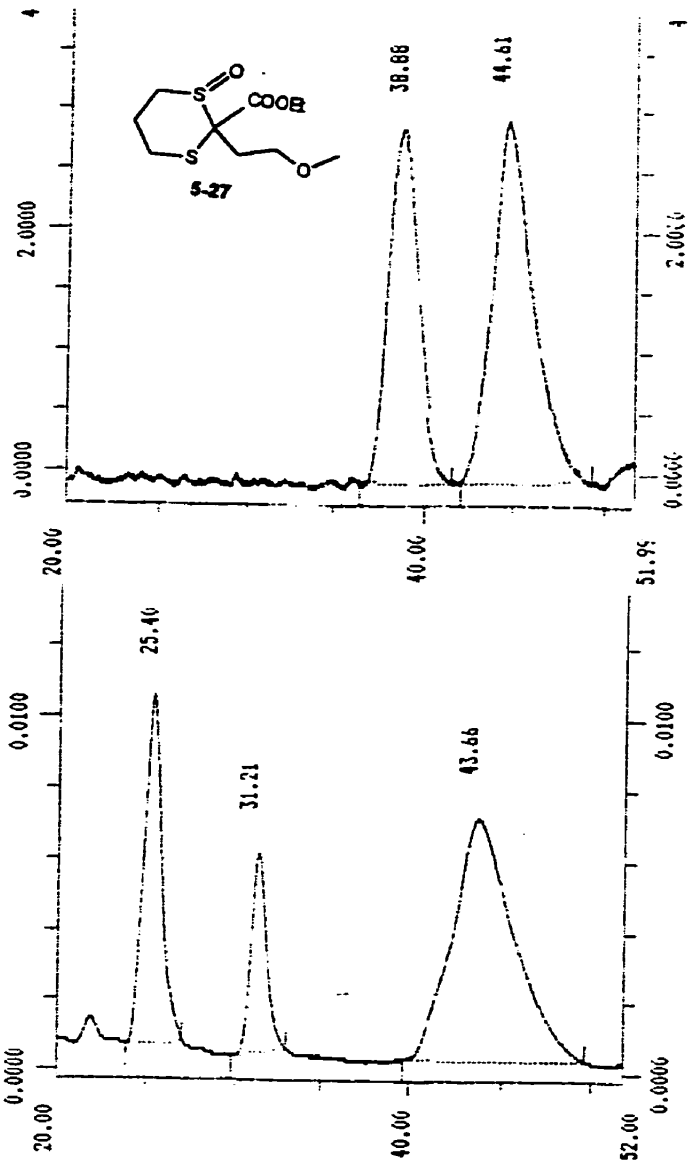




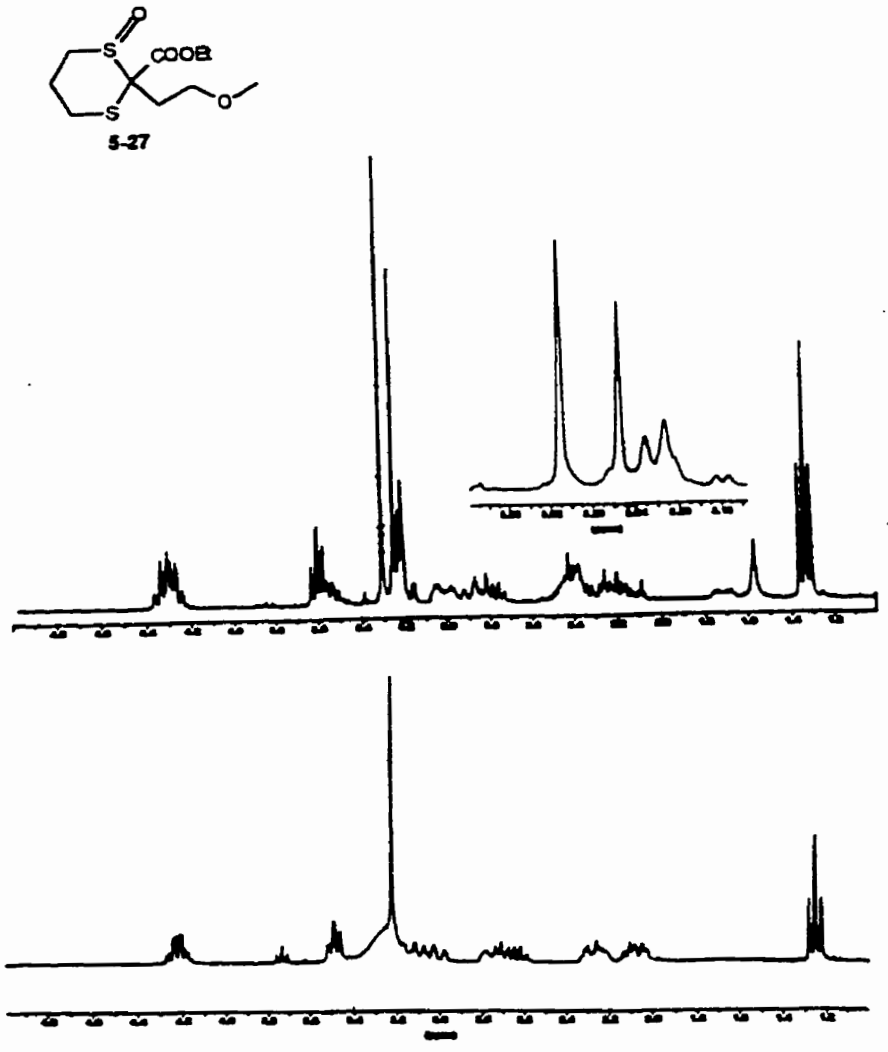
2-Oxa-6,10-dithiaspiro[4.5]decan-1-one 5-14



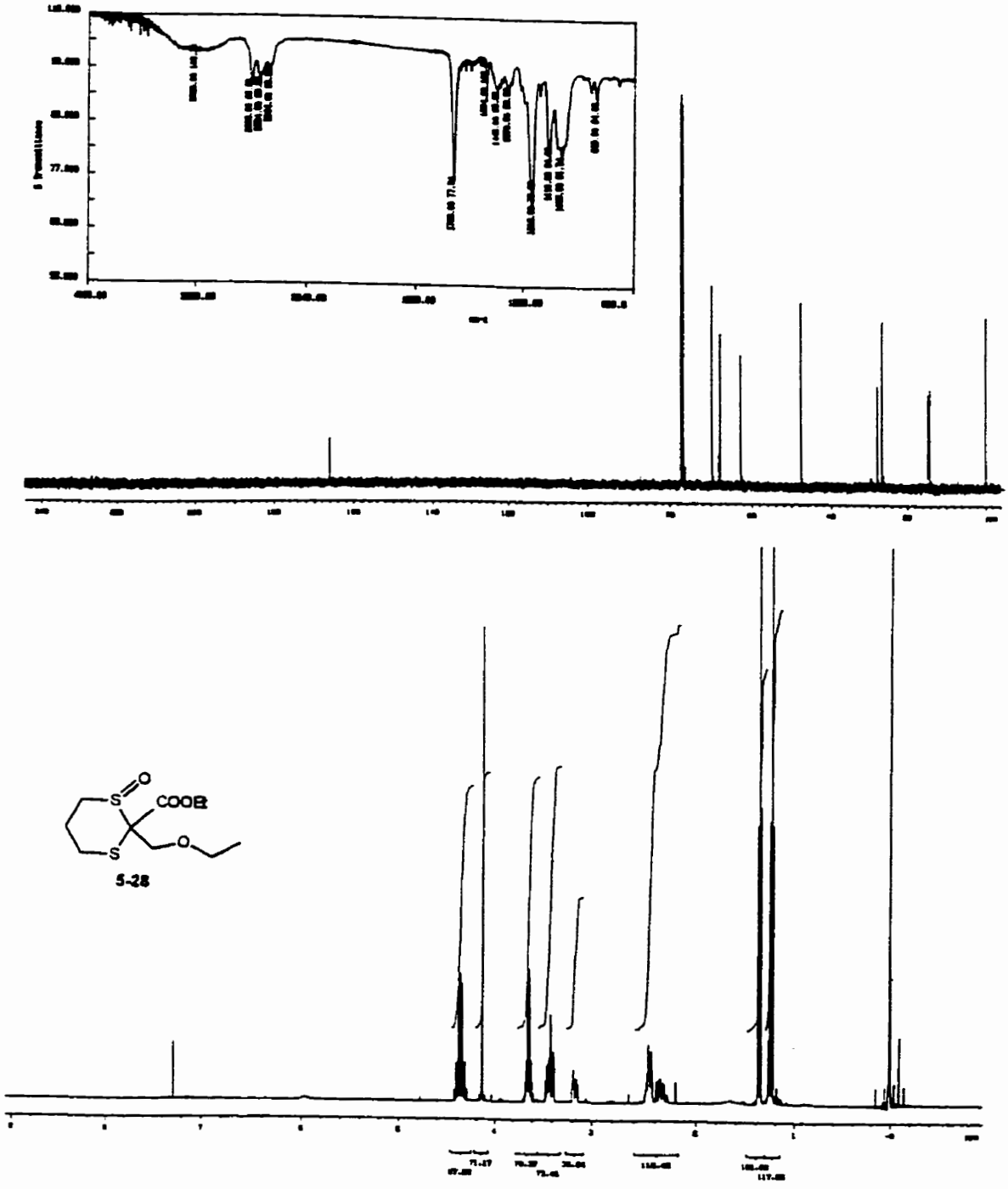
2-Methoxyethyl-1,3-dithiane-2-carboxylic acid ethyl ester 1-sulfoxide **5-27**



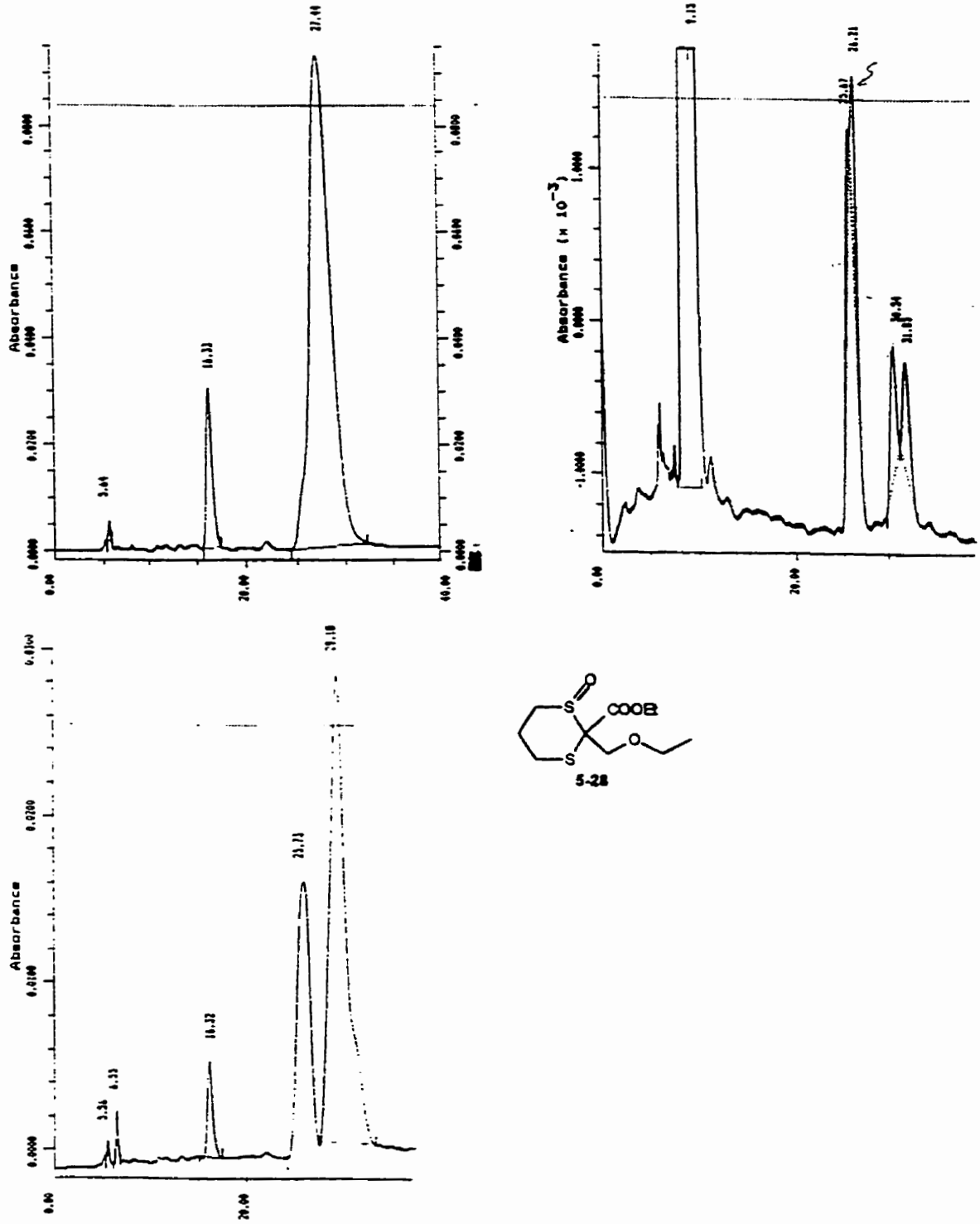
HPLC traces of 5-27 from chemical oxidation (upper) and yeast oxidation (lower)



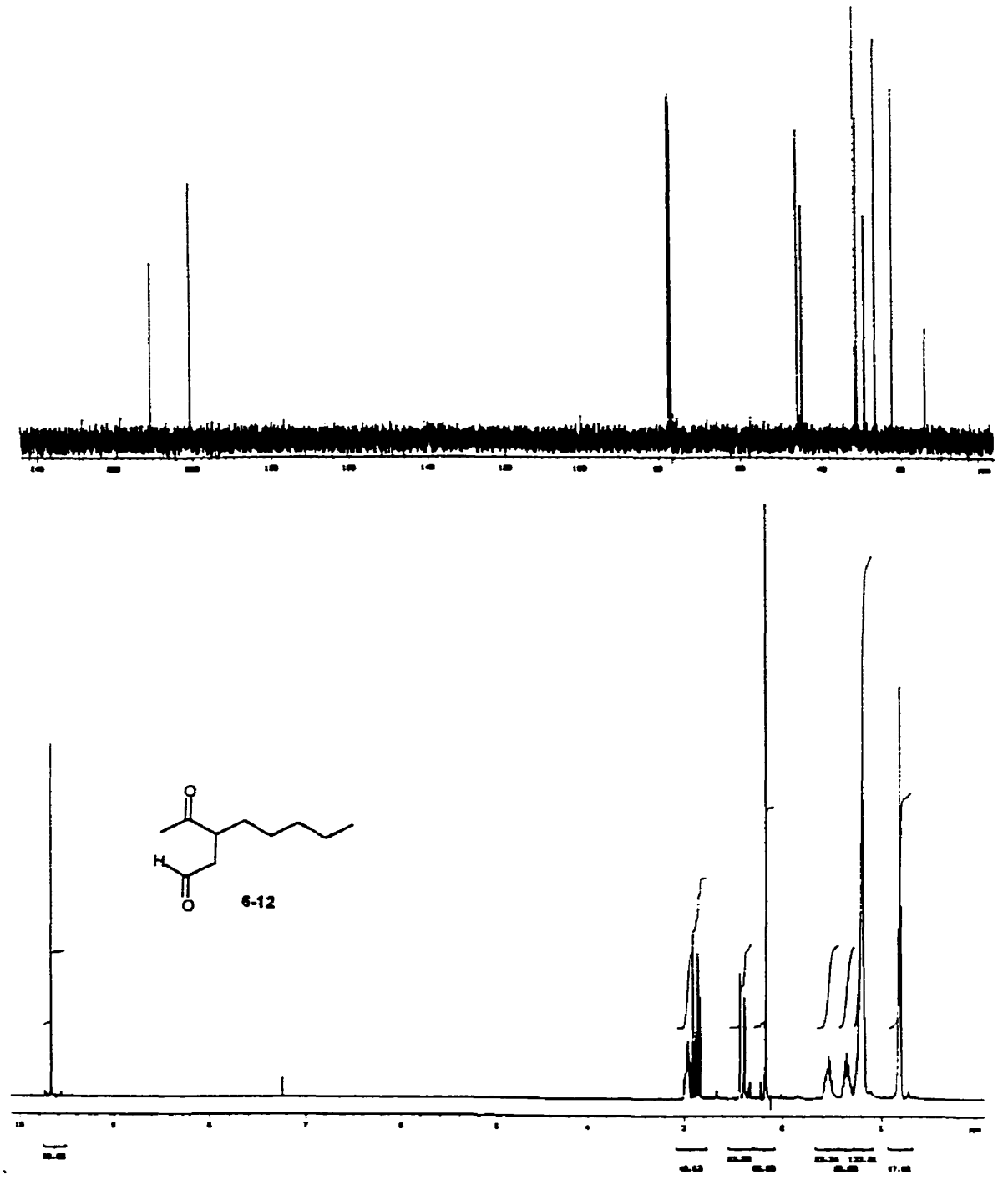
Determination of enantio- and diastereopurity of 5-27 by NMR experiments using shifting reagent TAE



2-Ethoxymethyl-1,3-dithiane-2-carboxylic acid ethyl ester 1-solfoxide **5-28**



HPLC traces of 5-28 from yeast (upper left), and chemical (upper right) oxidations and co-injection (lower)



3-Acetyloctanal 6-12