ENANTIOSELECTIVE CHEMOENZYMATIC SYNTHESIS OF OSELTAMIVIR (TAMIFLU)(R) INTERMEDIATES

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

HACI EŞİYOK

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOTECHNOLOGY

JANUARY 2008

Approval of the thesis:

ENANTIOSELECTIVE CHEMOENZYMATIC SYNTHESIS OF OSELTAMIVIR (TAMIFLU)(R) INTERMEDIATES

submitted by **HACI EŞİYOK** in partial fulfillment of the requirements for the degree of **Master of Science in Biotechnology Department, Middle East Technical University** by,

Prof. Dr. Canan Özgen _____
Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. Gülay Özcengiz _____
Head of Department, **Biotechnology**

Prof. Dr. Ayhan S. Demir
Supervisor, **Chemistry Dept.**, **METU** _____

Prof. Dr. Ufuk Bakır
Co-Supervisor, **Chemical Engineering Dept., METU** _____

Examining Committee Members:

Assoc. Prof. Dr. Özdemir Doğan _____
Chemistry Dept., METU

Prof. Dr. Ayhan S. Demir _____
Chemistry Dept., METU

Assoc. Prof. Dr. Nezire Saygılı _____
Faculty of Pharmacy, HU

Assoc. Prof. Dr. Emine Bayraktar _____
Chemical Engineering Dept., AU

Assoc.Prof. Dr. Nursen Çoruh _____
Chemistry Dept., METU

Date: 30.01.2008

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name : Hacı Eşiyok

Signature :

ABSTRACT

ENANTIOSELECTIVE CHEMOENZYMATIC SYNTHESIS OF OSELTAMIVIR (TAMIFLU)(R) INTERMEDIATES

Eşiyok, Hacı

M.S., Department of Biotechnology

Supervisor : Prof. Dr. Ayhan S. Demir

Co-Supervisor: Prof. Dr. Ufuk Bakır

January 2008, 72 pages

The objective of this presented study was to synthesize optically active compounds considered to be key intermediates in the synthesis of Oseltamivir (Tamiflu) by performing chemical and biotechnological methods. Thereof, the carboethoxy cyclohexenone skeleton first was synthesized utilizing easily available substances. The synthesis of alpha-hydroxy ketones in enantiomerically pure form offers a great importance in the synthesis of biologically active compounds. Toward this fact, the enantioselective synthesis of alpha-hydroxy carboethoxy cyclohexenone scaffold has been accomplished by following the routes which were manganese(III) acetate-mediated chemical oxidation followed by enzyme-mediated hydrolysis and additionally microbial direct biooxidation by whole cells of fungi expressly A. oryzae and A. flavus. A very satisfying results have been obtained by both of the methods.

Keywords: Alpha-Hydroxy Ketone, Manganese(III) Acetate, Enzymatic Kinetic Resolution, Microbial Hydroxylation, Fungi.

ÖZ

OSELTAMIVIR (TAMIFLU)(R) ANTİVİRAL İLAÇ HAMMADDELERİNİN KEMOENZİMATİK SENTEZLERİ

Eşiyok, Hacı
Yüksek Lisans, Biyoteknoloji Bölümü
Tez Yöneticisi : Prof. Dr. Ayhan S. Demir
Ortak Tez Yöneticisi: Prof. Dr. Ufuk Bakır

Ocak 2008, 72 sayfa

Bu çalışmanın amacı antiviral bir ilaç olan Oseltamivir (Tamiflu) sentezinde kullanılabilecek optikçe aktif hammaddelerin kimyasal ve biyoteknolojik yöntemlerle sentezlenmesidir. Bu nedenle ilk olarak karboetoksi siklohekzenon yapısı kolayca bulunabilen basit maddeler kullanılarak sentezlendi. Alfa-hidroksi ketonların enansiyomerikçe saf hallerinin eldesi biyolojik açıdan aktif bileşiklerin sentezinde büyük bir önem teşkil eder. Bu gerçeğe yönelik olarak, alfa-hidroksi karboetoksi siklohekzenon yapısının enansiyoseçici sentezi, mangan(III) asetat vasıtasıyla kimyasal oksidasyon ve beraberinde enzim yardımıyla hidroliz ve ayrıca A. oryzae ve A. flavus türündeki mantarların tüm hücreleri kullanılarak yapılan mikrobiyel biyooksidasyonla gerçekleştirildi. Her iki metodla da tatmin edici sonuçlar elde edildi.

Anahtar kelimeler: Alfa-Hidroksi Keton, Mangan(III) Asetat, Enzimatik Kinetik Resolüsyon, Mikrobiyel Hidroksilasyon, Mantar.

To My Family

ACKNOWLEDGMENTS

I would like to remark my feelings of appreciation and gratitude to my supervisor Prof. Dr. Ayhan S. Demir for his support, encouragement and guidance throughout the study.

I would like to thank to my co-supervisor Prof. Dr. Ufuk Bakır for her helps, and understanding.

My distinctive thanks extent to Peruze Ayhan for her endless support and suggestions during my study. I would like to thank also to my labmates Betül Sopacı, Umut Erkılıç, Umut Demirtaş and İlke Şimşek for their friendship.

I also express my sincere appreciation to all of the members of Demir's Research Group.

I am grateful to the Scientific and Technological Research Council of Turkey (TÜBİTAK) for their financial support during my study.

And I am indepted to my family…

TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

CHAPTER 1

INTRODUCTION

1.1 Bioconversions in Organic Chemistry

Incorporation of biotransformation steps including the use of microorganisms and/or isolated enzymes is increasingly being made use of both in industry and academic synthesis laboratories. Regio- and stereo-control in organic synthesis can be succeeded using an enzyme catalyzed reaction which is the fact primarily considered for consolidation of a biotransformation in a synthetic sequence. Biotransformations are becoming accepted as a method both for generating optically active compounds and for developing efficient pathways to desired target molecules. Biotransformations ensure an alternative to the chemical synthetic methodology that is sometimes competitive and therefore a section of tools available to the synthetic chemist [1].

The use of isolated enzyme systems or intact whole organisms for biotransformation applications has own advantages and disadvantages. Many isolated enzyme systems are commercially available or are relatively easy to isolate, at least in a crude form. They can be stable and easy to use by generally giving clean and single products. Despite the fact that many hydrolytic reactions do not need a co-factor, redox reactions in which co-factor is used are present. This gives rise to need for regeneration and thus complication of procedure. On the other hand, whole organisms do not have this disadvantage. They do tend to give more than one product, which may or may not be an advantage. However, they are often cheaper to use than isolated enzyme systems [2].

Biotransformations have a number of advantages as compared with traditional chemical methods. They are not only regio- and stereospecific but are also enantiospecific providing the production of chiral products from racemic mixtures. Conditions for biotransformations are mild and do not require the protection of other functional groups for most of the cases [2]. On the other hand, it is worthy to note that the choice between a biotransformation and chemical technology will be driven by the commercial performance of the selected synthetic strategy on a specific target. Many chemical reactions for which there is no equivalent biotransformation are present. There exists a number of chemical methods for introducing a chirality into a molecule in which biotransformations might not be competitive. However, biology might be useful in cases where there is no chemical solution or might allow extension of the arsenal of chemical transformations [3].

Biotransformation applications are mainly composed of hydrolytic transformations catalyzed by hydrolase type enzymes. They include ester and amide bonds transformations by using proteases, esterases or lipases. In addition to that the formation and/or cleavage of epoxides, nitriles and phosphate esters are other types of hydrolase enzymes applications. For instance, the microbial whole-cell biocatalyst, *Rhodococcus erythropolis AJ270*, catalyzed the biotransformation of 3-arylpent-4-enenitriles **(1)** by an enzyme couple nitrile hydratase/amidase under very mild conditions [4] (Figure 1).

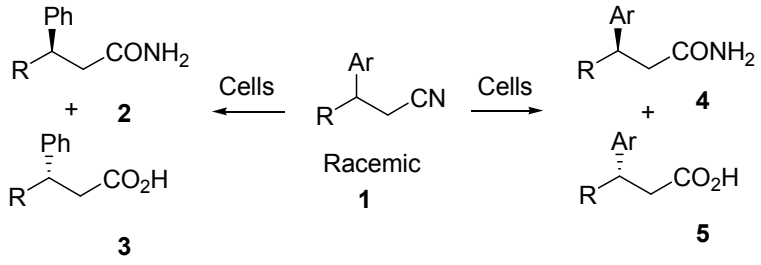


Figure 1 Whole cell catalyzed biotransformation of nitriles

The hydrolytic kinetic resolution of glycidaldehyde acetal derivatives **6** was examined by using the recombinant *Aspergillus niger* epoxide hydrolase as biocatalyst [5] (Figure 2). It was developed a method also that makes possible the specific incorporation of sterically demanding α-fluoroalkyl as well as α-methyl amino acids into P_1 position of peptides using commercially available proteases namely trypsin and α-chymotrypsin [6].

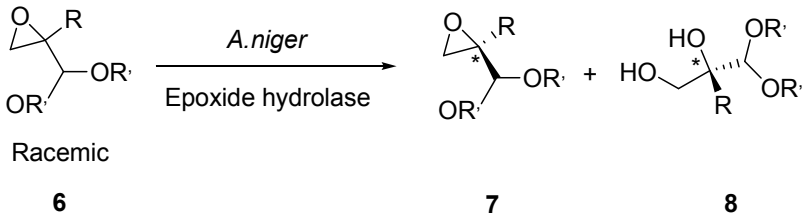


Figure 2 Epoxide hydrolase mediated enantioselective hydrolysis

The efficacy and high enantioselectivity of esterase producing strain *Trichosporon beigelli* in its crude native form has been effectively demonstrated on a broad spectrum of molecules, which include the alkyl ester of alcohols and carboxylic acids such as 1-aryl alkanols, non-steroidal anti-inflammatory drugs (NSAIDS) such as ibuprofen and a variety of thiocarboxylic acids [7]. Over the last few years, there has been a dramatic increase in the number of publications in the field of lipase-catalyzed reactions performed in common organic solvents, ionic liquids or even non-conventional solvents. A fairly large percentage of these publications have emerged from organic chemists who have recognized the potential of biocatalysis as a viable and popular technique in organic synthesis [8]. Figure 3 illustrates applications for certain types of reactions.

Dehydrogenases have been widely used for the reduction of carbonyl groups of aldehydes or ketones and of carbon-carbon double bonds. The significance of the use of these enzymes is that chiral product can potentially be obtained from a prochiral

substrate. Carbonyl reductases from various microorganisms have been used to prepare optically active alcohols from carbonyl compounds. It was reported that a versatile biocatalyst that shows high enantioselectivity for a variety of ketones, *Saccharomyces cerevisiae* carbonyl reductase (SCR), has been identified and utilized for enantioselective bioreductions [9]. Other functional group reductions include the reduction of carbon-carbon double bonds. Carbon-carbon double bond reductase has been isolated from the cells of baker's yeast [10]. The corresponding enzyme catalyzed the reduction of α,β-unsaturated ketones **9** and resulted in the formation of saturated (S)-ketone **10** selectively as can be seen from the Figure 4.

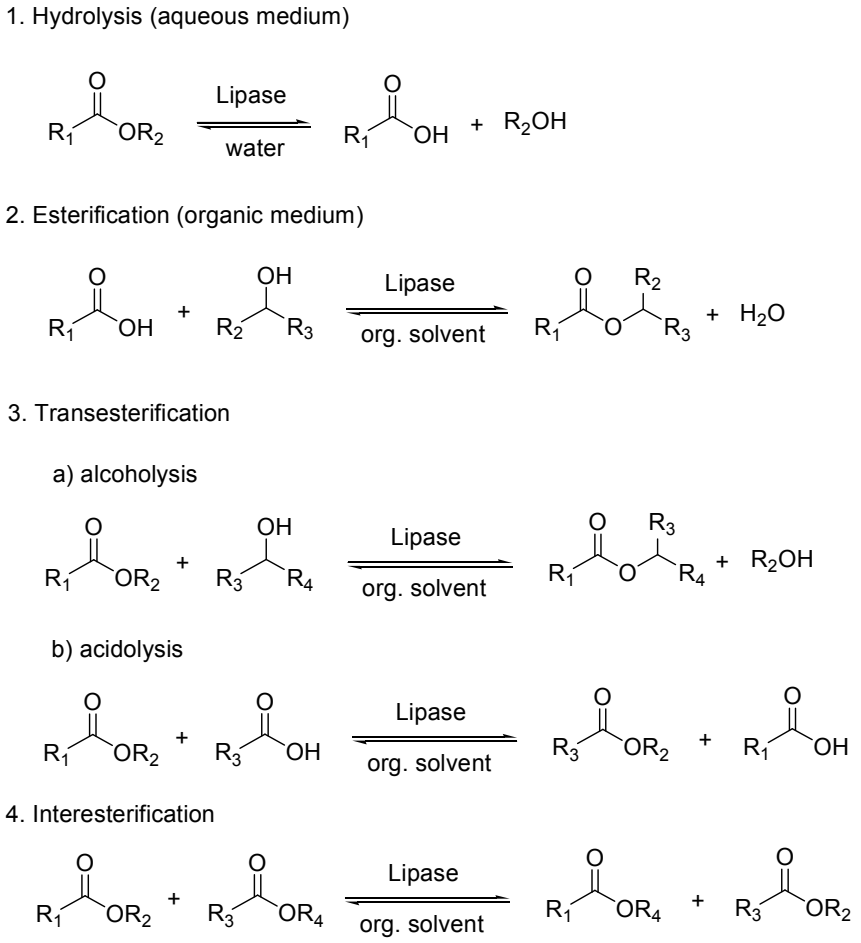


Figure 3 Some reactions catalyzed by lipases in aqueous and organic media

Oxygenases majorly lead to oxidation biotransformations by incorporating molecular oxygen into a molecule either by incorporation of one or both atoms of O_2 or by an electron-transfer oxygen donor process. It is important to note that direct oxyfunctionalization of unactivated organic substrates in a regio or enantioselective manner is a significant problem in organic synthesis however that can be overcome by use of biotransformation step. A study in which 3-α-hydroxysteroid dehydrogenase from a microorganism *Pseudomonas paucimobilis* catalyze the preparative scale and stereospecific oxidation of hydroxyl groups of several C-21 bile acids was reported [11]. The synthesis of optically pure 2-hydroxy acids by α-hydroxylation of long chain carboxylic acids with molecular oxygen was achieved by α-oxidase of peas (*Pisum satiuum*) [12]. Furthermore, an enzyme possessing both peroxidase and epoxidation activity was isolated from culture cells of *Nicotiana tabacum* for use in epoxidation of styrenes **11** [13] (Figure 5).

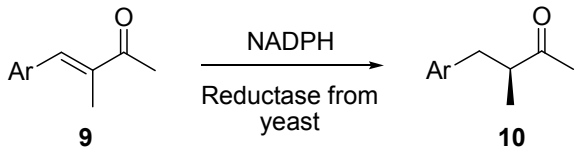


Figure 4 Asymmetric reduction of enone with a reductase from baker's yeast

In spite of these mentioned biooxidation applications, it is also needed to state another interesting study [14] in which the benzo(a)pyrene has been transformed into benzo[a]pyrene 1,6-3,6-and 6,12-quinones by purified extracellular laccase of *Pycnoporus cinnabarinus* by oxidation.

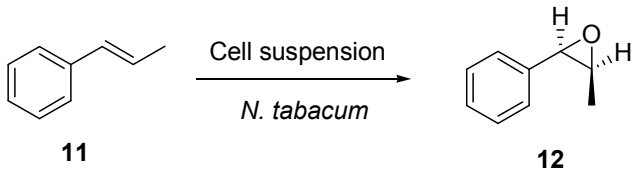


Figure 5 Epoxidation of styrene catalyzed by peroxidase from the cultured cells of *N. tabacum*

Other types of biotransformations develop an area of increasing impact. Isolation of new enzymes and control of existing enzymes under conditions enabling synthetically useful biotransformations gave rise to arise of new areas such as carbon-carbon bond formations. Enzymatic systems belonging to the class of lyases, which are capable of forming carbon-carbon bonds in a highly stereoselective manner are known and include reactions such as aldol condensation, acyloin reactions, Michael additions, Diels-Alder reaction and carboxylation. For example, it was presented that asymmetric synthesis of mixed benzoins, that is, benzoins with nonidentical aromatic moieties using an enzyme-catalyzed benzoin condensation was performed [15].

1.2 Chirality and Asymmetric Synthesis

The discovery of *chirality* that is formally defined as geometric property of rigid object of not being superimposable with its mirror image [16], during the last century, called for that chemists concern themselves with developing methods in order to obtain enantioenriched substances. The importance of chirality is emphasized by the fact that almost all natural products are chiral and that their physiological or pharmacological properties depend upon their recognition by chiral receptors. These chiral receptors interact only with molecules of the proper absolute configuration [17].

Organic compounds play an important part in modern life, not least in the area of pharmaceuticals, agrochemicals, and other materials which illustrate useful biological activity. Often such biological activity arises through the interaction of the organic compound with a biomolecule such as an enzyme or a receptor. As previously mentioned such sites of action are constructed from chiral building blocks such as amino acids or carbohydrates. Being natural chiral compounds, these building blocks are present as single enantiomers, and it follows of course that the resulting biomolecules are single enantiomers [18].

As many natural products exist as one stereoisomer, the term *asymmetric synthesis* is needed to prepare nature-identical material. Asymmetric synthesis can be defined as a reaction where an achiral unit in an ensemble of substrate molecules is converted by a reactant into a chiral unit in such a manner that stereoisomeric products are formed in unequal amounts. The fact that nature produces a wide variety of chiral compounds, asymmetric synthesis is strictly required to obtain enantiomerically pure compounds [19].

1.2.1 Chirality-Activity, Structure-Activity Relationships

Enantiomers of any biologically active compound have identical physical and chemical properties in an achiral environment, however in a chiral environment, one enantiomer may display different chemical and pharmacological behavior than the other enantiomer and so likely to interact differently with the natural biomolecule [16]. The enantiomers probably show different levels of biological activity and could also exhibit quite different types of activity since it is clear that living systems are themselves chiral. As it is considered the effect, the two enantiomers should be seemed as two distinct compounds [18].

One enantiomer usually is more active than the other. This proves the fact that use of racemic biologically active compounds is clearly undesirable only one of the enantiomers possesses the desired beneficial activity however both enantiomers can have the risk of undesirable activity that is side effect. In addition, the side effects

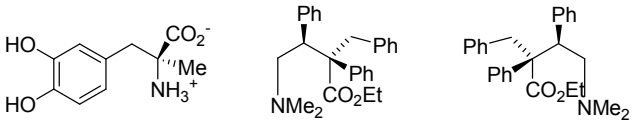
could be different for each enantiomer. The risk of side effects is far predominated by the positive effect for the active enantiomer whereas the inactive enantiomer provides little or no benefit, but does carry the risk [18].

For a chiral biologically active compound the following possibilities present [18]:

- Only one of the enantiomers is active, the other being devoid of activity.
- Both enantiomers are active, but they have very different potencies.
- Both enantiomers have similar or equal activity.
- Both enantiomers are active, but the type of activity is different.

The first two situations are prevalent for instance L-enantiomer of hypertensive agent α-methyldopa **(13)** is active but D-enantiomer is not. It is rare that both enantiomers have similar potency however examples are known for the case in which the enantiomers have different activities. Propoxyphene is interesting in that both enantiomers have useful but different biological activities. The D-enantiomer that is given a trade name Darvon ® **(14)** is an analgesic, on the other hand, the antipode **(15)** (Novrad ®) shows antitussive properties but no analgesic effect (Figure 6) [18].

For a pharmaceutical as biologically active compound it is useful to illustrate a hypothetical interaction between a chiral drug and its chiral binding site in Figure 7 [16] to view the difference between two enantiomers of a drug. The figure demonstrates that the active enantiomer has a three dimensional structure that allows drug domain A to interact with binding site domain a, B to interact with b, and C to



L-alpha-methyldopa **13** | Darvon (R) **14** | Novrad (R) **15**

Figure 6 Examples for the chiral biologically active compounds

interact with c. In contrast, the inactive enantiomer can not be aligned to bind the same three sites simultaneously. The difference in three-dimensional structure allows the active enantiomer to bind and have a biological effect, whereas the inactive enantiomer can not do that.

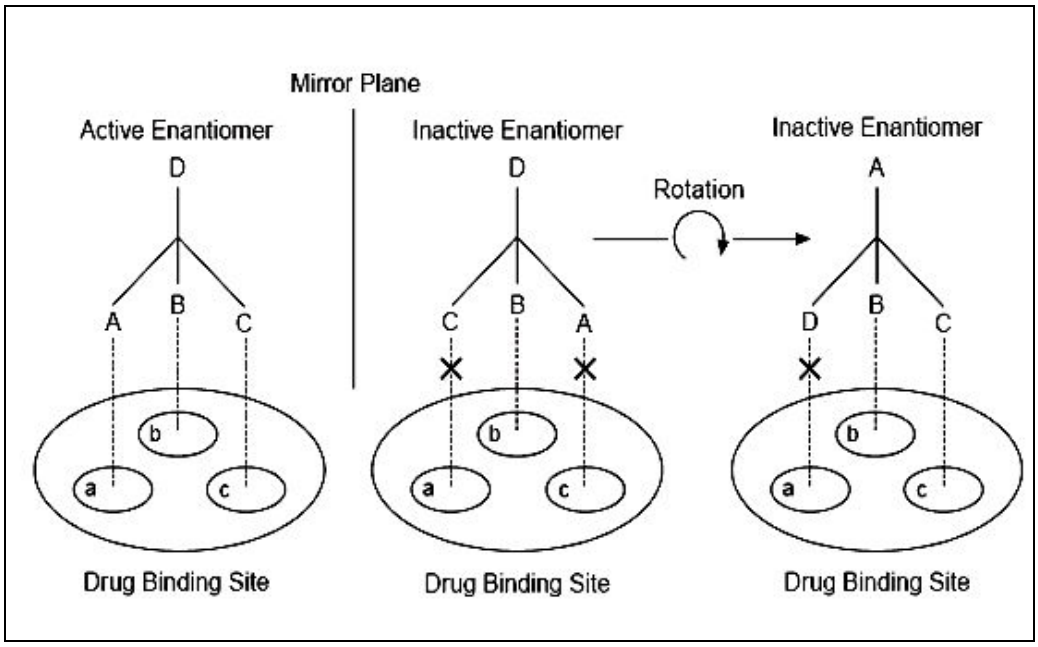


Figure 7 The hypothetical interaction between the two enantiomers of a chiral drug and its binding site

In some cases, the portion of a molecule containing the chiral center(s) may be in a region that does not play a role in the molecule's ability to interact with its target. In these cases, the individual enantiomers may possess very similar or even equivalent pharmacology at their target site. Even in these cases, the enantiomers may differ in their metabolic profiles as well as their affinities for other receptors, transporters, or enzymes [16].

1.2.2 Methods for the Synthesis of Chiral Compounds

Enantioselective synthesis can be obtained in such a way that at least one of the agents in the system must be chiral. It is known that there are two major methods to achieve this objective namely: Resolution or Asymmetric Synthesis which includes the use of a chiral starting material, chiral auxiliaries or reagents [19].

1.2.2.1 Resolution

The separation of an optically inactive enantiomeric pair into its constituent optically active enantiomers is called *resolution* and a chiral agent of some sort is required in the process [20]. Although this method often is seen as the last way out of an enantioselective synthesis, and the least glamorous, there are many circumstances when it is worth looking into a resolution method. Until recently, the use of a chiral natural product as starting material or a resolution were the only two methods available for the preparation of a chiral material [19].

As a general way, the first step in the resolution of racemic mixture is the conversion of the racemic mixture into a diastereomeric mixture by means of a reaction with an optically active substance. Since the diastereomers will have different physical properties, they might be separated, for example, by recrystallization. Once this is achieved, the next step is the reverse reaction to give back the original enantiomers. The whole process is then repeated until the optical rotation reaches a maximum [21].

1.2.2.1.1 Kinetic Resolution

Kinetic resolution involves using a chiral catalyst or reagent to promote selective reaction of one enantiomer over the other giving a mixture of enantioenriched starting material and product, and the desired component is then isolated as can be seen from the Figure 8 [22].

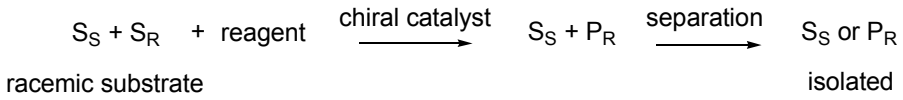


Figure 8 Catalytic kinetic resolution

The theoretical yields for such resolutions are usually 50%. If the *undesired* resolution byproduct can be racemized or come back to the desired enantiomer, then this can improve the yield, and thus the practicality, of the resolution process, provided the additional cost in time and materials does not eclipse the cost of the initial resolution [22]. In some special circumstances, it is possible to induce substrate racemization under the conditions of resolution. It then becomes possible in principle to convert essentialy 100% of the racemate to the desired product. Such processes constitute a very special subclass of kinetic resolution reactions known as *dynamic kinetic resolution* [22].

The variety of enzyme-catalyzed kinetic resolutions of enantiomers reported in recent years is enormous. Similar to asymmetric synthesis enantioselective resolutions are carried out in either hydrolytic or esterification-transesterification modes. Both modes have advantages and disadvantages. Hydrolytic resolutions that are carried out in a predominantly aqueous medium are usually faster and, as a consequence, require smaller quantities of enzymes. On the other hand, esterifications in organic solvents are experimentally simpler procedures, allowing easy product isolation and reuse of the enzyme without immobilization [23].

Although enzyme-catalyzed kinetic resolutions have been known for a long time, the use of chemical catalysts to perform kinetic resolutions is a relatively new research topic. The first example of a successful kinetic resolution using a chemical catalyst, although in stoichiometric amounts, was published in 1981 by Sharpless and his research group (Figure 9) [24].

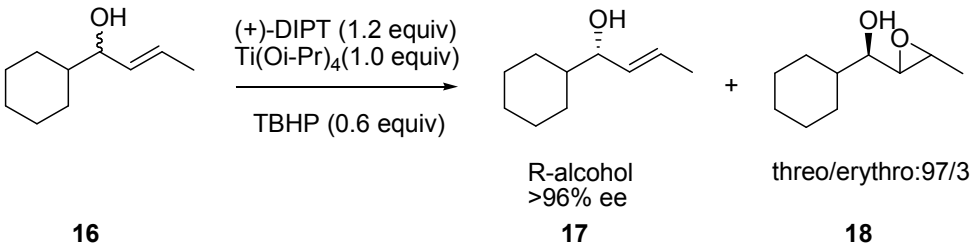


Figure 9 Kinetic resolution of allylic alcohol **16** as reported by Sharpless

1.2.2.1.2 Other Methods Available For Resolution

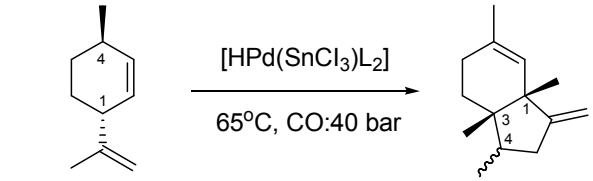
In order to achieve a resolution, there has been wide variety of methods either chemical such as chromatographic separation, selective crystallization of a single isomer, fractional crystallization, selective isomer crystallization by entrainment and selective extraction with a chiral host compound or biological, for instance, enzymatic resolution and catalytic antibodies. These mentioned methods have to be tried to obtain for the particular system in use. The functional groups present within the subject molecule often determine which of the methods stated can be utilized. Cost and the scale are other major factors [19].

1.2.2.2 Asymmetric Synthesis

Chiral center in a molecule can be generated by being aware of on the synthesis to attain desired molecule by considering three major options which are chiral starting material, chiral reagent either chemical or biological, and chiral environment [19]. Chirality can also be introduced in a temporary manner through the use of chiral auxiliary that can be considered as a sub-class of chiral substrates. Each approach has advantages and disadvantages and these must be envisioned on a case-by-case basis in order to increase the chance of success in synthetic plan [19].

1.2.2.2.1 Chiral Starting Material

A substrate bearing one or more asymmetric centers which react with an achiral reagent in the presence of an achiral catalyst can be a source of stereodifferentiation to gain stereoselectivity [25]. It was published that cyclocarbonylation of (1R, 4R)-isolimonene **(19)** (Figure 10) in which the stereogenic center C_4 is far from C_1 that bears the isopropenyl group was accomplished. The reaction was catalyzed by palladium(II) precursors containing the non-chiral ligands 1,4-bis(diphenylphosphino)butane (dppb), or 1,1'-bis(diphenylphosphino)ferrocene (dppf) [26].



(1R, 4R)-isolimonene

19 **20**

Figure 10 Cyclocarbonylation reaction of (1R, 4R)-isolimonene **(19)**

1.2.2.2.2 Chiral Reagents

As can be seen in many of the ways, this is the approach of choice as nature utilizes this methodology through enzymes. The reagent must be selective both in terms of induction and functional group specificity. The need for protection should be considered carefully since the fact that this gives rise to the introduction of extra steps. In addition, chiral reagent should allow for the expensive cost component to be recycled, if necessary, or have a very high turnover number. As deciding the use of any chiral reagent, chemical or biological, in any of the enantioselective synthesis, some of the issues have to be compared properly, for instance, throughput, catalyst

cost, selectivity, yield, isolation and processing, operating conditions and catalyst design [19].

1.2.2.2.3 Chiral Environments

The environment of a chemical reaction can be made a chiral. The majority of the examples in this class use chiral solvents or additives. To affect the differentiation of the free energies of the diastereomeric transition states, and therefore enable useful induction, these agents must be closely associated with the reaction center [19]. In most cases, this has not been fruitful, as in the use of chiral solvents, but some reactions that use chiral ligands do provide good enantiomeric excess values [27].

1.3 Synthesis of α-Hydroxy Ketones

The availability of efficient methods for the construction of enantipure α-hydroxy carbonyl compounds is of considerable interest since this structural array is featured in many biologically relevant molecules [28]. Compounds containing this moiety are indispensable building blocks for the asymmetric synthesis of natural products [28] including antitumor agents, antibiotics, pheromones, and sugars due to their versatile functional groups which can be easily transformed to other functionalities, for examples, diols, halo or amino derivatives, and epoxides that can be seen from the Figure 11 [29].

1.3.1 Chemical Methods

As it is stated previously, chiral α-hydroxy ketones are important building blocks for the asymmetric synthesis of natural products and medicines, consequently, numerous studies have aimed at their stereoselective synthesis [30]. α-Hydroxy ketones are generally prepared by one of the following methods such as α-hydroxylation by treatment of their enolate forms with a molybdenum peroxide reagent in THF-hexane at -70^{o}C [31], α-hydroxylation of silyl enol ethers with *m*-chloroperbenzoic acid [32] that is known as Rubottom reaction, or with certain other oxidizing agents, for

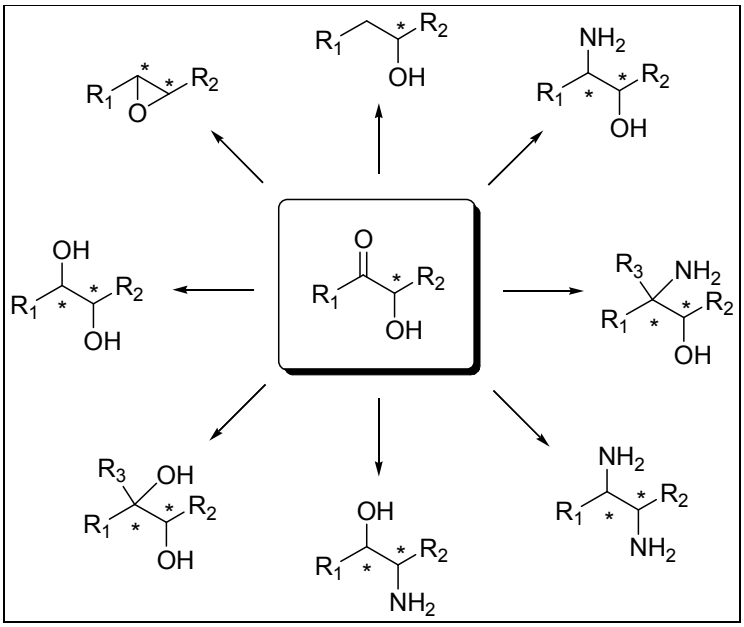


Figure 11 α-Hydroxy ketones as building blocks for several active compounds

example, 2-sulfonyloxaziridine [33]. It is also known that there has been considerable interest in the development of direct methods for the synthesis of α-hydroxy ketones using nontoxic hypervalent iodine reagents. Recently, it was developed a microwave-assisted clean, simple and convenient method for the synthesis of α-hydroxy ketones [34]. The direct formation of acyloins from olefins via *ketohydroxylation* that is a process in which three C-O bonds are formed in one step by using certain oxidizing agents like $KMnO_4$ or osmium and ruthenium salts (OsO_4, $OsCl_3$, RuO_4, $RuCl_3$) seems another efficient alternative [35].

1.3.1.1 Manganese(III) Acetate Mediated Oxidation of Enones

Synthesis of α-hydroxy ketones can be accomplished by another useful method in which manganese(III) acetate ($Mn(OAc)_3$) is utilized as oxidizing agent. It is a versatile reagent and has various type of application in organic chemistry and one of them is that the oxidation of enones **21** yielding α′-acetoxyenones [36]. Once the α′-acetoxyenone **22** has been synthesized, enzyme-mediated kinetic resolution results in

the formation of α′-hydroxy-ketone **23** derivatives of corresponding acetoxyenones (Figure 12). It was reported that many of the chemoenzymatic synthesis of pharmacologically interesting compounds were synthesized in optically pure form, such as (R)-2-hydroxypropiophenones [37], and both enantiomers of 4-hydroxycyclohex-2-ene-1-one [38].

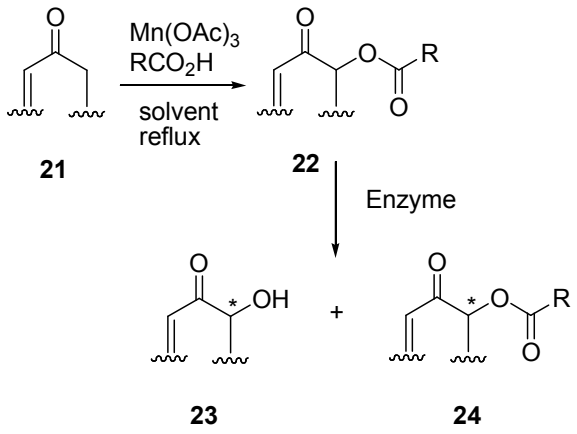


Figure 12 $Mn(OAc)_3$ oxidation followed by enzymatic kinetic resolution yields α′-hydroxy and α′-acetoxy ketones

1.3.2 Biotechnological Methods

The synthesis of chiral α-hydroxy ketones can be performed by using another methodology namely biotechnology. As it was previously stated that chiral α-hydroxy ketones has great synthetic importance in organic and pharmaceutical chemistry, for instance, the synthesis of vitamin E and antifungals [39]. Another well known example is (R)-phenylacetylcarbinol **(26)** (PAC). For many decades, this pre-step for the synthesis of (1R,2S)-ephedrine **(27)** has been obtained by biotransformation of benzaldehyde **(25)** using fermenting yeast [40] as can be seen from Figure 13.

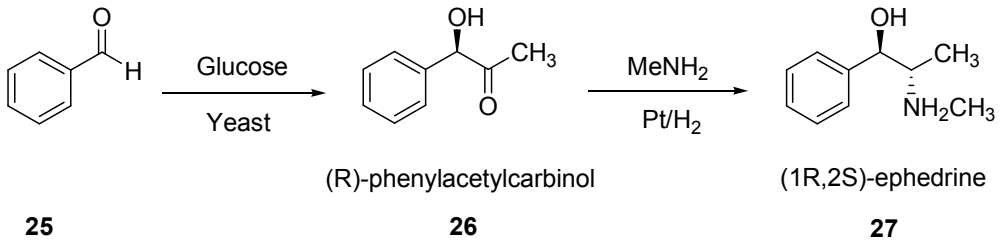


Figure 13 Synthesis of (1R,2S)-ephedrine **(27)**

Chiral α-hydroxy ketones (acyloins) can be obtained by a number of enzymes under relatively mild conditions via a polarity change (umpolung) on the carbonyl carbon in order to form an acylanion equivalent synthon. Several enzymes, such as acetohydroxyacid synthase (AHAS), benzaldehyde lyase (BAL), benzoylformate decarboxylase (BFD), phenylpyruvate decarboxylase (PhPDC), and pyruvate decarboxylase (PDC) catalyze desired reaction. These enzymes all rely on a cofactor, namely thiamine pyrophosphate (TPP) **(28)** (Figure 14). The cofactor thiamine pyrophosphate (TPP) or thiamine diphosphate (TDP), a natural thiazolium salt has three distinctive units, which include a pyrophosphate part, a thiazolium core and pyrimidine unit. It acts by a covalent interaction with the substrate. TPP is mainly engaged in a variety of carbon-carbon bond forming reactions, in which each unit has a special role in enzymatic catalysts [29].

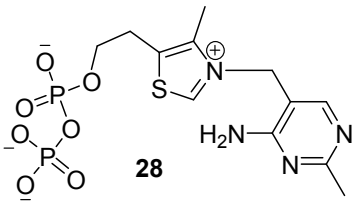


Figure 14 Thiamine pyrophosphate (TPP) structure

1.4 Fungi Mediated Conversions: Fungi as Chemical Reagents

Fungi are eukaryotic organisms, that is, membrane-bound nuclei containing several chromosomes and cytoplasmic organelles. They typically grow as filaments, termed hyphae with apical growth in contrast to many other filamentous organisms like filamentous green algae. Fungi are heterotrophs. In other words, they need preformed organic compounds as energy sources and also as carbon skeletons for cellular synthesis. They have distinctive range of wall components which typically including chitin and glucans. Fungi have haploid nuclei-that is an important difference from almost all other eukaryotes. In addition, they produce spores by both sexual and asexual means [41]. Yeasts, molds, mushrooms are examples of fungi.

Fungi have many traditional roles in biotechnology but also some novel roles, and there is major scope for their future commercial development. For example, fungi are used to produce several traditional foods and beverages including alcoholic drinks and bread. Several metabolites of both primary and secondary metabolites are produced commercially from fungal cultures such as secondary metabolites of penicillins and griseofulvin. In spite of these, it is important to note that fungi have many internal enzymes and enzymic pathways that can be exploited for the bioconversion of many organic compounds [41].

Fungi and their enzymes have been used to functionalize nonactivated carbon atoms, to introduce centers of chirality into optically inactive substrates, and to carry out optical resolutions of racemic mixtures. The types of chemistries catalyzed by microbial fungi catalysts can be outlined as hydrolysis, oxidations, reductions and other additional chemistries (e.g. acyloin condensation, isomerization, decarboxylation, phosphorylation) [42].

Stereo- and regioselective hydrolysis are among the most widely applied fungi-mediated bioconversion reactions. Application of hydrolysis for stereoselective resolutions of many esters, epoxides, nitriles and amides have been covered in many studies [42]. Asymmetric hydrolysis of 1,2-epoxyoctane to 1,2-octanediol was

studied and excellent enantioselectivity for 1,2-epoxyoctane was reported [43]. The enantioselective biohydrolysis of various substituted alkyl-epoxides by using seven different fungi in high enantiomeric purity were described [44]. In another study, the enzymatic kinetic resolution of 2-hydroxymethyl-2,5-dihydrofuran and pyrrole derivatives has been studied. Acetylation of primary alcohols as well as the hydrolysis and alcoholysis of the corresponding acetates were investigated in the presence of different lipases extracted from different kinds of fungus [45].

Fungi-mediated biooxidation reactions have also many of the examples in literature. For instance, baeyer-villiger biooxidation of prochiral 3-substituted cyclobutanones were carried out using the fungus *Cunninghamella echinulata*. β-substituted γ-butyrolactones were obtained in high enantiomeric excess (ee ≥ 98%) [46]. The fungus *Beauveria bassiana* was employed to hydroxylate pyrimidine heterocycles regioselectively. Carbamate was hydroxylated by *B.bassiana* at the C_5 position of the pyrimidine ring [47].

Stereospecific bioreduction reactions have become a key approach for asymmetric synthesis of chiral alcohols in pharmaceutical and other industries, and are among the most widely used microbial reactions in synthetic organic chemistry [42]. Specific examples can be stated as chemoenzymatic synthesis of optically active (R)-(+)-2-methylbutan-1-ol in which the key step involved a reduction catalyzed by baker's yeast was performed [48]. The synthon was used in the synthesis of (R)-10-methyldodecan-1-yl acetate, the chiral methyl-branched pheromone of *Adoxophyes* sp. In spite of this, fungi mediated bioreduction of benzil **(29)** to benzoin **(30**,**32)**, as well as benzoin to hydrobenzoin **(31)** was accomplished (Figure 15) [49].

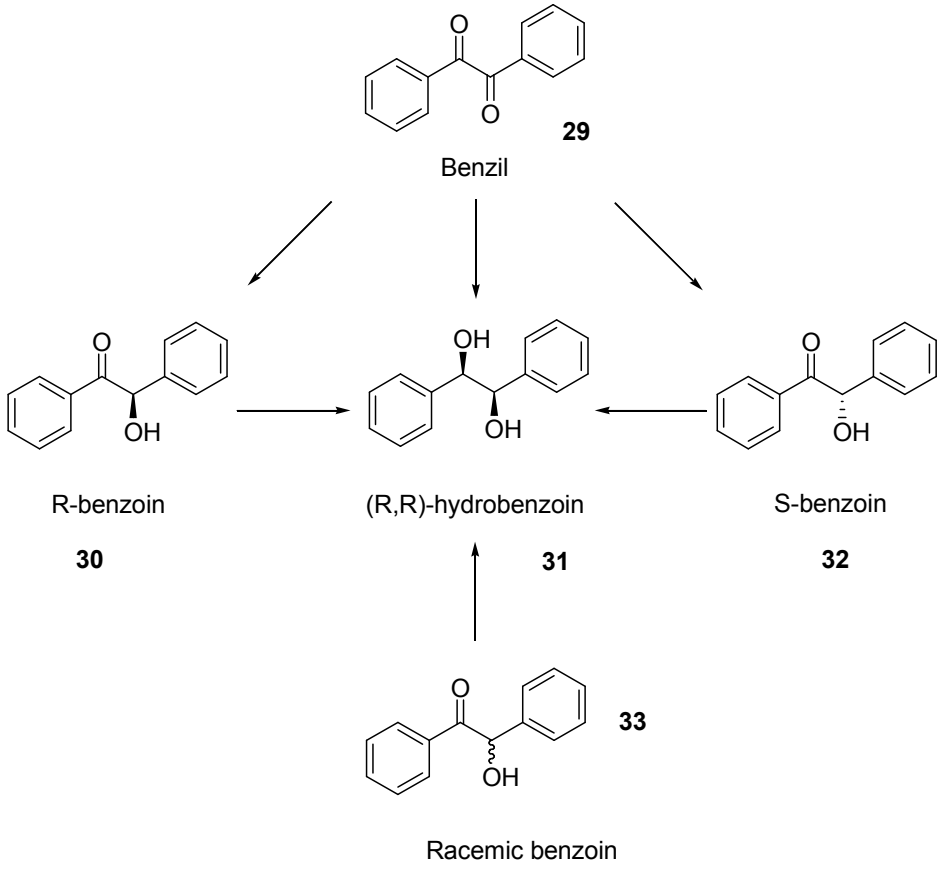


Figure 15 Fungi mediated conversion of benzil to benzoin and hydrobenzoin

Several additional biocatalytic chemistries have been of key importance to products. Asymmetric microbial acyloin condensation was discovered in 1921 and utilized since 1934 in the synthesis of the natural (1R,2S)-ephedrine **(27)**. In this thiamine pyrophosphate **(28)**-mediated process, benzaldehyde **(25)** is added to fermenting yeast and reacts with acetaldehyde, generated from glucose by the biocatalyst, to yield (R)-1-phenyl-1-hydroxy-2-propanone **(26)**. The enzymatically induced chiral center helps in the asymmetric, reductive (chemical) condensation with methylamine to yield (1R,2S)-ephedrine **(27)** (Figure 13). Substituted benzaldehyde derivatives react in the same manner. Similar asymmetric aldol condensations have been developed for the synthesis of unusual sugars for pharmaceutical and materials applications [42].

1.5 Oseltamivir Phosphate (TAMIFLU)

Oseltamivir phosphate **(34)** (Figure 16) is a prodrug which is hydrolyzed hepatically to the active metabolite **(35)**, the free carboxylate of oseltamivir (GS4071). It is a potent inhibitor of influenza A and B neuraminidase commercially developed. It is orally administered for the treatment and prevention of influenza infections [50]. It was developed by Gilead Sciences and is currently marketed by Hoffman-La Roche (Roche) under the trade name Tamiflu®. It is used worldwide and the recent spread of the avian virus H5N1 has prompted governments to stockpile Tamiflu as a precautionary measure against an influenza pandemic. However, the high cost of the drug makes it difficult for developing countries to stockpile Tamiflu [51].

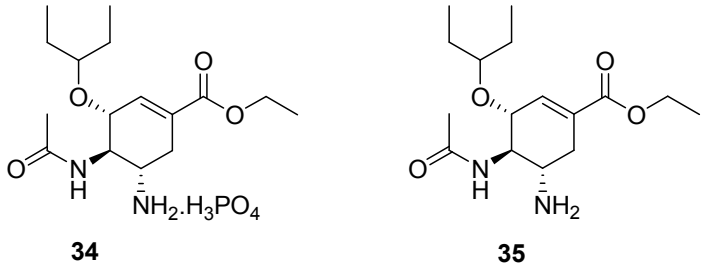


Figure 16 Oseltamivir phosphate **(34)** and its active metabolite **(35)**

Commercial production of oseltamivir starts from the biomolecule shikimic acid **(36)** harvested from Chinese star anise. Large scale production of the drug is limited by the scarcity of this synthetic precursor. It was reported that US government plan to store 300 million doses of Tamiflu, 23 tons of bulk drug substance and that would be required (assuming a yield of 35% from shikimic acid to oseltamivir phosphate and a dose of 75 mg), equivalent to about 840 tons of star anise [52].

The needs for the US alone clearly require an alternative source of shikimic acid. This was partially addressed by Frost and co-workers [53]. They were able to genetically modify *E.coli* to produce shikimic acid by recombinant microbial

biocatalysis. Currently, the fermentation approach supplies approximately 30% of the present requirements [52].

The current manufacturing process is illustrated in Figure 17. The synthesis proceeds through intermediate **37**, which is regioselectively reduced to epoxide **38** [54]. The process involves the use of potentially hazardous azide **39**. Overall yield was mentioned as 27-29%, however the dependence on using azide chemistry in order to convert **38** into oseltamivir phosphate **(34)** was considered a weakness in this first generation manufacturing process [52].

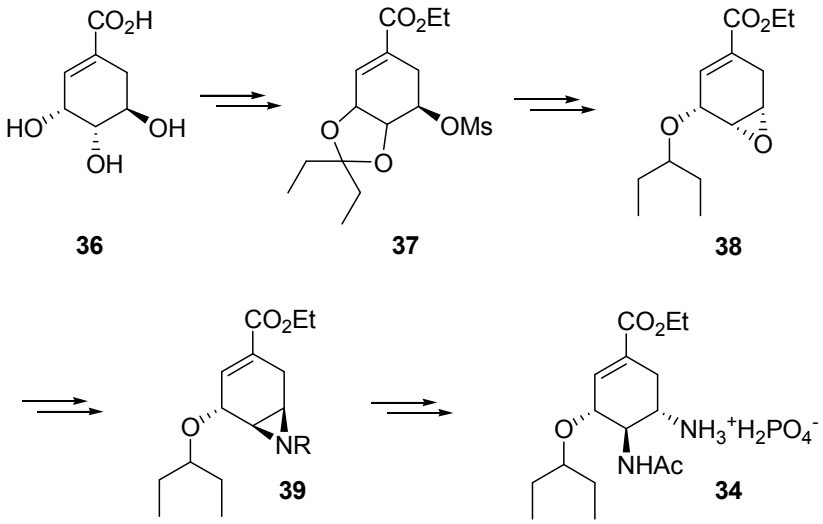


Figure 17 Key intermediates to oseltamivir phosphate from shikimic acid

The reaction sequence described in Figure 18 represents a new, unprecedented azide-free transformation of an **38** into a 1,2-diamino compound **44** avoiding reduction and hydrogenation conditions [55]. Overall yield was increased up to 35-38% compared with previous methodology shown in Figure 17.

The synthesis starts from (-)-shikimic acid. The 3,4-pentylidene acetal mesylate **(37)** is prepared after three steps: esterification with ethanol and thionyl chloride,

ketalization with p-toluenesulfonic acid and 3-pentanone and mesylation with triethylamine and methanesulfonyl chloride. The corresponding epoxide **38** is formed under basic conditions with potassium bicarbonate. The epoxide is opened with allyl amine by using magnesium bromide diethyl etherate as lewis acid to yield the corresponding 1,2-aminoalcohol **42**. The deprotected 1,2-aminoalcohol **43** is obtained by reduction on palladium which is promoted by ethanolamine and after acidic work-up. The aminoalcohol is converted into corresponding allyl-diamine **44** after four steps. Selective acylation with acetic anhydride yields the desired N-acetylated product **45** which is then deallylated in order to get freebase of oseltamivir, which is converted to the desired oseltamivir phosphate **(34)** by treatment with phosphoric acid.

Some of the new approaches for the synthesis of Tamiflu have been reported from the academic chemists. Corey and co-workers reported an interesting approach (Figure 19) employing 1,3-butadiene **(46)** and trifluoroethyl acrylate **(47)** as raw materials [56]. Butadiene reacts in an asymmetric Diels-Alder reaction with trifluoroethyl acrylate catalyzed by oxazaborolidine CBS catalyst **(48)**. This cycloaddition was followed by amidation, iodo-lactamization, and protection to yield **50**, dehydrohalogenation of which followed by allylic bromination yielded **52**. Base-promoted dehydrohalogenation gave **53**, and its regio- and stereoselective bromo-amidation yielded **54**. Sequential dehydrohalogenation gave an intermediate aziridine, which was regioselectively opened with 2-pentanol to furnish **55**. After that, deprotection of **55** produced oseltamivir phosphate **(34)** in 11 chemical steps and about 30% overall yield.

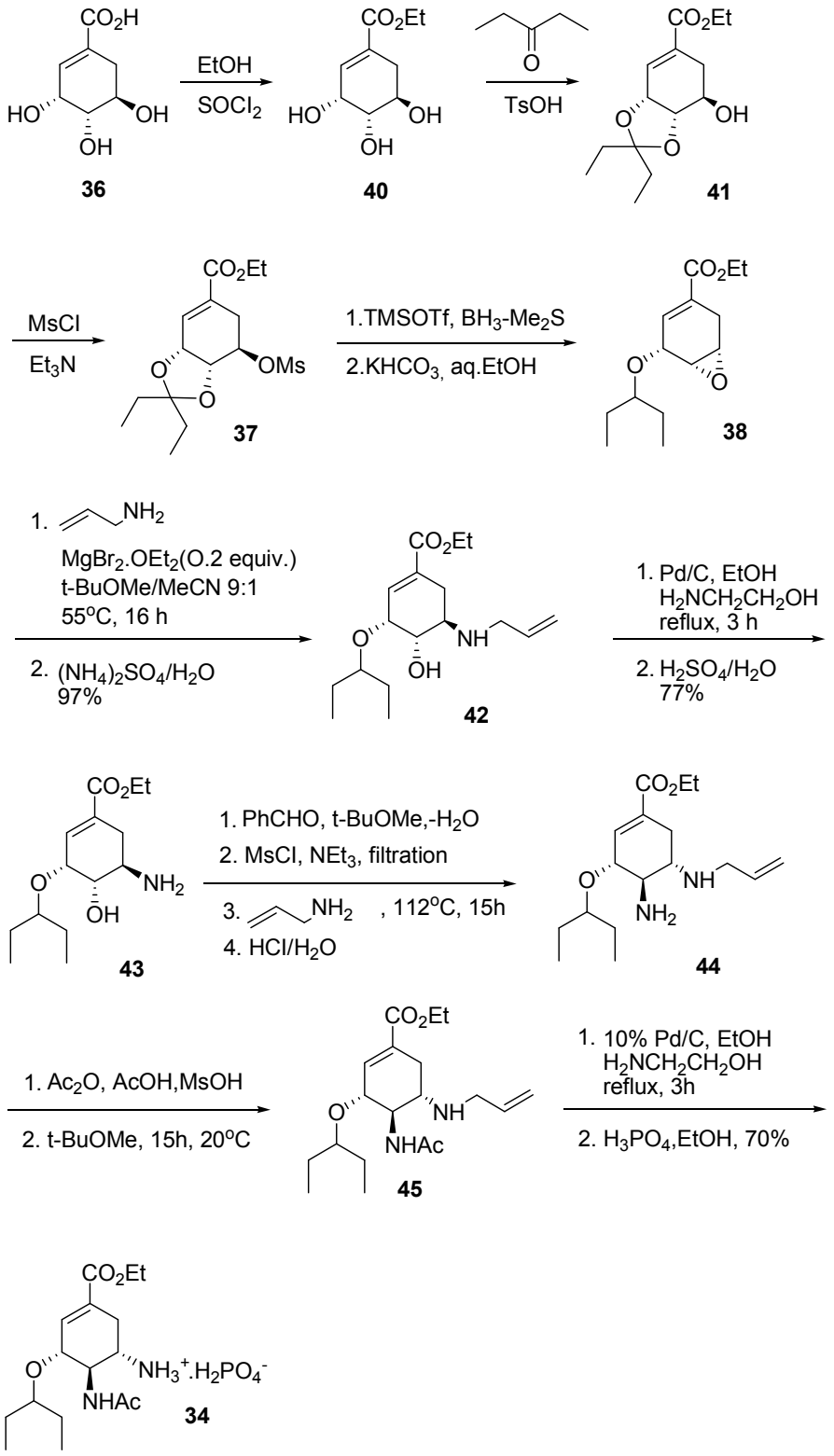


Figure 18 A new azide-free production of oseltamivir starting from shikimic acid

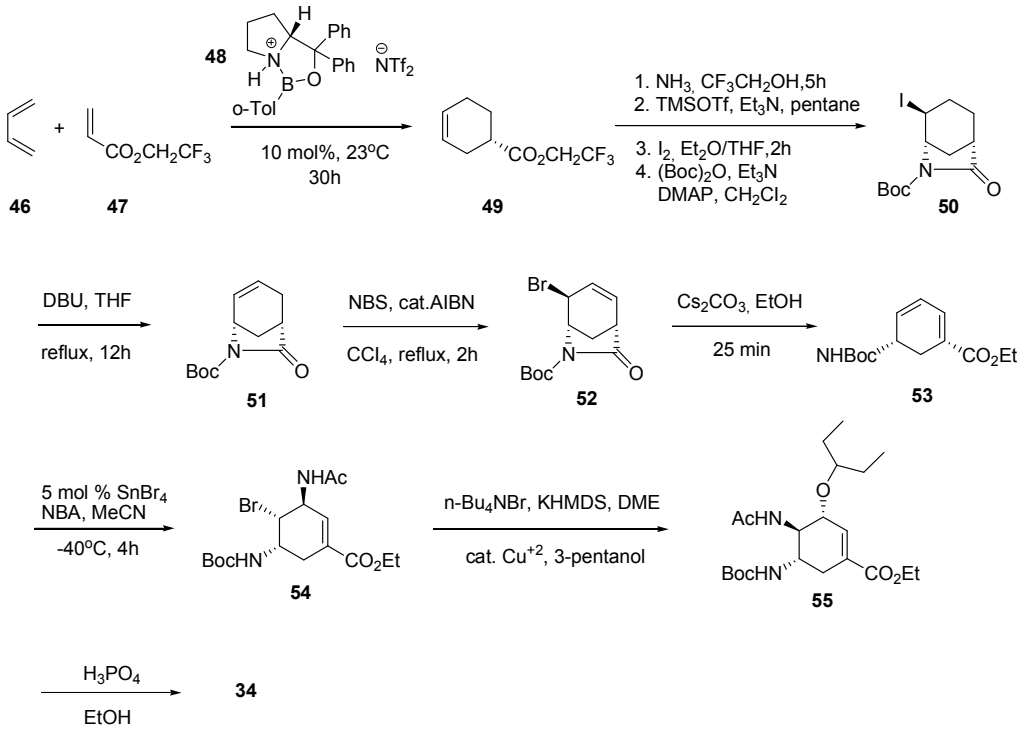


Figure 19 Corey and co-worker's approach

Shibasaki and co-workers also published an enantioselective approach to oseltamivir by using another cheap and widely available raw material, 1,4-cyclohexadiene as illustrated in Figure 20 [57]. Acylaziridine **56** opening is achieved by the use of Y^{III} catalyst and chiral ligand **57** with 91% ee. After some functional manipulations and allylic oxidation in two steps using selenium dioxide and Dess-Martin periodinane yielded **60**. Ni-catalyzed cyanation yielded **61**, which was then stereoselectively reduced to **62**. The crucial ether bond was formed by a Mitsunobu reaction, and then standard required manipulations yielded **34** in 17 steps and overall yield of 1%.

Cong and Yao also reported a synthesis of the active pharmaceutical ingredient by using a cheap and widely available chiral raw material, L-serine [58]. The synthesis is based on a ring-closing metathesis reaction, catalyzed by second-generation Ru carbene species.

Shibasaki and co-workers recently reported a new catalytic asymmetric synthesis of Tamiflu which is mainly based on cyanophosphorylation of enone skeleton and allylic substitution with an oxygen nucleophile [59]. This study was followed by another synthetic route by the same group. In this work, synthesis of Tamiflu was achieved in 12 steps using the Diels-Alder reaction and Curties rearrangement as key steps [60].

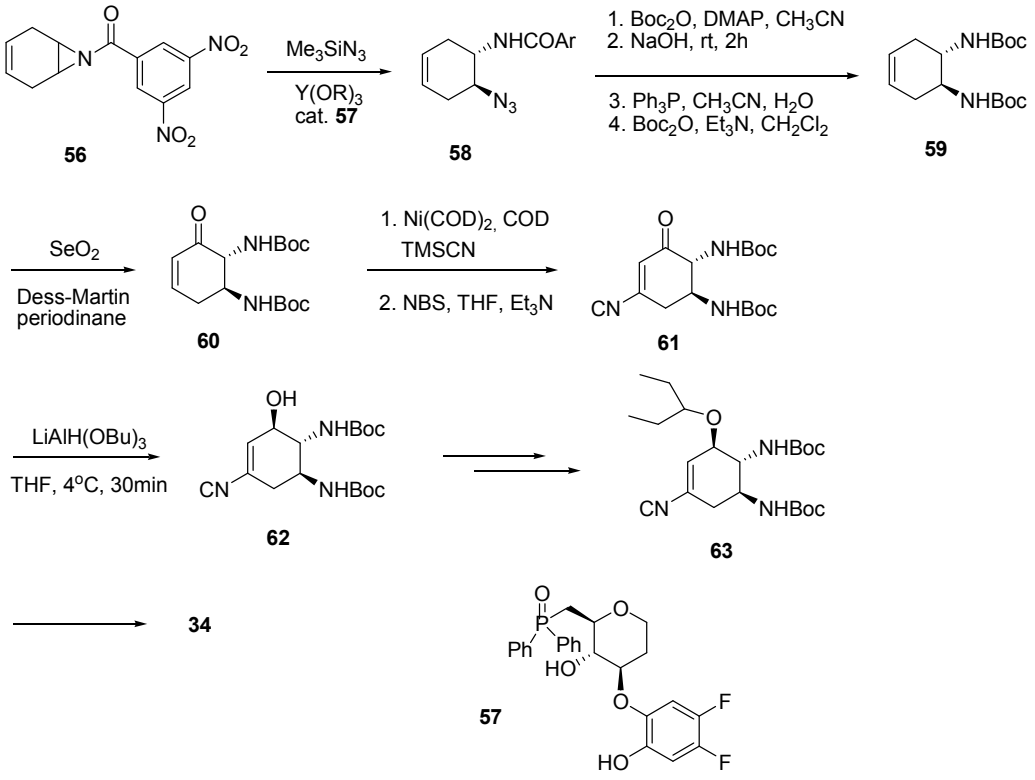


Figure 20 Shibasaki and co-worker's approach

Another interesting study was published by Fang and co-workers [61]. They synthesized the Tamiflu and its phosphonate congener and the guanidine analogues with reasonably high yields (5.2-13.5%).

In spite of these approaches, Fukuyama and co-workers reported recently a new synthetic way to get oseltamivir starting from pyridine and acrolein [51]. The synthesis commences with the asymmetric Diels-Alder reaction like Corey's starts.

1.6 Aim of the Work

The enantioselective chemoenzymatic synthesis of oseltamivir's intermediates was aimed in this study. Influenza remains a major health problem for humans and animals. Oseltamivir is an antiinfluenza drug and a competitive inhibitor of both influenza A and B neuraminidase. It is orally administered for the treatment and prevention of influenza infections [51]. For these reasons, the asymmetric synthesis of oseltamivir's intermediates has a great importance in terms of development of concise methodologies to yield this biologically active molecule. It is therefore required to synthesize firstly the carboethoxy cyclohexenone skeleton **68** that exists in the structure of drug, commencing from cheap and readily available substances. Synthetic sequence is considered to start with cyanohydrin formation from cyclohexanone **(64)** and is followed by acid-catalyzed oxidation and then esterification to yield **66**. Base-catalyzed elimination and allylic oxidation furnish **68**. Next, the chiral center is considered to be generated on the α-carbon of enone structure **68** to attain **69** either by chemical oxidation in which $Mn(OAc)_3$ is used as an oxidant, followed by enzyme-mediated hydrolysis, or by direct microbial biooxidation in which fungi are utilized as biocatalyst (Figure 21).

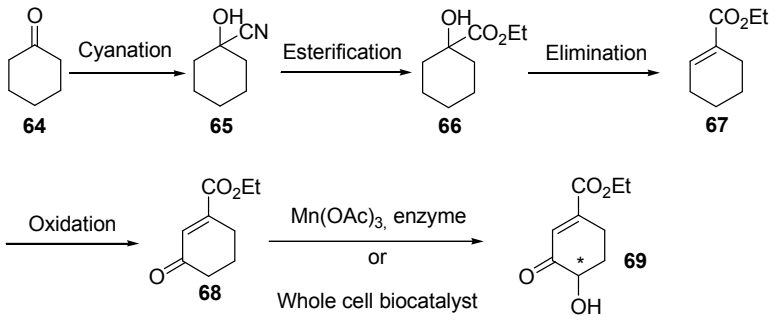


Figure 21 Aim of the work

CHAPTER 2

RESULTS AND DISCUSSION

2.1 Perspective of the Work

Oseltamivir is one of the most promising therapeutics for the treatment of the H5N1 avian influenza virus and is employed in the inhibition of neuraminidase (NA), which is a major surface glycoprotein expressed by both influenza A and B viruses [62], as it was pointed out previously.

The synthesis of novel precursors possessing potential such that they can be utilized in the total synthesis of oseltamivir, carry a great importance in order to design and develop new routes to synthesize oseltamivir. A molecule, ethyl 4-hydroxy-3-oxocyclohex-1-enecarboxylate **(69)**, has been considered a valuable key intermediate that can be employed in the oseltamivir total synthesis and for this reason, a possible synthetic pathway has been figured out in Figure 21 starting from easily available compounds to get this molecule.

The synthesis was started with cyanohydrin formation from cyclohexanone **(64)** to furnish cyclohexanone cyanohydrin **(65)** which was hydrolyzed to carboxylic acid in acidic condition and at high temperature. After esterification in order to get ethyl 1-hydroxycyclohexanecarboxylate **(66)**, base-catalyzed elimination yielded 1-carboethoxycyclohexene **(67)**. Next, allylic oxidation assisted to form 1-carboethoxy cyclohexene-3-one **(68)**. Asymmetric center was generated on the α-carbon of enone **68** by both of the strategies stated earlier namely, manganese (III) acetate-mediated α′-acetoxylation followed by stereoselective enzyme-mediated hydrolysis, and fungi-mediated direct microbial biooxidation.

Manganese(III) acetate ($Mn(OAc)_3$) is a versatile reagent that can be utilized in the oxidation of enone in order to yield α′-acetoxyenone structure [36]. This racemic enone scaffold was then hydrolyzed enantioselectively by lipase type enzymes isolated from different microorganisms in order to get enantioenriched α′-acetoxyenone and α′-hydroxyenone.

Lipase-catalyzed asymmetric access to enantiomerically pure compounds is a rapidly growing field in synthetic organic chemistry [63]. Lipases have proven particularly to be useful for asymmetric synthesis because of their abilities to discriminate between enantiotopic ester and hydroxyl groups. Lipase-catalyzed kinetic resolutions are often practical for the preparation of optically active pharmaceuticals [23].

Fungi-mediated bioconversion reactions have been widely used in recent years as they are easy to handle, environmentally benign and inexpensive. Fungi mediated biooxidation reactions have interesting examples in literature as stated earlier [46,47]. In this study, many of the readily available fungi belonging to *Aspergillus* and *Fusarium* genera have been screened for their ability to catalyze oxidation of carboethoxy enone structure **68** at α′-position to furnish **69**. It was determined that two *Aspergillus* species namely, *A. oryzae*, *A. flavus*, had enzyme system that catalyzed desired reaction which was α′-hydroxylation.

A chemoenzymatic synthetic pathway shown in Figure 22 to attain ethyl 4-hydroxy-3-oxocyclohex-1-enecarboxylate **(69)** have been developed starting from easily available compounds. It is believed that this enantioenriched compound **69** might be a key intermediate in concise alternative methods to synthesize oseltamivir.

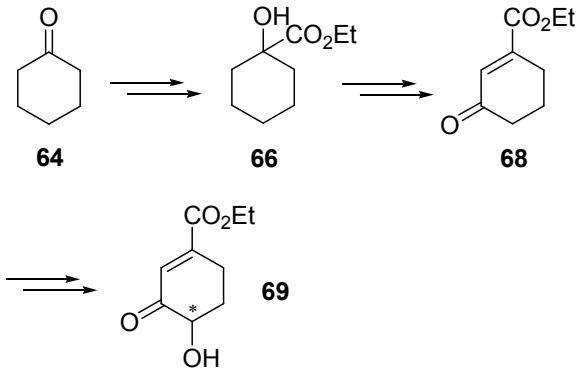


Figure 22 Synthetic route to intermediate **69**

2.2 Retrosynthetic Analysis of Oseltamivir

The structure of oseltamivir actually corresponds to the ethyl ester of the carbocyclic sialic acid analogue that was originally designed as a transition state based neuraminidase inhibitor [64]. It possesses a hydrophobic moiety that forms a strong bond with the interior cleft of neuraminidase and thus inactivating it [65]. In order to get this deserving trisubstituted cyclohexene ethylcarboxylate structure, the retrosynthetic analysis outlined in Figure 23 can be proposed.

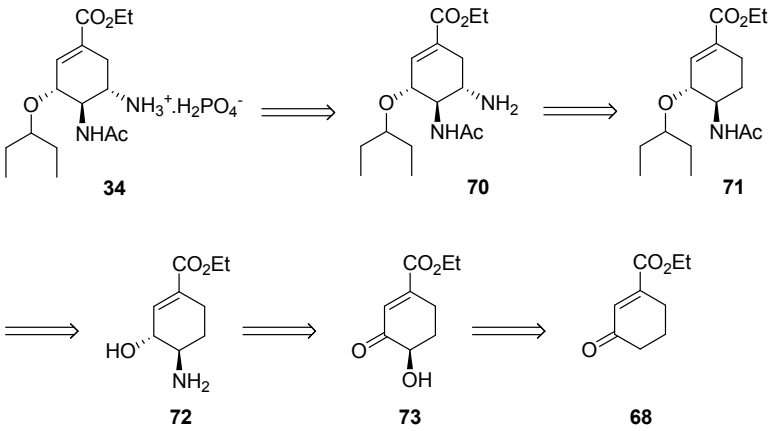


Figure 23 Retrosynthesis of Oseltamivir

The molecule 1-carboethoxycyclohexene-3-one **(68)** seems a potential starting material for the synthesis of **34** after retrosynthetic analysis. This valuable precursor could be converted into α′-hydroxyenone **73** either by $Mn(OAc)_3$ mediated acetoxylation followed by enzyme mediated hydrolysis or direct microbial α′-hydroxylation. The cyclic trans amino alcohol **72** could be derived from **73** by regioselective reduction and amine formation. A molecule **71**, a precursor of **70**, could be constructed from **72** by selective acylation and hydrophobic moiety in **71** could be generated by pentyloxylation. Finally, oseltamivir phosphate **(34)** could be derived from the diamino compound **70** as a salt.

2.3 Synthesis of Enone Structure

The synthesis (Figure 22) of enone structure **68** considered to be the starting point in the route to oseltamivir, have been commenced with the synthesis of cyclohexanone cyanohydrin **(65)** starting with cyclohexanone **(64)** as can be seen from the Figure 24.

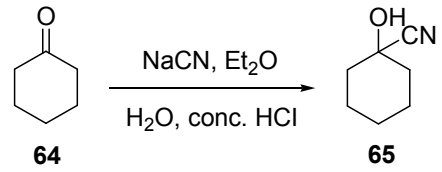


Figure 24 Synthesis of cyclohexanone cyanohydrin

In a three-necked round bottom flask cyclohexanone, diethylether and water were placed and then sodium cyanide was added all at once with vigorous stirring. The flask was surrounded by an ice-salt bath. When most of the sodium cyanide was dissolved and the temperature of the mixture was fallen to 5^oC, concentrated hydrochloric acid was added from the dropping funnel at such a rate that the temperature remained between 5^oC and 10^oC. It is important to keep the temperature

of mixture in this range since this acid-catalyzed cyanide addition is an exothermic reaction. After all the acid has been added, the cooling bath was removed and the stirring was continued for 2 hours. Then, the mixture was allowed to settle. Organic layer was separated and the aqueous layer was washed with an additional amount of water to dissolve the salts. Aqueous solution was extracted four times with 50mL portions of ether that was combined with original ether layer. Ether was then evaporated and cyanohydrin product was attained with 88% yield as colourless oil. It should be noted that the mentioned preparation was conducted in a hood to avoid exposure to the poisonous hydrogen cyanide that was evolved.

The synthesis of cyclohexanol-1-ethylcarboxylate **(66)** was considered to be accomplished by using ethanolic concentrated HCl solution (20% v/v) under reflux. However, this was not the case. For this reason, another methodology was conceived. It was decided that cyanohydrin could be hydrolyzed to corresponding carboxylic acid **74** in acidic conditions and at high temperature (Figure 25), then **74** could be esterified to the desired cyclohexanol-1-ethylcarboxylate **(66)** as illustrated in Figure 26.

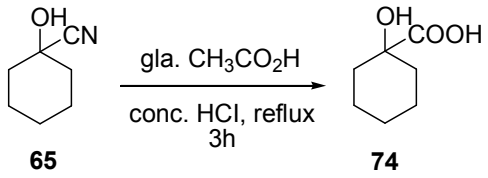


Figure 25 Synthesis of 1-hydroxycyclohexanecarboxylic acid

The cyclohexanone cyanohydrin **(65)** was refluxed with glacial acetic acid and concentrated HCl for 3 hours. The solution was then concentrated in vacuo, the residue dissolved in chloroform, washed with water and purified by means of the sodium salt using NaOH solution. Solution was then acidified by concentrated HCl.

Chloroform extraction and then evaporation of chloroform gave white granules of the desired product **74** with an isolated yield of 60%.

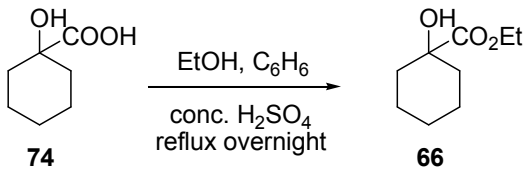


Figure 26 Synthesis of cyclohexanol-1-ethylcarboxylate

In order to obtain **66**, catalytic amount of concentrated H_2SO_4 was added to a stirred solution of **74** and ethanol in benzene and the mixture was refluxed overnight. After that, the solvent was removed in vacuo and the residue dissolved in ether. The unchanged acid was filtered off, the solution was washed with sodium hydrogencarbonate solution to eliminate any unreacted acid residue. After required separation and purification the **66** was gathered with 65% yield.

It was decided that 1-carboethoxycyclohexene **(67)** can be synthesized from previously obtained **66** by first chlorination with thionyl chloride ($SOCl_2$) and then elimination catalyzed by pyridine as a weak base as shown in Figure 27. It should be noted that care must be taken since the temperature was rised rapidly while addition of $SOCl_2$ to the reaction mixture and some hazardous gases SO_2 and HCl might be evolved. After workup, the desired product **67** was obtained in 86% yield. It was also considered to do this elimination in acidic media that concentrated H_2SO_4 and 85% H_3PO_4 were used separately, however, in these cases the product yields were very low and many of the byproducts were observed.

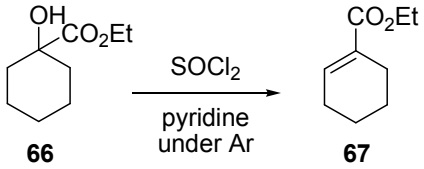


Figure 27 Synthesis of 1-carboethoxycyclohexene

The oxidation of olefin **67** to an α,β-unsaturated ketone **68** has been accomplished by utilizing chromium trioxide (CrO_3) as an oxidant in acetic acid (AcOH). The procedure [66] published before was applied. While the addition of CrO_3 to reaction mixture in which olefin was dissolved in AcOH and small amount of water, the temperature started to increase so it was maintained at 40^{o}C by external cooling. The isolated product **68** was obtained in 57% yield. The reaction pattern can be summarized in Figure 28 as follows.

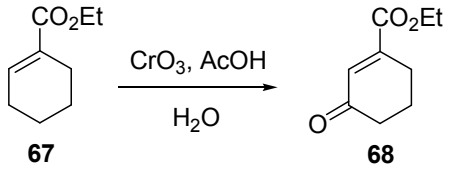


Figure 28 Synthesis of 1-carboethoxy-cyclohexene-3-one

2.4 Oxidation of Enone by Chemoenzymatic Method

Selective α′-acetoxylation of α,β-unsaturated ketones enables key precursors for pharmaceutically important compounds and useful chiral ligands [67]. Many of the successful α′-acetoxylation in which manganese (III) acetate have been utilized as an oxidant, of a great variety of substrates have been reported in literature and today, $Mn(OAc)_3$ mediated acetoxylation is one of the most useful methods for the synthesis of α′-acetoxy α,β-unsaturated ketones [68].

It is clear that the enone structure **68** previously synthesized could be transformed into corresponding α′-acetoxy enone by the use of $Mn(OAc)_3$. In order to do that first $Mn(OAc)_3$ was synthesized starting from commercially available $Mn(OAc)_2.4H_2O$. Powdered $Mn(OAc)_2.4H_2O$ was added to glacial acetic acid and waited for 1 hour for stirring. To well stirred mixture, $KMnO_4$ powder was added in small portions and waited for 1-2 hours. The mixture was allowed to cool room temperature and small amount of water was added. After 24 hours precipitate was collected on a glass filter by washing with glacial acetic acid. Then, it was dried over P_2O_5 under high vacuum to remove water. Finally, $Mn(OAc)_3$ was obtained as a dark brown colored crystall.

It should be noted that there have been some problems associated with the use of $Mn(OAc)_3$ due to inconsistent results in literature, and unclear synthetic and mechanistic aspects of $Mn(OAc)_3$ mediated oxidation of enones. However, Demir and co-workers [68] solved these misunderstandings. In their report, the procedure for direct acetoxylation of enones has been improved by the use of acetic acid as a co-solvent from a synthetic point of view and by use of as low as 1.25 equiv. $Mn(OAc)_3$ which adds to economy of the process. The reaction was carried out in a AcOH-benzene (1:10) mixture. For these reasons, based on a procedure reported by Demir and co-workers [68] 4-acetoxy-1-carboethoxy-cyclohexene-3-one **(***rac*-**75)** has been synthesized as a racemate that is illustrated in Figure 29.

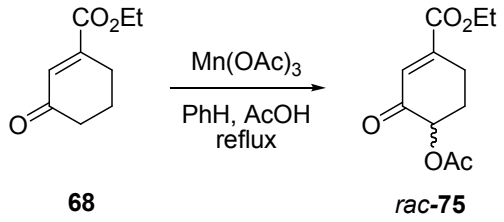


Figure 29 Synthesis of 4-acetoxy-1-carboethoxy-cyclohexene-3-one

The role of acetic acid could be related to an increased solubility of $Mn(OAc)_3$ in the reaction mixture however, acetic anhydride could be also used instead of acetic acid. Although benzene was employed as a solvent, cyclohexane and MeCN could be utilized for the presented reaction in Figure 29 [68].

It was waited for a time at which no further change was observed in reaction mixture therefore the reaction was terminated after 17 hours reflux. The crude products were purified by flash column chromatography using EtOAc-hexane (1:5) as eluent. 4-acetoxy-1-carboethoxy-cyclohexene-3-one **(***rac*-**75)** was obtained with 98% yield and no other products were observed. That seems a very efficient reaction in terms of yield and selectivity.

The mechanism for the oxidation of enones to α′-acetoxyenones was not fully described in literature. Several mechanisms were proposed for this oxidation. One of them declared that α′-acetoxyenones could be generated by the formation of a metal enolate followed by acetate transfer as can be seen from the Figure 30 (path a), analogous to the lead(IV) acetate oxidation [69]. However, since the oxidation of carbonyl compounds with $Mn(OAc)_3$ was reported to involve an α-oxo radical resulting from the oxidation of an enol or enolate anion by $Mn(OAc)_3$ [70], it is viable that this reaction also proceeds *via* the formation of an α-oxo radical followed by ligand transfer (Figure 30 (path b)) to yield the corresponding acetoxy enone.

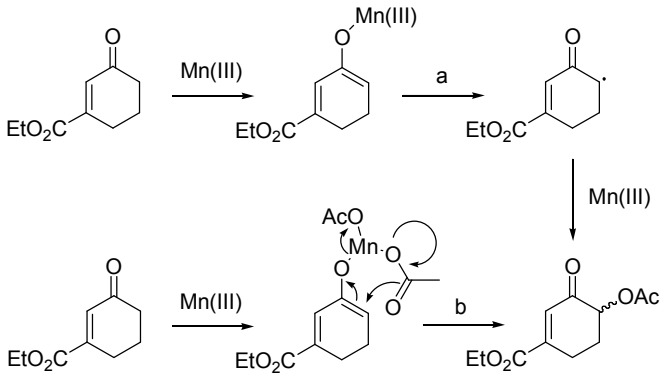


Figure 30 Proposed mechanisms for selective α'-acetoxylation

The structure of *rac*-**75** has been confirmed by NMR and GC-MS analysis. ^{1}H NMR spectrum (Figure 48) of the corresponding compound showed that singlet at 6.7 ppm results from olefinic proton. Doublet of doublet at 5.25 shows a proton that is attached to α-carbon (CH) and coupled with two different protons at β-position. Quartet at 4.21 ppm is due to the two equal methylene (CH_2) protons in ethyl ester moiety. Multiplets at 2.83 ppm, 2.61 ppm, and 2.25 ppm are due to the two methylene γ-protons and one methylene β-proton, respectively. Singlet at 2.1 ppm proves the presence of three methyl (CH_3) protons of the acetoxy group. Additionally, a signal due to one of the β-proton splitted as multiplet at 2.05 ppm overlaps the singlet at 2.1 ppm. Triplet at 1.3 ppm indicates the three methyl protons in the ethyl ester group.

In addition to that, ^{13}C NMR spectrum (Figure 49) illustrated that there is a carbonyl carbon peak at 194 ppm. The peaks at 169 and 165 ppm illustrate the presence of other carbonyl carbons attached to oxygens. Olefinic carbons give signals at 148 and 131 ppm. The peak at 73 ppm is due to the tertiary methine carbon. The carbon attached to oxygen in ester moiety gives a signal at 61 ppm and the rest of the four peaks between 27 and 14 ppm support the presence of sp^3 hybridized saturated four carbon atoms.

Furthermore, GC-MS analysis revealed that molecular ion peak (M^+) is observed at mass to charge ratio (m/z) of 226 which is the molecular weight of desired acetoxy enone molecule *rac*-**75**. Base peak is at m/z 43 shows the presence of acetyl group and removal of this side group from molecule predominates the other peaks. Other related peaks and their relative abundances are possessed in Figure 50. These characterization procedures revealed the fact that the synthesis of the desired acetoxy enone *rac*-**75** has been accomplished.

It is required to state that lipases have enormous potential in chemical synthesis because of several reasons: (1) They are stable in organic environment; (2) they possess broad substrate specificity; (3) they exhibit high regio- and enantioselectivity. A number of lipases have been isolated from fungi and bacteria

and characterized [71]. Lipases are used to prepare enantiomerically pure esters, and alcohols. The study presented in here constitutes a good example for the aforementioned statement such that several lipase type enzymes were screened for enantioselective hydrolysis of acetoxy enone *rac*-**75** in order to yield enantioenriched acetoxy and hydroxy enone **69**.

In a typical experiment shown in Figure 31, for enzymatic hydrolysis, the racemic acetoxy enone **75** was dissolved in DMSO and then phosphate buffer (pH: 6.9) was added. The mixture was then stirred at room temperature in the presence of enzyme. The reaction was monitored by TLC. As approximately 50% conversion was attained, the reactions were terminated. The crude product was separated by preparative TLC to afford enantioenriched acetoxy enone **75** and hydroxy enone **69**.

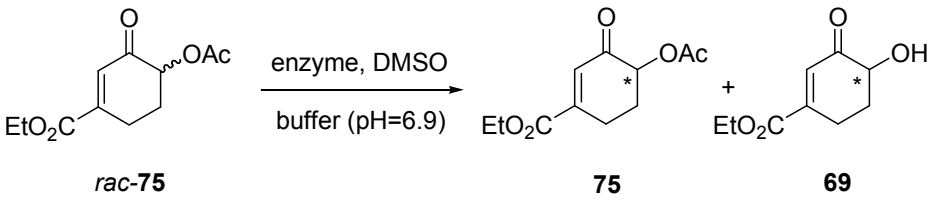


Figure 31 Enzyme-mediated hydrolysis of acetoxy enone *rac*-**75**

The structure of hydroxy enone compound **69** has been determined by the use of previously stated techniques; NMR and GC-MS. ^{1}H NMR spectrum (Figure 51) of corresponding hydroxy enone showed the fact that singlet at 6.7 ppm results from olefinic proton at α-position. Quartet at 4.2 ppm is resulted from two methylene protons attached to ester group and they were coupled with saturated methyl protons of ethyl ester moiety. Doublet of doublet at 4.1 ppm is due to the proton attached to methine carbon which is adjacent to carbonyl and hydroxy groups and it appears that it is coupled with two different protons at β-position. Proton of hydroxy group can be seen at 3.45 ppm as a singlet. Multiplet at 2.8 ppm is due to one of the γ-proton adjacent to olefin. Another methylene proton adjacent to double bond give multiplet

at 2.55 ppm. Multiplet at 2.4 ppm is because of one of the methylene proton at β-position that is splitted by one geminal proton, one methine proton at α-position and two unequal vicinal protons adjacent to double bond. The presence of other methylene proton can be figured out as doublet (due to one geminal and two vicinal protons) of quartet (due to vicinal proton at α-position) at 1.8 ppm. Triplet at 1.3 ppm proves the presence of the three methyl protons coupled with two methylene protons attached to oxygen in the ethyl ester group.

^{13}C NMR spectrum (Figure 52) of the corresponding compound illustrated that there have been nine different carbon atoms such that the signal at 200 ppm is due to the carbonyl carbon adjacent to double bond at α-position. The other carbonyl carbon adjacent to both double bond and oxygen in ester moiety gives a signal at 165 ppm. The sp^2 hybridized tertiary carbon at α-position gives a signal 150 ppm and the presence of the other sp^2 hybridized quaternary carbon can be realized by a signal at 130 ppm. The peak at 73 ppm is due to the sp^3 hybridized carbon at α-position. Methylene carbon (CH_2) adjacent to oxygen gives a signal at 62 ppm whereas the presence of saturated methylene carbon at β-position can be seen as a signal at 31 ppm. Another sp^3 hybridized carbon adjacent to double bond gives a signal at 25 ppm and a signal at 14 ppm is because of methyl carbon in ester group.

Additionally, it is required to mention the GC-MS analysis such that molecular ion (M^+) peak can be realized at m/z 184. Base peak in the spectrum can be seen as a m/z 112 that might be generated by the removal of ester moiety from the molecule and that seems possible since the resulting enone can be rearranged and stabilized itself as enol. Other related peaks and their relative abundances are demonstrated in Figure 53. These informations stated above possess that the hydroxy enone structure **69** has been attained.

Once the kinetic resolution of acetoxy enone to hydroxy enone have been accomplished, it was required to determine the enantiomeric excess values of acetoxy and hydroxyl enones. They were assigned by HPLC using chiral column

(Chiralpak AS-H column, UV detection at 254 nm, eluent hexane/2-propanol = 85/15, flow 0.7 mL/min 20^{o}C, using racemic compounds as references). Racemic hydroxy enone **69** synthesized from racemic acetoxy enone **75** with K_2CO_3/MeOH according to previously reported procedure [72]. The HPLC chromatogram of racemic acetoxy enone **75** is demonstrated in Figure 32.

The retention times for two enantiomers of acetoxy enone molecule were recorded as 15.4 and 18.0 min., respectively, as can be seen from the Figure 32. In addition to that information, it is needed to note that the retention times for two enantiomers of corresponding hydroxy enone were determined as 18.7 amd 23.9 min. as illustrated in Figure 33.

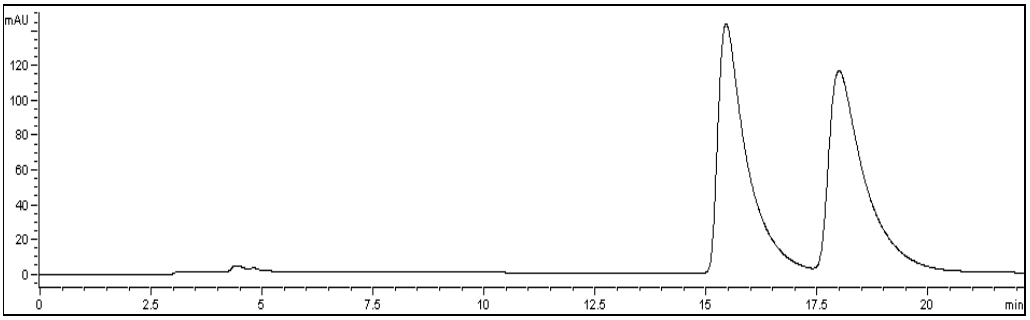


Figure 32 HPLC chromatogram of racemic acetoxy enone **75**

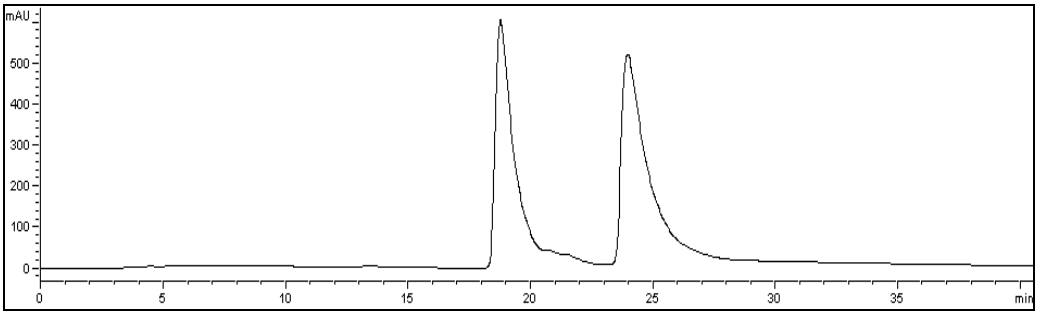


Figure 33 HPLC chromatogram of racemic hydroxy enone **69**

It has been employed a series of lipases for screening the enantioselective hydrolysis of racemic acetoxy enone **75** as it was stated before. Table 1 exhibits results of this enzyme-mediated hydrolysis of *rac*-**75**.

The results tabulated in Table 1, showed that CCL (see Figure 34 and Figure 35 for acetoxy and hydroxy enones, respectively), Lipase OF, and Lipase MY30 furnished the best results for both enantioenriched acetoxy (88 – 94% ee) and hydroxy enones (88 – 92% ee). The other results also seem reasonably good for acetoxy enone (95% ee) and hydroxy enone (91 – 97% ee) separately. Sih and co-workers [73] introduced a parameter describing the selectivity of enzymatic kinetic resolution as the dimensionless *Enantiomeric Ratio* (E) which remains constant through out the reaction and is only determined by the environment of the system. It can be seen from the Table 1, unacceptable selectivity results (E=8-11) have been attained for practical purposes in addition to the good selectivities (E=32-95) that have been achieved with the lipases tested for the enzymatic resolution.

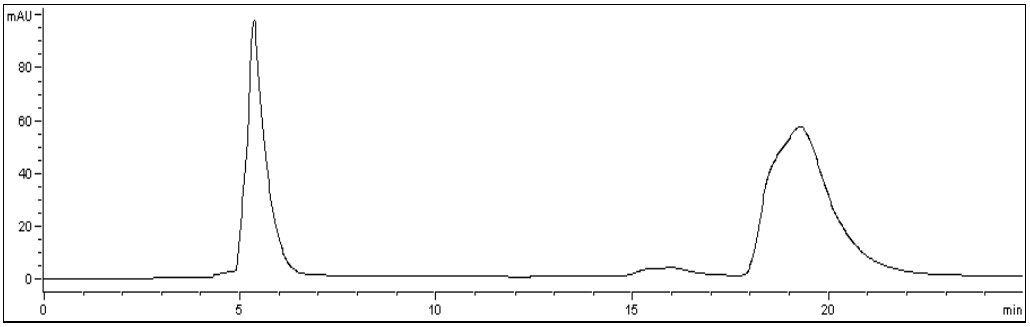


Figure 34 HPLC chromatogram of acetoxy enone **75** hydrolyzed by CCL

Table 1 Enzymatic hydrolysis of 4-acetoxy-1-carboethoxy-cyclohexene-3-one **(75)**

Entry	Enzyme	Time (h)	Conversion (%)[a]	Acetate (ee%)	Alcohol (ee%)	E[b]
1	CCL[d]	24	50	92	92	8
2	Lipase OF[c]	3	52	94	88	55
3	Lipase MY30[c]	17	49	88	90	55
4	Lipase QLM[c]	3	63	94	55	11
5	Lipase SL[c]	3	68	95	45	9
6	PRL[d]	74	32	43	91	32
7	HPL[d]	74	28	38	97	95
8	MML[d]	97	32	44	93	43

[a] Conversion (c) = $ee_s/(ee_s + ee_p)$

[b] See Ref. 73,

Enantiomeric ratio (E) = $\ln[(1- ee_s)/(1+ ee_s/ ee_p)] / \ln[(1+ ee_s)/(1+ ee_s/ ee_p)]$

[c] MEITO SANGYO Co., Ltd., Tokyo, Japan

[d] Commercially available lipases

CCL (*Candida cylindracea* lipase)

PRL (*Penicillium roqueforti* lipase)

HPL (*Hog pancreas* lipase)

MML (*Mucor javanicus* lipase)

The absolute configurations of the enantioenriched acetoxy and hydroxy enones were not determined. However, it is known that they can be assigned with the help of X-ray analysis or 2D-NMR. Additionally, their absolute configurations can be determined in such a way that they are converted to compounds whose absolute configurations are known.

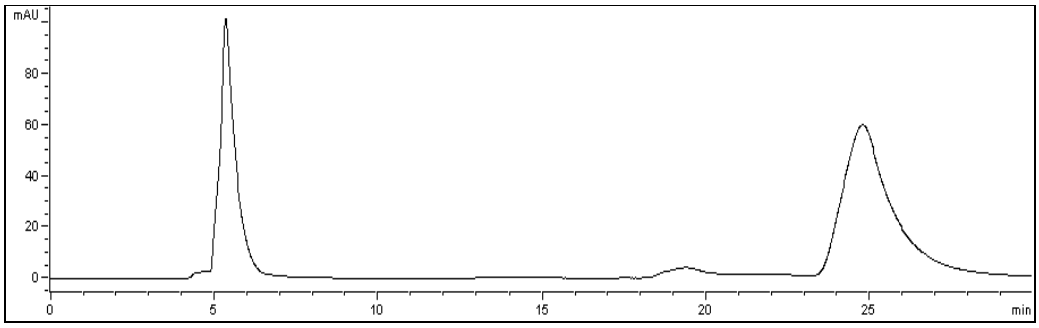


Figure 35 HPLC chromatogram of hydroxy enone **69** yielded by CCL

2.5 Microbial Direct Oxidation Reactions

The addition of molecular oxygen to organic molecules using biological systems is an important tool in organic chemistry. Microbial hydroxylation is an established technique for the syntheses of steroids and alkaloids, and has been used to prepare derivatives of many complex natural products including ascomycin, artemether, paclitaxel and quinidine [74]. In the light of these informations, it was considered that direct oxidation of previously synthesized enone **68** can be accomplished utilizing microorganisms as biocatalysts, which would be a shortcut route when compared with the Manganase(III) acetate mediated acetoxylation coupled with enzymatic hydrolysis.

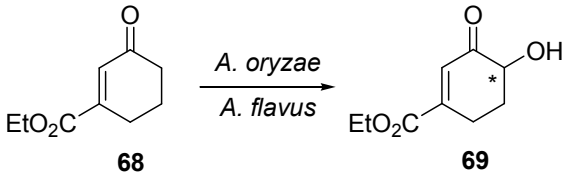
Seven different fungi selected from *Aspergillus* and *Fusarium* species were targeted in order to find out their hydroxylation potential. Strains of *A. niger* 200910, *A. oryzae* OUT5048, *A. flavus* 200120, *F. roseum* OUT4019, *F. solani* OUT4021, *F. anguioides* OUT4017, *F. bulbigenum* OUT4115 were utilized. *A. niger* 200910 and *A. flavus* 200120 were obtained from TÜBİTAK MAM while other microorganisms used in this study were generous gifts from Osaka University Culture Collection.

As a general procedure, screening and time-course experiments were performed in conical Erlenmeyer flasks (250mL) containing 100mL growth medium which was Malt/Glucose/Yeast/Peptone (MGYP): 3g/L malt; 10g/L glucose; 3g/L yeast extract;

5g/L peptone. Before each experiment, cultures of microorganisms were aseptically transferred to conical Erlenmeyer flasks and they were kept on a rotary shaker (160 rpm) at 25^{o}C for 4 days for *Fusarium* species (average 5mg of dry cell weight) and at 37^{o}C for 2 days for *Aspergillus* species to acquire biomass. For *Aspergillus* species, the mycelium from 100mL of cultivation medium (average 20g of wet cell weight) was filtered off, and then resuspended in 100mL of the freshly prepared growth medium. After that, 1mL of mixture (substrate with a concentration of 20mg/mL in DMSO) without prior sterilization, was added to the growth media. It was sufficient to use solvent (DMSO) to dissolve the substrate since it was not soluble in growth medium. At the desired time intervals, that is, in each 2 hours after substrate addition, the reactions were monitored by using 2mL portions of the substrate mixture which were extracted with 0.5mL EtOAc. The reactions were monitored by TLC and they were terminated when no further product formation was observed in the reaction medium by addition of 50mL EtOAc to each mediums. Reaction mediums were extracted three times with 50mL portions of EtOAc. Combined organic phases were dried over $MgSO_4$ and solvent was removed by evaporation. The crude products were separated by flash column chromatography to afford the desired hydroxy enone product **69**.

It should be noted that enzyme systems of the fungi namely; *A. oryzae* OUT5048, and *A. flavus* 200120 catalyzed the enantioselective hydroxylation of 1-carboethoxy-cyclohexene-3-one **(68)** to yield 4-hydroxy-1-carboethoxy-cyclohexene-3-one **(69)** as shown in Figure 36. Other microorganisms, *A. niger* 200910, *F. roseum* OUT4019, *F. solani* OUT4021, *F. anguioides* OUT4017, and *F. bulbigenum* OUT4115 failed to mediate this transformation.

Control experiments were carried out without microorganisms (*A. oryzae* OUT5048, and *A. flavus* 200120) to verify the stability of the substrate or just with the microorganism (i.e., without substrate) to verify the normal products of microbial metabolism. These control experiments were performed with the same way stated for standard procedure above and no oxidation products could be observed in control flasks.



68 **69**

Figure 36 Whole-cell mediated hydroxylation

The hydroxy enone was not the only product attained. Two more byproducts which were not characterized, were observed. It is important to note that special care must have been given to separate the desired product from byproducts and unreacted substrate. EtOAc-hexane (1:3) solvent system has been utilized as eluent in flash column chromatography.

Formation of the desired product; hydroxy enone **69,** attained by microbial hydroxylation was first controlled by TLC, by comparing with previously synthesized hydroxy enone *via* $Mn(OAc)_3$ acetoxylation followed by enzyme-mediated hydrolysis. And it was realized that they were the same products. Identification of the product has been progressed with ^{1}H-NMR analysis and corresponding spectrum revealed the synthesis of the desired hydroxy enone product obtained by microbial hydroxylation by both of the microorganisms, *A. oryzae* OUT5048, and *A. flavus* 200120.

Enantiomeric excess (ee) values of the produced hydroxy enone **69** were determined by chiral HPLC analysis. Chiralpak AS-H column eluted with hexane/2-propanol (85/15) and 0.7 mL/min flow rate, has been utilized. UV detector at 254 nm was used to monitor the relative abundances of enantiomers. The results of HPLC analysis are illustrated in Table 2 including yields of the reactions.

The results possess the fact that fungi-mediated direct hydroxylation of 1-carboethoxy-cyclohexene-3-one **(68)** at α´-position has been accomplished. Reaction times and enantiomeric excess values (>99-97%ee) for both of the fungi are very satisfactory whereas isolated yields of the desired product are needed to be improved.

The yields of the reaction for the direct microbial hydroxylation can be further improved by optimization of the reaction conditions. As these are whole-cell mediated reactions, selection of the appropriate growth medium would be benefical for both increasing the amount of biomass thus increasing the rate of the reaction and for changing the metabolism of the microorganism giving rise to increase in the production of the enzyme or enzyme systems for the desired reaction.

Another important parameter for an increased yield is pH of the medium. pH is a detrimental factor for enzymatic activity. Medium pH can be adjusted so that the desired enzyme or enzyme systems operate at maximum rate.

Physiological state of the microorganism is also thought to be effective. Growing cell, wet cell (also noted as stationary cell), crude cell (prepared by cracking of the cellular biomass) would show different yields. This is expected since the cells at these physiological state would differ in the metabolism. The use of growing cell or wet cell, for example, for the substrates which have mass limitations, i.e., substrate can not be transported into intracellular medium of the cell (in the case of reactions catalyzed by intracellular enzymes), will be unwanted whereas the use of crude cell preparations will be preferred in these cases. In addition, no studies were performed to clarify whether the results depended on the age of the culture or not.

It is known that the main parameter for carrying out oxido-reduction reactions should be the aeration conditions. Oxidation should require aerobic conditions in order to allow the respiratory chain to provide oxidized NAD^+ [75]. Therefore, oxidation reactions were performed in Erlenmeyer flasks under an air atmosphere.

The fungi are relatively fast growing and more tolerant to high concentrations of substrate. That makes possible the direct addition of substrate which was previously dissolved in a solvent dimethylsulfoxide (DMSO), to the reaction media. DMSO is a common solvent used in organic chemistry and enables homogeneous condition for the reaction to proceed because of its high miscibility in both aqueous and organic phases. In addition to that, it is harmless to microorganisms used as biocatalysts.

Table 2 Microbial hydroxylation of 1-carboethoxy-cyclohexene-3-one **(68)** by fungi *A. oryzae* OUT5048 and *A. flavus* 200120

Entry	Microorganism	Time(h)	Yield(%)[a]	ee(%)
1	*A. oryzae* OUT5048[b]	24	31	>99
2	*A. flavus* 200120[c]	24	17	97

[a] Isolated yield after column chromatography eluted with EtOAc/hexane (1:3)

[b] Osaka University Culture Collection, Japan

[c] Tübitak MAM Culture Collection

It is evident that the reaction times are very applicable compared to other fungi-mediated biotransformation reactions in literature. The rapid decrease in the concentration of enone **68** or rapid conversion during *A. oryzae* OUT5048 and *A. flavus* 200120 cultivation can be explained not only by metabolic conversion, but also by adsorption onto (or absorption into) the fungal mycelium. Attaching a polar (oxygen) anchor to a highly lipophilic molecule that is then transported through the cell wall by an active carrier system is well-known microbial detoxification mechanism [76].

Discussion regarding the physiology of enone biotransformation is controversial. No matter how, there exists a fact that enzyme-catalyzed introduction of oxygen into molecules can be accomplished by a multitude of enzymes, such as hydrolases, peroxidases, laccases and monooxygenases.

It is required to mention that microbial hydroxylation of the enone **68** at α′-position generated a chiral center and this is so crucial since introducing hydroxyl groups at

non-activated carbon centers regio- and stereoselectively is a difficult reaction to perform by standard chemical methods. Additionally, predicting the reaction site and stereoselectivity of microbial hydroxylations is a major unresolved problem that continues to attract research interest as reported by Wong and co-workers [74]. Several groups have studied this problem with various microbial systems and substrates and active site models have been proposed for *Bauveria bassiana*, *Calonectria decora*, *Absidia blakesleeana*. In these studies, monocyclic and polycyclic substrates are frequently selected as targets for microbial hydroxylation. It is important to note that microbial hydroxylation may be useful technique for the selective modification of these compounds since their preparation by standard chemical methods is not straightforward, and the resulting products are often useful intermediates to pharmaceutical compounds or natural products.

The last but not least, failure in some organisms namely *A. niger* 200910, *F. roseum* OUT4019, *F. solani* OUT4021, *F. anguioides* OUT4017, and *F. bulbigenum* OUT4115 used to mediate desired microbial hydroxylation of enone **68** may be explained by an enzyme active site model that an electron rich center in the substrate initially binds to the enzyme's active site followed by hydroxylation at a carbon atom located a specific distance away based on report published by Fonken and co-workers [77].

CHAPTER 3

EXPERIMENTAL

3.1 Materials and Equipments

^{1}H-NMR and ^{13}C-NMR spectra were recorded with Brucker-Spectrospin DPX-400, Ultra Shield, High Performance Digital FT-NMR spectrometer using tetramethylsilane (TMS) as internal standard and deuterochloroform ($CDCl_3$) as solvent.

Mass spectra were recorded with Thermo Quest GC-MS in which Phenomenex Zebron capillary GC column (60m length, 0.25mm ID, 0.25μm film thickness) was utilized.

Enantiomeric excess (ee) values were determined by using HPLC in which chiral column (Chiralpak® AS-H (0.4cm Ø x 1cm)) was used.

Flash column chromatography was performed by using Merck Silica Gel 60 (particle size: 40-63μm, 230-400 mesh ASTM).

Solvents used in the extractions were removed by using rotary evaporator.

The chemicals used in the study were purchased from Sigma, Aldrich, Fluka, and Acros Organics.

3.2 General Procedures

3.2.1 Synthesis of 1-hydroxycyclohexanecarbonitrile (65)

In a three-necked round bottomed flask 8.7mL cyclohexanone ($C_6H_{10}O$), 12.5mL of ether (Et_2O), 20mL of water (H_2O) and 10.3gr sodium cyanide (NaCN) were added. 50mL of dropping funnel containing 7.1mL of concentrated hydrochloric acid (HCI) was placed on the top of the flask. The apparatus was assembled in a well-ventilated hood. The flask was surrounded by an ice-salt bath, NaCN was added all at once with vigorous stirring. When most of the NaCN has dissolved and the temperature of the mixture has fallen to 5^oC, concentrated HCl was added from the dropping funnel at such a rate that the temperature remains between 5^oC and 10^oC. After all the acid has been added, the cooling bath was removed and vigorous stirring continues for 2 hours. The mixture was allowed to settle. Upper layer was ether (organic) and lower layer was water (aqueous) phase. Water phase was extracted four times with ether. Organic phase was dried over $MgSO_4$, and concentrated in vacuo to afford a colourless oil with 88% yield. $C_7H_{11}NO$: 125.1 g/mol.

Spectral data for 1-hydroxycyclohexanecarbonitrile **(65)**;

^{1}H-NMR (400 MHz, $CDCl_3$): δ ppm 1.30 (m, 1H, CH_2)
1.63 (m, 5H, CH_2)
1.80 (m, 2H, CH_2)
2.07 (m, 2H, CH_2)

^{13}C-NMR (100 MHz, $CDCI_3$): δ ppm 22.5 (2 CH_2)
24.5 (CH_2)
37.9 (2 CH_2)
69.4 (quarternary C)
121.5 (C≡N)

3.2.2 Synthesis of 1-hydroxycyclohexanecarboxylic acid (74)

The 1-hydroxycyclohexanecarbonitrile **65** (4.6mL) was refluxed with glacial acetic acid (CH_3CO_2H) (70mL) and concentrated HCl (70mL) for 5 hours. The solution was then concentrated in vacuo, the residue dissolved in chloroform ($CHCI_3$), washed with water, and purified by means of the sodium salt using 200mL 1N sodium hydroxide (NaOH) solution. Solution was then acidified by concentrated HCI such that pH was about 1.0. Resulting mixture was extracted with chloroform and organic phase was concentrated in vacuo to attain white granules of desired product with 60% yield. $C_7H_{12}O_3$: 144.2 g/mol

Spectral data for 1-hydroxycyclohexanecarboxylic acid **(74)**;

^{1}H-NMR (400 MHz, $CDCl_3$): δ ppm 1.25 (m, 1H, CH_2)
1.50-1.70 (m, 7H, CH_2)
1.75 (m, 2H, CH_2)

^{13}C-NMR (100 MHz, $CDCI_3$): δ ppm 21.0 (2 CH_2)
25.1 (CH_2)
34.5 (2 CH_2)
73.4 (quarternary C)
182.4 (CO_2H)

3.2.3 Synthesis of ethyl 1-hydroxycyclohexanecarboxylate (66)

To a stirred solution of 1-hydroxycyclohexanecarboxylic acid **(74)** (2g=14mmol) and ethanol (EtOH)(1.7 mL=29mmol) in benzene (C_6H_6)(6mL), 0.06mL concentrated sulfuric acid (H_2SO_4) was added and the mixture was refluxed overnight. The solvent was removed in vacuo and the residue dissolved in ether. Then, unchanged acid was filtered off and the solution washed with sodium hydrogen carbonate ($NaHCO_3$) solution. Resulting organic phase was dried over magnesium sulfate ($MgSO_4$) and concentrated in vacuo. The residue was purified by flash column

chromatography eluted with ether/petroleum ether (1:1) to afford light yellow oil ethyl 1-hydroxy cyclohexane carboxylate in 65% yield. $C_9H_{16}O_3$: 177.2 g/mol

Spectral data for ethyl 1-hydroxycyclohexanecarboxylate **(66)**;

^{1}H-NMR (400 MHz, $CDCl_3$): δ ppm 1.30 (t, J= 7.1 Hz, 3H, CH_3)
1.53-1.80 (m, 10H, CH_2)
2.86 (broad s, 1H, OH)
4.21 (q, J= 7.1 Hz, 2H, CH_2)

^{13}C-NMR (100 MHz, $CDCI_3$): δ ppm 14.2 (CH_3)
21.1 (2 CH_2)
25.2 (CH_2)
34.7 (2 CH_2)
61.3 (CH_2 attached to oxygen)
73.2 (quarternary C)
177.1 (carbonyl carbon, CO_2Et)

3.2.4 Synthesis of ethyl cyclohex-1-enecarboxylate (67)

A 50mL reaction flask equipped with an argon and a magnetic stirrer was charged with 0.8mL ethyl 1-hydroxy cyclohexane carboxylate **(66)** and 0.5mL pyridine (C_5H_5N). 1.0mL thionyl chloride ($SOCl_2$) were added carefully due to evolution of SO_2 and HCI gases. Temperature was controlled by an ice bath while addition of $SOCI_2$. Then, the reaction mixture was stirred with room temperature under argon for 1 hour. This mixture was quenched by pouring into ice water and extracted into ether. The organic layer was dried over $MgSO_4$ and evaporated. Resulting mixture was purified by flash column chromatography eluted with ether/petroleum ether (1:3) to attain ethyl cyclohex-1-enecarboxylate **(67)** in 86% yield as colourless oil. $C_9H_{14}O_2$: 154.2 g/mol

Spectral data for ethyl cyclohex-1-enecarboxylate **(67)**;

^{1}H-NMR (400 MHz, $CDCl_3$): δ ppm 1.20 (t, J= 7.1 Hz, 3H, CH_3)
1.50-1.78 (m, 8H, CH_2)
4.21 (q, J= 7.1 Hz, 2H, CH_2)
6.87 (m, 1H, olefinic C-H)

^{13}C-NMR (100 MHz, $CDCI_3$): δ ppm 14.3 (CH_3)
21.4 (CH_2)
24.0 (CH_2)
25.6 (CH_2)
36.8 (CH_2)
59.8 (CH_2 attached to oxygen)
130.5 (quarternary C)
138.9 (olefinic C)
167.0 (carbonyl carbon, CO_2Et)

3.2.5 Synthesis of ethyl 3-oxocyclohex-1-enecarboxylate (68)

In a two-necked round bottom flask, 1.00g (10mmol) chromium trioxide (CrO_3) was added over 5min. to a well stirring solution of 1mL (1.02g) ethyl cyclohex-1-enecarboxylate **(67)** in 7mL of acetic acid and 0.15mL water while the reaction temperature was maintained at 40^{o}C by external cooling. After another 60min. at 40-50^{o}C, the mixture was cooled by addition of ice, neutralized cautiously with a concentrated solution of potassium hydroxide (KOH), and extracted with ether. The ether extract was washed with saturated $NaHCO_3$, water, and brine, and dried over $MgSO_4$. The organic phase was then concentrated in vacuo, and the residue was purified by column chromatography eluted with ether/petroleum ether (1:5) to get ethyl 3-oxocyclohex-1-enecarboxylate **(68)** in 57% yield as slightly yellow oil. $C_9H_{12}O_3$: 168.2 g/mol

Spectral data for ethyl 3-oxocyclohex-1-enecarboxylate **(68)**;

^{1}H-NMR (400 MHz, $CDCl_3$): δ ppm 1.28 (t, J= 7.1 Hz, 3H, CH_3)
2.00 (m, J= 6.5 Hz, 2H, CH_2)
2.36 (t, J=6.5 Hz, 2H, CH_2)
2.51 (t, J=6.5 Hz, 2H, CH_2)
4.21 (q, J= 7.1 Hz, 2H, CH_2)
6.65 (s, 1H, olefinic C-H)

^{13}C-NMR (100 MHz, $CDCI_3$): δ ppm 14.1 (CH_3)
22.2 (CH_2)
24.8 (CH_2)
37.6 (CH_2)
61.4 (CH_2 attached to oxygen)
132.9 (quarternary C)
148.7 (olefinic C)
166.1 (carbonyl carbon, CO_2Et)
199.2 (carbonyl carbon)

Mass: m/z (relative abundances) 168(M^+)(88); 140(83); 123(69); 112(100); 95(82); 84(100); 67(85); 55(75); 41(86); 39(100); 29(83)

3.2.6 Synthesis of (±)-4-acetoxy-1-carboethoxy-cyclohexene-3-one (75)

A solution of 5mmol (0.84g) of ethyl 3-oxocyclohex-1-enecarboxylate **(68)** and 6.25mmol (1.45g) of $Mn(OAc)_3$ in 50mL of benzene and 5mL of acetic acid was stirred under reflux during which the dark brown color of $Mn(OAc)_3$ disappeared by time which was also monitored by TLC. After almost all starting material was consumed, the reaction mixture was diluted with ether and washed with brine. Resulting organic phase was dried over $MgSO_4$ and concentrated under vacuum. Crude products were purified by column chromatography using EtOAc/Hexane (1:5) as eluent to attain 4-acetoxy-1-carboethoxy-cyclohexene-3-one **(75)** in 98% yield.

HPLC: Chiralpak AS-H column, UV detection at 254 nm, eluent: hexane/2-propanol = 85:15 flow 0.7 mL/min, 20°C, R_f for **(±)-75**: 15.4 min and 18.0 min. $C_{12}H_{16}O_4$: 224.2 g/mol

Spectral data for 4-acetoxy-1-carboethoxy-cyclohexene-3-one **(75)**;

^{1}H-NMR (400 MHz, $CDCl_3$): δ ppm 1.28 (t, J= 7.1 Hz, 3H, CH_3)
2.05 (m, 1H, CH_2)
2.10 (s, 3H, CH_3)
2.25 (m, 1H, CH_2)
2.61 (m, 1H, CH_2)
2.83 (m, 1H, CH_2)
4.21 (q, J= 7.1 Hz, 2H, CH_2)
5.25 (dd, J=11.1, 5.3 Hz, 1H, CH)
6.70 (s, 1H, olefinic C-H)

^{13}C-NMR (100 MHz, $CDCI_3$): δ ppm 14.1 (CH_3)
20.6 (CH_2)
24.5 (CH_3)
27.9 (CH_2)
61.7 (CH_2 attached to oxygen)
73.3 (tertiary carbon CH)
131.7 (quarternary C at β-position)
148.3 (olefinic C at α-position)
165.4 (carbonyl carbon, CO_2Et)
169.5 (carbonyl carbon, CO_2Me)
193.7 (carbonyl carbon)

Mass: m/z (relative abundances) 226(M^+)(15); 184(50); 155(20); 140(40); 127(33); 112(80); 99(12); 83(36); 53(31); 43(100); 29(50)

3.2.7 Synthesis of 4-hydroxy-1-carboethoxy-cyclohexene-3-one (69)

3.2.7.1 Enzymatic Kinetic Resolution of (±)-4-acetoxy-1-carboethoxy-cyclohexene- 3-one

To a stirred solution of (±)-4-acetoxy-1-carboethoxy-cyclohexene-3-one (3μL) in dimetylsulfoxide (DMSO) (100μL) and phosphate buffer (pH 6.9, 300μL), enzyme (0.5 mg) was added in one portion and the reaction mixture was stirred at room temperature. Conversion was monitored by TLC up to 50% conversion. The reaction mixture was extracted with EtOAc, dried over $MgSO_4$, concentrated and purified by preparative TLC eluted with EtOAc/Hexane (1:3) to afford 4-hydroxy-1-carboethoxy-cyclohexene-3-one **(69)**. HPLC: Chiralpak AS-H column, UV detection at 254 nm, eluent: hexane/2-propanol = 85:15 flow 0.7 mL/min, 20^oC, R_f for **(±)-69**: 18.7 min and 23.9 min. $C_9H_{12}O_4$: 184.2 g/mol

3.2.7.2 Direct Microbial Oxidation of ethyl 3-oxocyclohex-1-enecarboxylate (68)

Erlenmeyer flasks (250mL) containing 100mL Malt/Glucose/Yeast/Peptone (MGYP): 3g/L malt; 10g/L glucose; 3g/L yeast extract; 5g/L peptone, growth medium was sterilized in autoclave at 121^oC for 15 min. Cultures of microorganisms were aseptically transferred to conical Erlenmeyer flasks and they were kept on a rotary shaker (160 rpm) at 37^oC for 2 days for *Aspergillus* species namely *A. oryzae* OUT5048 and *A. flavus* 200120 to acquire biomass. The mycelium from 100mL of cultivation medium (average 20g of wet cell weight) was filtered off, and then resuspended in 100mL of the freshly prepared growth medium (MGYP). 1mL of substrate solution (20mg/mL in DMSO) was added to the growth medium without prior sterilization. The reactions were monitored by TLC and they were terminated after 24 hours of substrate addition. After filtration, the filtrate was extracted with three times 50mL portions of EtOAc, dried over $MgSO_4$, concentrated and purified by column chromatography (EtOAc/Hexane 1:3) to afford 4-hydroxy-1-carboethoxy-cyclohexene-3-one in 17-31% yields.

Spectral data for 4-hydroxy-1-carboethoxy-cyclohexene-3-one **(69)**;

^{1}H-NMR (400 MHz, $CDCl_3$): δ ppm 1.28 (t, J= 7.1 Hz, 3H, CH_3)
1.80 (dq, J = 13.0 , 5.0 Hz, 1H, CH_2)
2.40 (m, 1H, CH_2)
2.55 (m, 1H, CH_2)
2.80 (m, 1H, CH_2)
3.45 (s, 1H, OH)
4.12 (dd, J=13.0, 5.0 Hz, 1H, CH)
4.23 (q, J= 7.1 Hz, 2H, CH_2)
6.75 (s, 1H, olefinic C-H)

^{13}C-NMR (100 MHz, $CDCI_3$): δ ppm 14.1 (CH_3)
24.6 (CH_2)
30.7 (CH_2)
61.7 (CH_2 attached to oxygen)
72.9 (tertiary carbon CH)
130.1 (quarternary C at β-position)
150.0 (olefinic C at α-position)
165.4 (carbonyl carbon, CO_2Et)
200.3 (carbonyl carbon)

Mass: m/z (relative abundances) 184(M^+)(7); 155(12); 140(39); 137(14); 112(100); 96(25); 84(85); 67(18); 55(31); 39(62); 29(81)

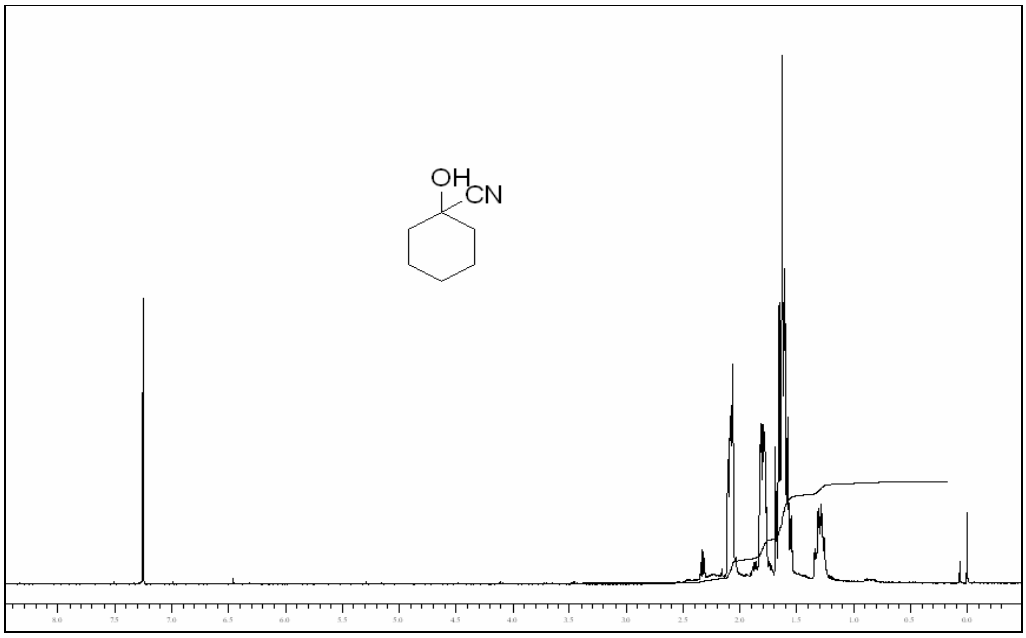


Figure 37 ^{1}H-NMR spectrum of 1-hydroxycyclohexanecarbonitrile

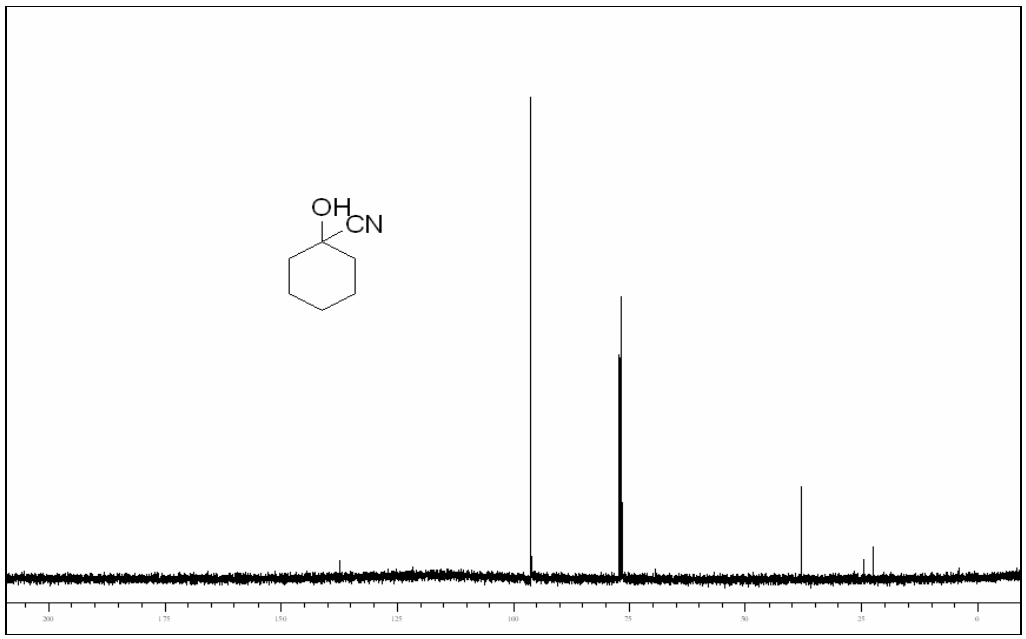


Figure 38 ^{13}C-NMR spectrum of 1-hydroxycyclohexanecarbonitrile

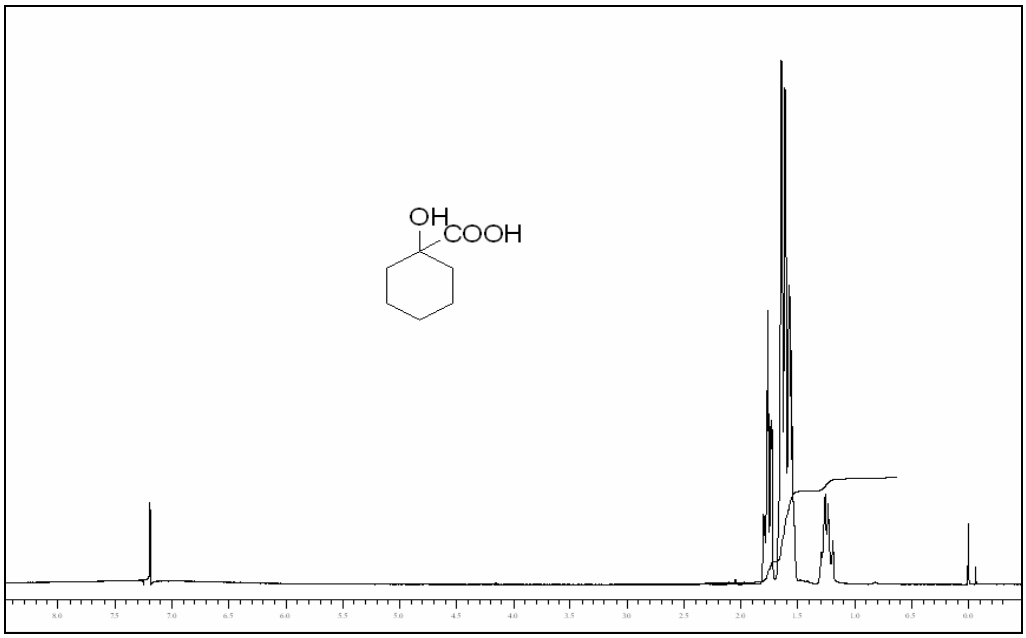


Figure 39 ^{1}H-NMR spectrum of 1-hydroxycyclohexanecarboxylic acid

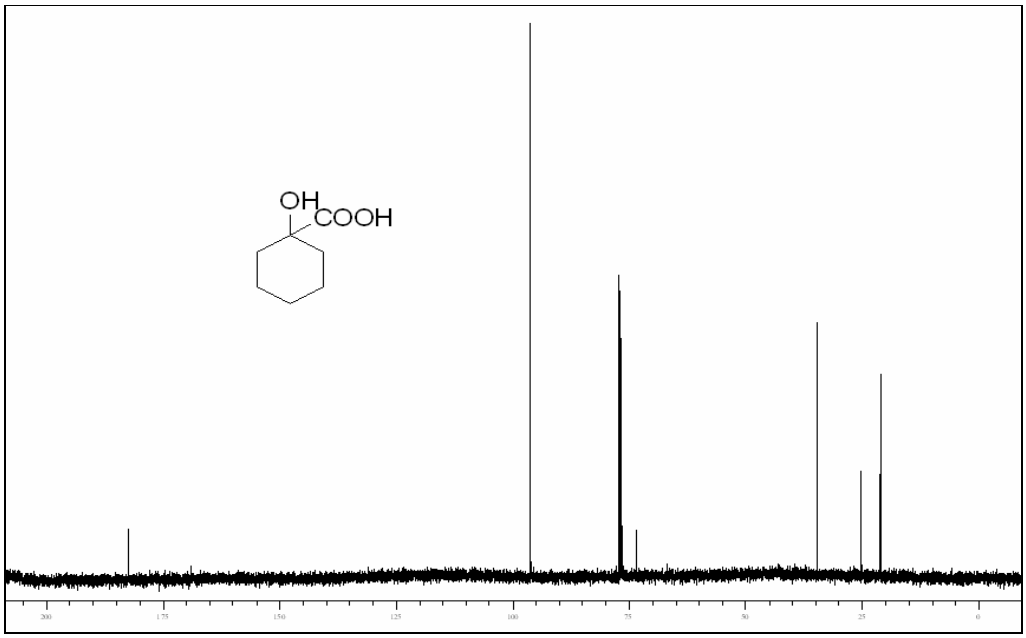


Figure 40 ^{13}C-NMR spectrum of 1-hydroxycyclohexanecarboxylic acid

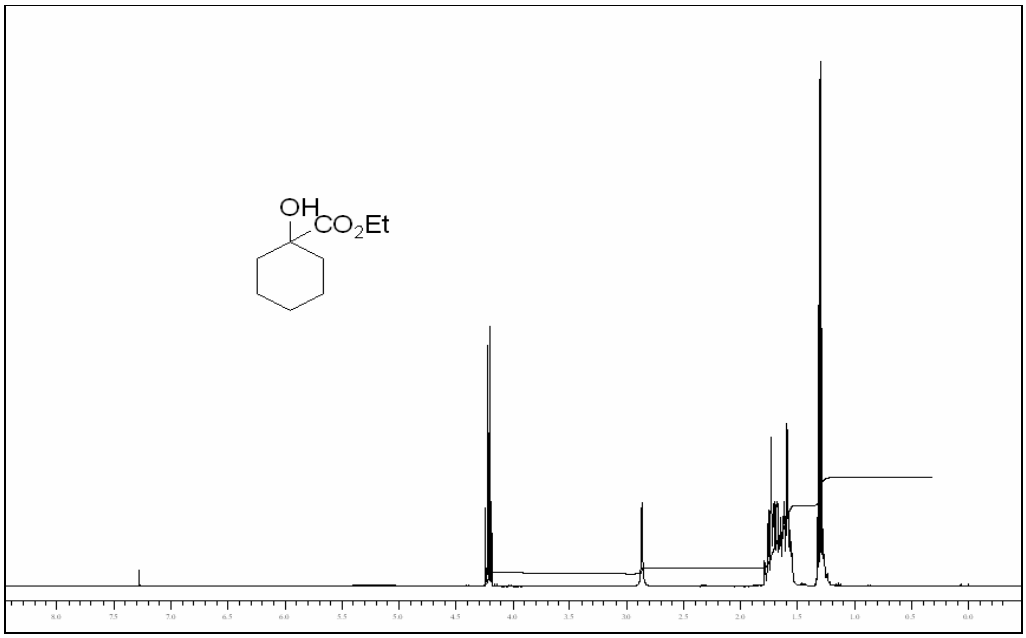


Figure 41 ^{1}H-NMR spectrum of ethyl 1-hydroxycyclohexanecarboxylate

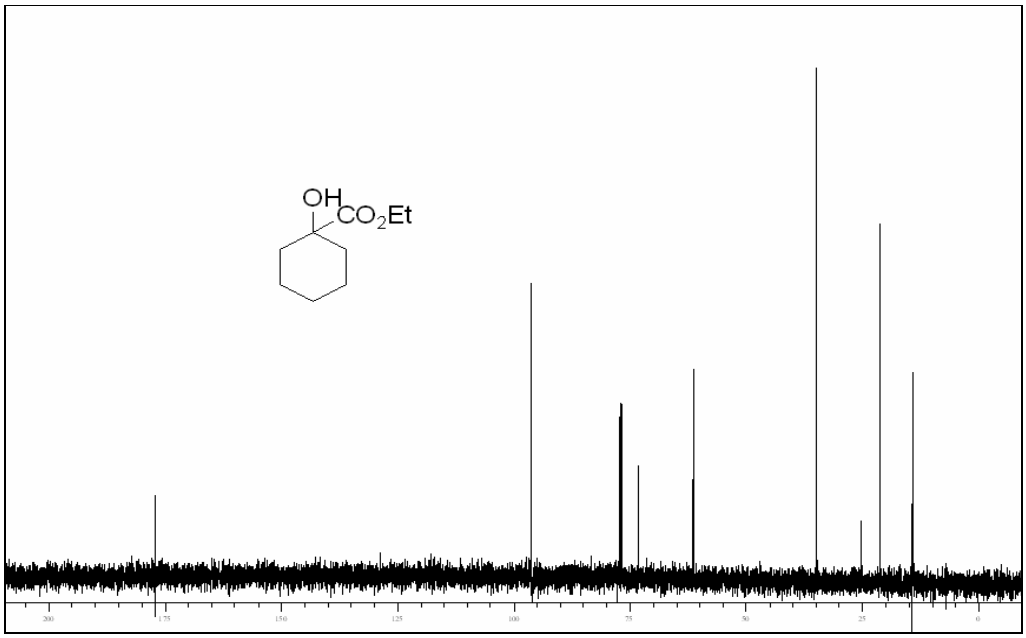


Figure 42 ^{13}C-NMR spectrum of ethyl 1-hydroxycyclohexanecarboxylate

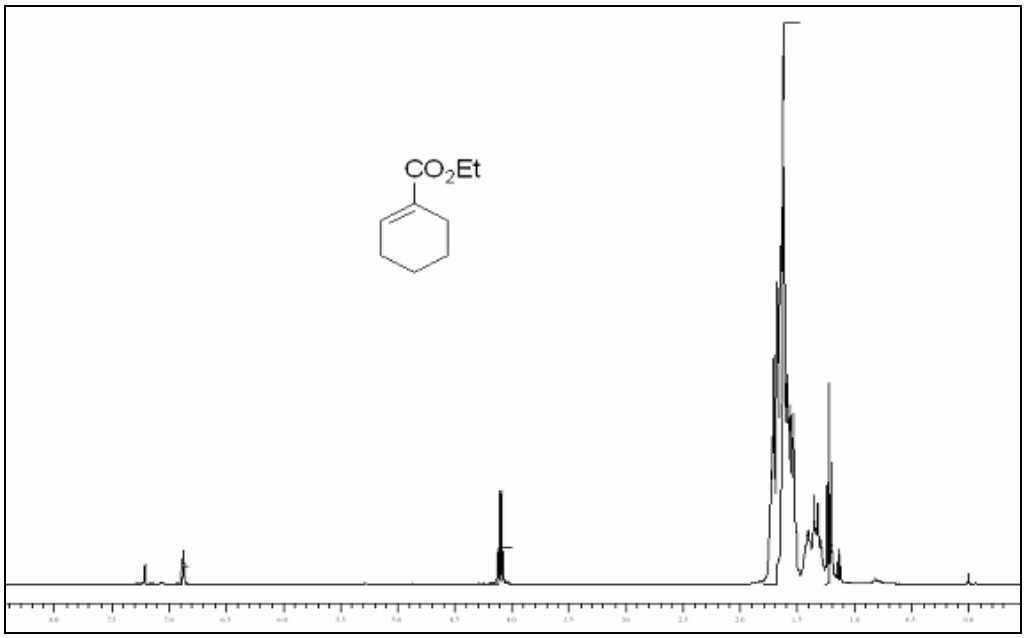


Figure 43 ^{1}H-NMR spectrum of ethyl cyclohex-1-enecarboxylate

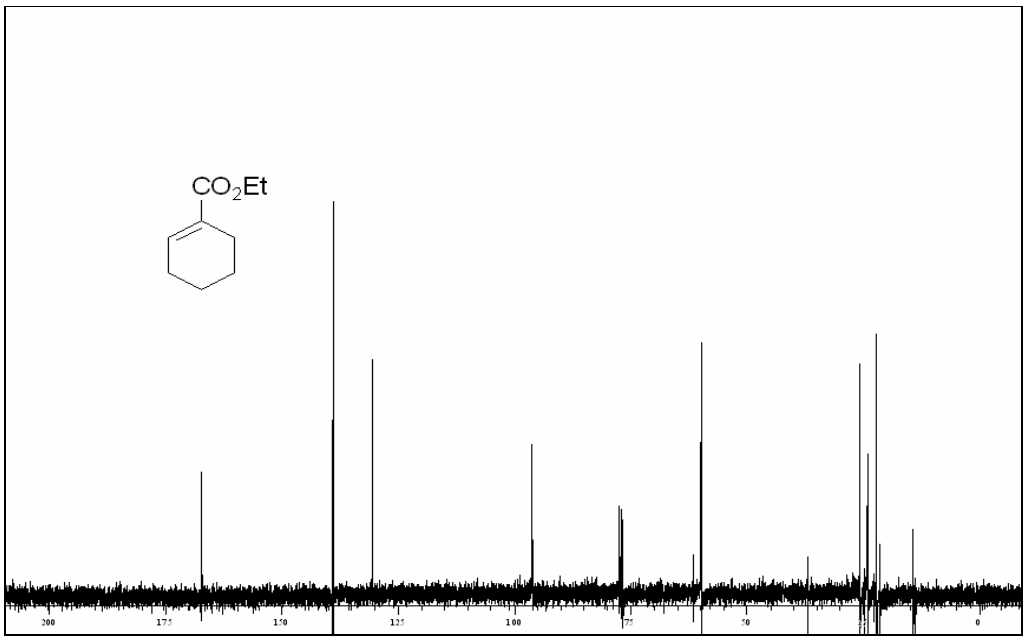


Figure 44 ^{13}C-NMR spectrum of ethyl cyclohex-1-enecarboxylate

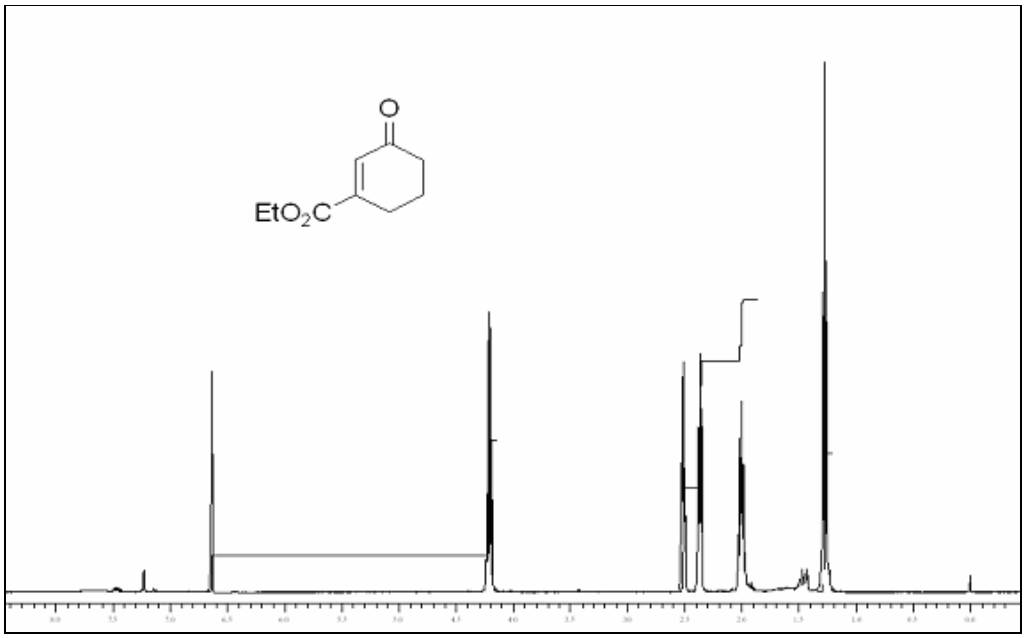


Figure 45 ^{1}H-NMR spectrum of ethyl 3-oxocyclohex-1-enecarboxylate

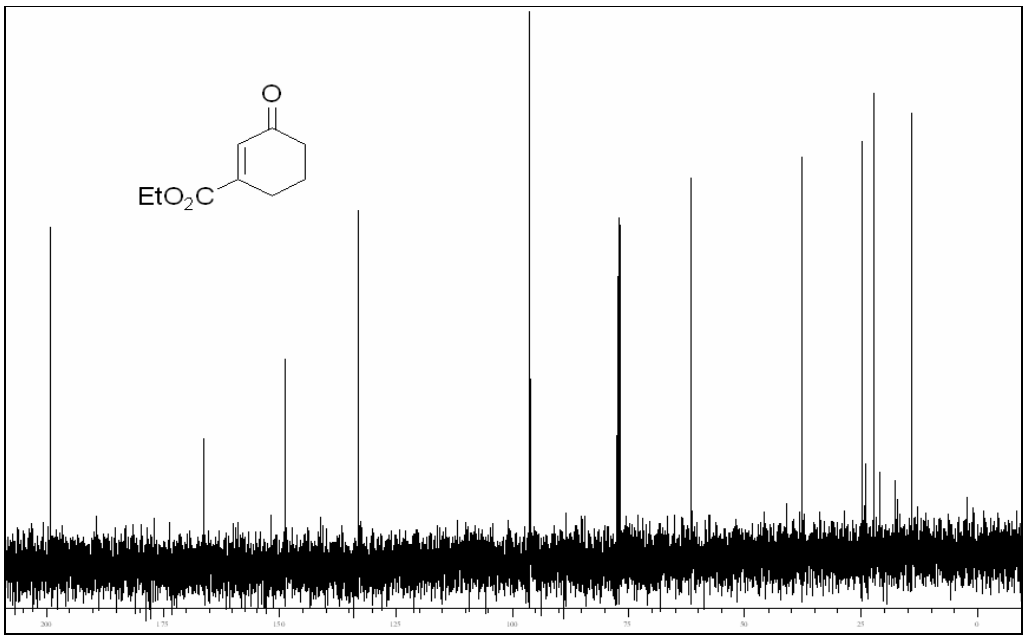


Figure 46 ^{13}C-NMR spectrum of ethyl 3-oxocyclohex-1-enecarboxylate

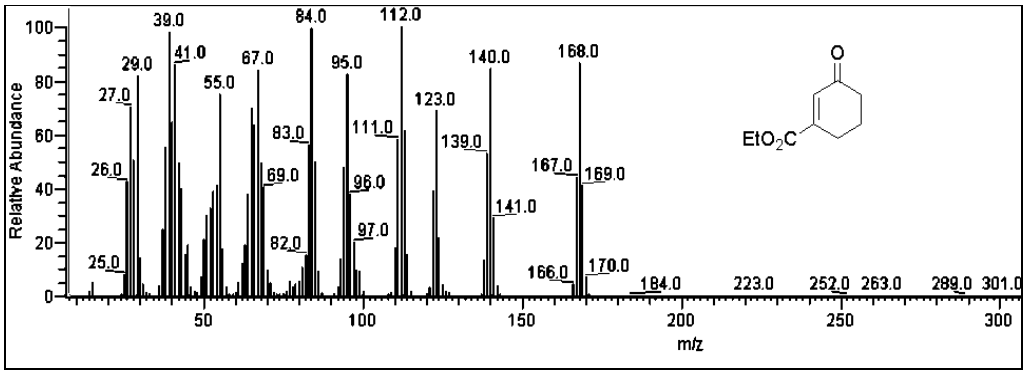


Figure 47 Mass spectrum of ethyl 3-oxocyclohex-1-enecarboxylate

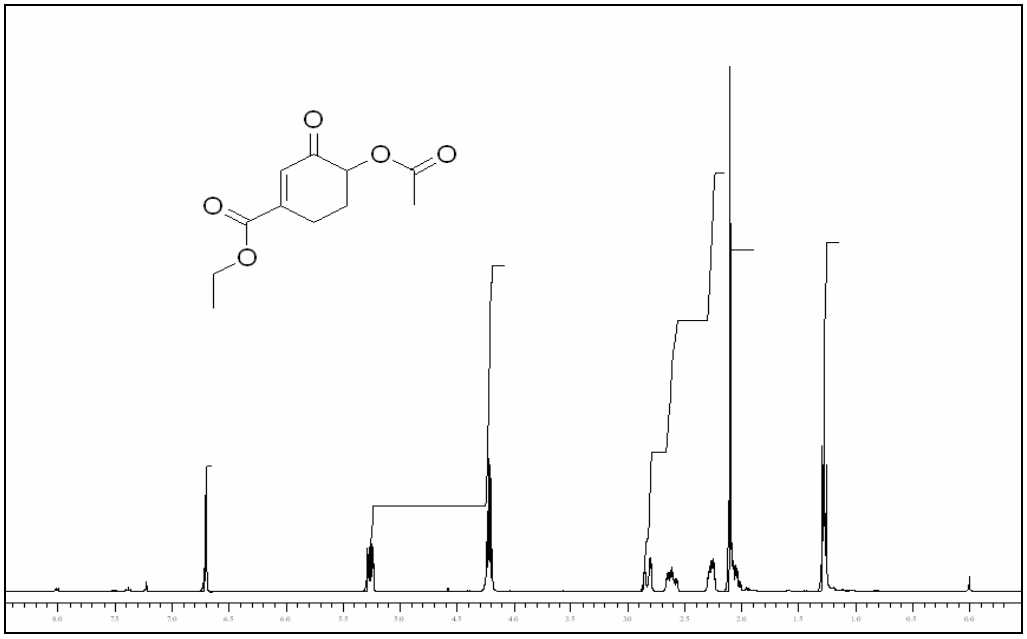


Figure 48 ^{1}H-NMR spectrum of 4-acetoxy-1-carboethoxy-cyclohexene-3-one

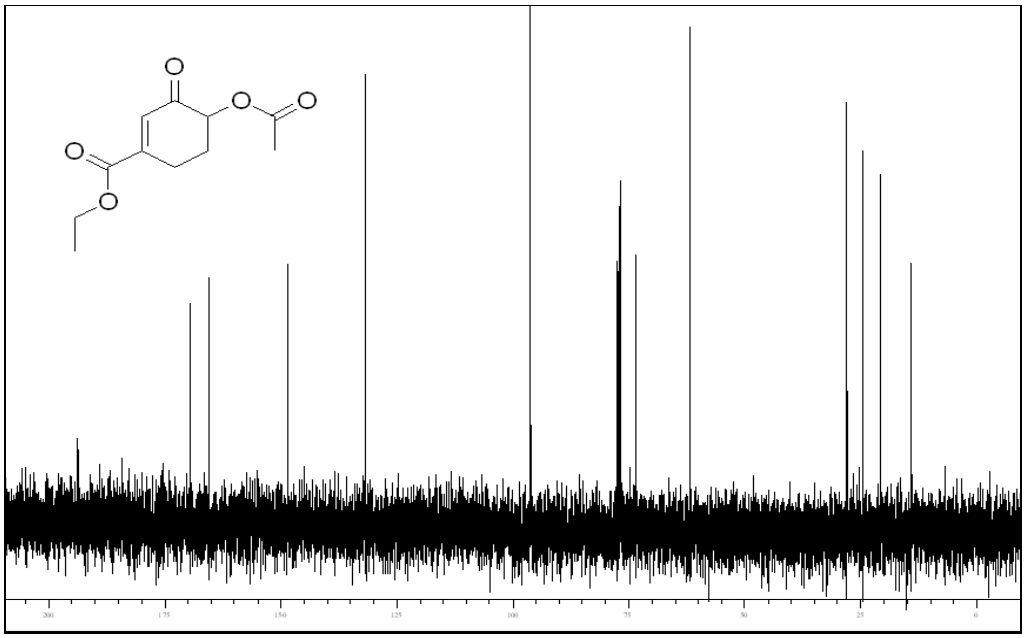


Figure 49 ^{13}C-NMR spectrum of 4-acetoxy-1-carboethoxy-cyclohexene-3-one

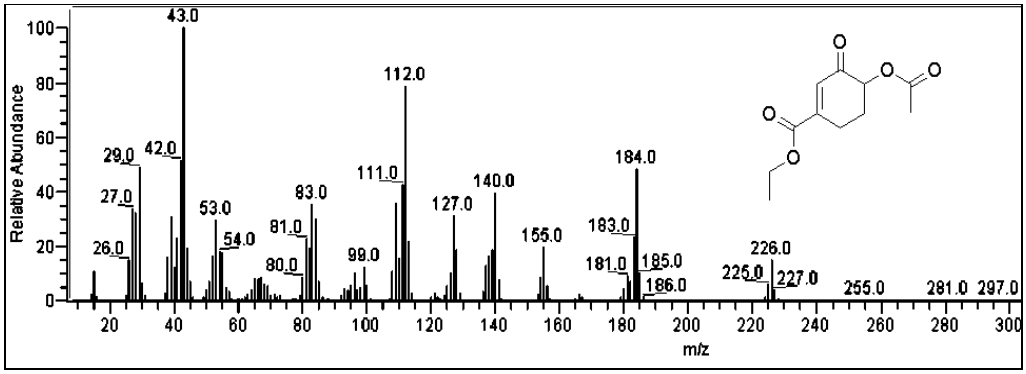


Figure 50 Mass spectrum of 4-acetoxy-1-carboethoxy-cyclohexene-3-one

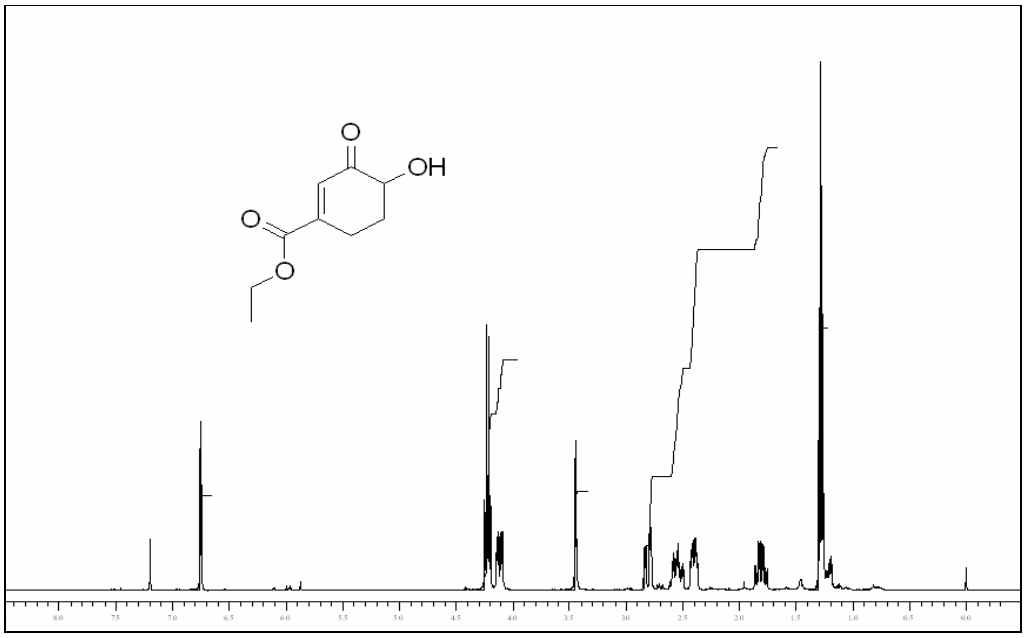


Figure 51 ^{1}H-NMR spectrum of 4-hydroxy-1-carboethoxy-cyclohexene-3-one

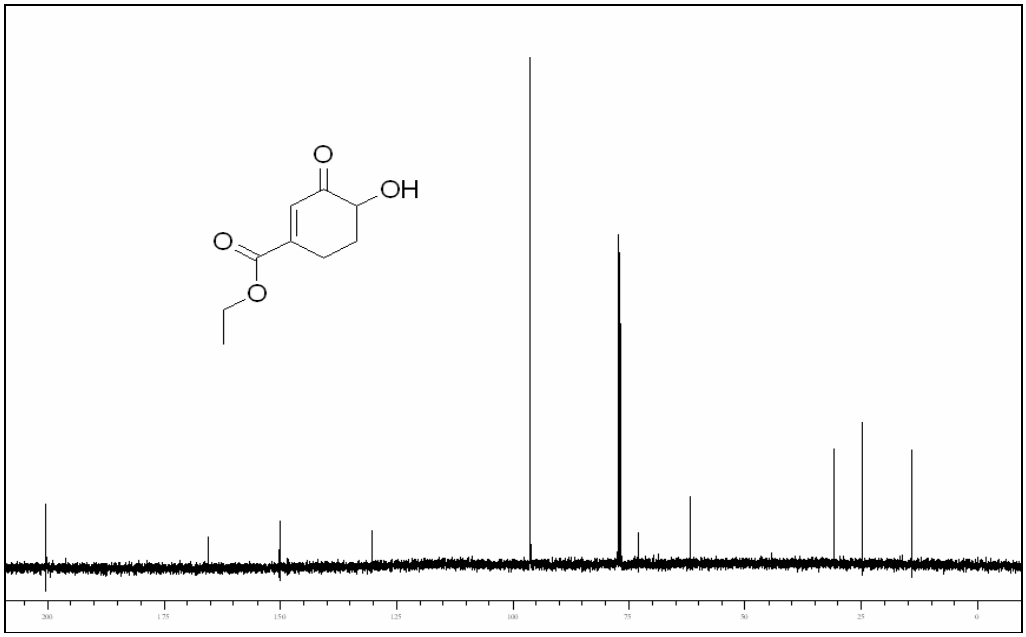


Figure 52 ^{13}C-NMR spectrum of 4-hydroxy-1-carboethoxy-cyclohexene-3-one

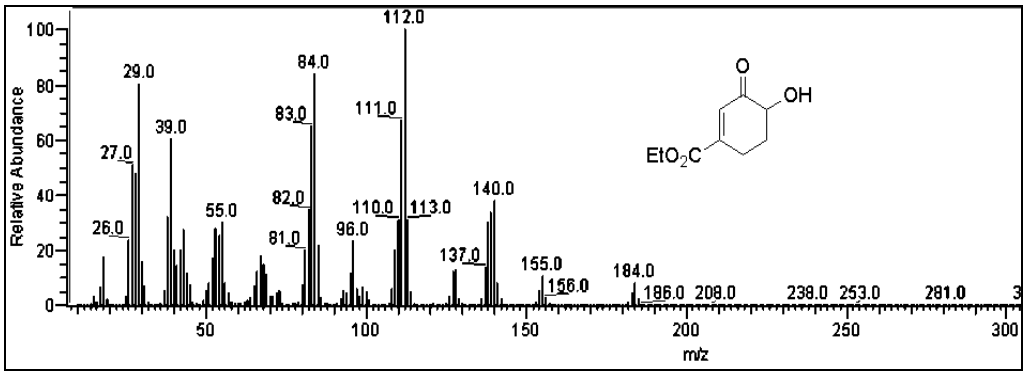


Figure 53 Mass spectrum of 4-hydroxy-1-carboethoxy-cyclohexene-3-one

CHAPTER 4

CONCLUSION

The study presented herein described the first enantioselective chemoenzymatic synthesis of 4-hydroxy-1-carboethoxy-cyclohexene-3-one **(69)** which was thought as a valuable intermediate in the total synthesis of Oseltamivir (Tamiflu) that is a therapeutic used in treatment of infections due to influenza A and B virus. The synthetic pathway commenced with the synthesis of ethyl 3-oxocyclohex-1-enecarboxylate **(68)**, which has been envisioned as potential starting compound in the synthesis of Tamiflu after retrosynthetic analysis, starting from simple and easily available substances. Then, two different routes have been followed to attain **69**. $Mn(OAc)_3$-mediated acetoxylation of **68** pursued by enzyme-mediated enantioselective kinetic resolution furnished great enantiomeric excess (ee) values for both acetoxy **75** (88 – 94%) and hydroxy enones **69** (88 – 92%ee). Besides, whole cells of fungi specifically *A. oryzae* OUT5048 and *A. flavus* 200120 catalyzed the direct oxidation of **68** to yield **69** enantioselectively (>99-97% ee). It is believed that the synthesized α´-hydroxy enone **69** structure in optically pure form can be utilized for the synthesis of biologically active compound Oseltamivir.

REFERENCES

1. Loughlin, W.A. *Bioresour. Technol.*, **2000**, *74*, 49.

2. Hanson, J.R. *An Introduction to Biotransformations in Organic Chemistry*, **1995**, W.H. Freeman and Comp.Ltd.

3. Meyer, H.P.; Leresche, J.E. *Org. Process Res. Dev.*, **2006**, *10*, 573.

4. Wang, M.X.; Gao, M.; Wang, D.X. ; Zheng, Q.Y. *J.Org.Chem.*, **2006**, *71*, 9532.

5. Furstoss, R.; Doumeche, B.; Archelas, A. *Adv. Synth. Catal.*, **2006**, *348*, 1948.

6. Koksch, B.; Thust, S. *J. Org. Chem.* , **2003**, *68*, 2290.

7. Taneja, S.C.; Koul, S.; Koul, J.L.; Singh, B.; Kapoor, M.; Parshad, R.; Manhas, K.S.; Qazi, G.N. *Tetrahedron: Asymmetry*, **2005**, *16*, 2575.

8. Ghanesm, A. *Tetrahedron*, **2007**, *63*, 1721.

9. Ema, T.; Yagasaki, H.; Okita, N.; Takeda, M.; Sakai, T. *Tetrahedron*, **2006**, *62*, 6143.

10. Kawai, Y.; Hayashi, M.; Inaba, Y.; Saitou, K.; Ohno, A. *Tetrahedron Lett.*, **1998**, *39*, 5225.

11. Bianchini, E.; Chinaglia, N.; Dean, M.; Giovannini, P.P.; Medici, A.; Pedrini, P.; Poli, S. *Tetrahedron*, **1999**, *55*, 1391.

12. Adam, W.; Boland, W.; Schreier, J.H.; Humpf, H.U.; Lazarus, M.; Saffert, A.; Möller, C.R.J.; Schreier, P. *J. Am. Chem. Soc.*, **1998**, *120*, 11044.

13. Hirata, T.; Izumi, S.; Ogura, M.; Yawata, T. *Tetrahedron*, **1998**, *54*, 15993.

14. Rama, R.; Mougin, C.; Boyer, F.D.; Kollmann, A.; Malosse, C.; Sigoillot, J.C. *Biotechnol. Lett.*, **1998**, *20*,1101.

15. Müller, M.; Dünkelmann, P.; Jung, D.K.; Nitsche, A.; Demir, A.S.; Siegert, P.; Lingen, B.; Baumann, M.; Potti, M. *J. Am. Chem. Soc.*, **2002**, *124*, 12084.

16. McConathy, J.; Owens, M.J. *J. Clin. Psychiatry*, **2003**, *5*, 70.

17. Penne, J.S. *Chiral Auxiliaries and Ligands in Asymmetric Synthesis*, **1995**, John Wiley&Sons, Inc., New York.

18. Procter, G. *Asymmetric Synthesis*, **1996**, Oxford University Press, New York.

19. Ager, D.J.; East, M.B. *Asymmetric Synthetic Methodology*, **1996**, CRC Press, Florida.

20. Gutsche, C.D.; Pasto, D.J. *Fundamentals of Organic Chemistry*, **1975**, Prentice Hall Inc., New Jersey.

21. Le Noble, W.J. *Highlights of Organic Chemistry*, **1974**, Marcel Dekker, Inc. New York.

22. Jacobsen, E.N.; Keith, J.M.; Larrow, J.F. *Adv. Synth. Catal.*, **2001**, *343*, 5.

23. Zaks, A. *Kirk-Othmer Encyclopedia of Chemical Technology*, **2007**, John Wiley&Sons, Inc.

24. Sharpless, K.B.; Martin, V.S.; Woodard, S.S.; Katsuki, T.; Yamada, Y.; Ikeda, M. *J. Am. Chem. Soc.*, **1981**, *103*, 6237.

25. Kalck, P.; Urritigoity, M. *Coord. Chem. Rev.*, **2004**, *248*, 2194.

26. Lacaze-Dufaure, C.; Lenoble, G.; Urritigoity, M.; Gorrichon, J.P., Mijoule, C., Kalck, P. *Tetrahedron: Asymmetry*, **2001**, *12*, 185.

27. Mukaiyama, T.; Soai, K.; Sato, T.; Shimizu, H.; Suzuki, K. *J. Am. Chem. Soc.*, **1979**, *101*, 1455.

28. Davis, F.A.; Chen, B.C. *Chem. Rev.*, **1992**, *92*, 919.

29. Demir, A.S.; Ayhan, P.; Sopaci, S.B. *Clean: Soil, Air, Water*, **2007**, *35(5)*, 406.

30. Sharpless, K.B.; Hashiyama, T.; Morikawa, K. *J. Org. Chem.*, **1992**, *57*, 5067.

31. Vedejs, E.J. *J. Am. Chem. Soc.*, **1974**, *96*, 5944.

32. Rubottom, G.M.; Vazquez, M.A.; Pelegrina, D.R. *Tetrahedron Lett.*, **1974**, 4319.

33. Davis, F.A.; Sheppard, A.C. *J. Org. Chem.*, **1987**, *52*, 954.

34. Horiuchi, C.A.; Utsukihara, T.; Nakamura, H.; Watanabe, M. *Tetrahedron Lett.* **2006**, *47*, 9359.

35. Plietker, P. *Tetrahedron: Asymmetry*, **2005**, *16*, 3453.

36. Demir, A.S.; Emrullahoğlu, M. *Curr. Org. Synth.*, **2007**, *4*, 223.

37. Demir, A.S.; Hamamcı, H.; Şeşenoğlu, Ö.; Aydoğan, F.; Çapanoğlu, D.; Neslihanoğlu, R. *Tetrahedron: Asymmetry*, **2001**, *12*, 1953.

38. Demir, A.S.; Şeşenoğlu, Ö. *Org. Lett.*, **2002**, *4*, 2021.

39. Iding, H.; Siegert, P.; Mesch, K.; Pohl, M, *Biochim. Biophys. Acta*, **1998**, *1385*, 307.

40. Hildebrandt, G.; Klavehn, W. *German Patent No: 548 459*, **1932**.

41. Deacon, J. *Fungal Biology*, **2006**, Blackwell Publishing, 4th edition, Edinburgh.

42. Michels, P.C.; Khmelnitsky, Y.L.; Rich, J.O. *Kirk-Othmer Encyclopedia of Chemical Technology*, **2007**, John Wiley&Sons, Inc.

43. Botes, A.L.; Weijers, C.A.G.M.; van Dyk, M.S. *Biotechnol. Lett.* , **1998**, *20*, 421.

44. Moussou, P.; Archelas, A.; Furstoss, R. *Tetrahedron*, **1998**, *54*, 1563.

45. Schieweck, F.; Altenbach, H.J. *Tetrahedron: Asymmetry*, **1998**, *9*, 403.

46. Alphand, V.; Mazzini, C.; Lebreton, J.; Furstoss, R. *J. Mol. Catal. B: Enzym.*, **1998**, *5*, 219.

47. Gotor, V.; Quiros, M.; Liz, R.; Frigola, J.; Fernandez, R. *Tetrahedron*, **1998**, *53*, 6421.

48. Geresh, S.; Valiyaveettil, T.J.; Lavie, Y.; Shani, A. *Tetrahedron: Asymmetry*, **1998**, *9*, 89.

49. Demir, A.S.; Hamamci, H.; Ayhan, P.; Duygu, A.N.; İgdir, A.C.; Capanoglu, D. *Tetrahedron: Asymmetry*, **2004**, *15*, 2579.

50. Harrington, P.J.; Brown, J.D.; Foderaro, T.; Hughes, R.C. *Org. Process Res. Dev.*, **2004**, *8*, 86.

51. Fukuyama, T.; Satoh, N.; Akiba, T.; Yokoshima, S. *Angew. Chem. Int. Ed.*, **2007**, *46*, 5734.

52. Farina, V.; Brown, J.D. *Angew. Chem. Int. Ed.*, **2006**, *45*, 7330.

53. Frost, J.W.; Oraths, K.M.; Knop, D.R. *J. Am. Chem. Soc.*, **1999**, *121*, 1603.

54. Federspiel, M.; Fischer, R.; Hennig, M.; Mair, H.-J.; Oberhauser, T.; Rimmler, G.; Albiez, T.; Bruhin, J.; Estermann, H.; Gandert, C.; Göckel, V.; Götzö, S.; Hoffmann, U.; Huber, G.; Janatsch, G.; Lauper, S.; Röckel-Stabler, O.; Trussardi, R.; Zwahlen, A.G. *Org. Process Res. Dev.*, **1999**, *3*, 266.

55. Karpf, M.; Trussardi, R. *J. Org. Chem.*, **2001**, *66*, 2044.

56. Corey, E.J.; Yeung, Y-Y.; Hong, S. *J. Am. Chem. Soc.*, **2006**, *128*, 6310.

57. Shibasaki, M.; Fukuta, Y.; Mita, T.; Fukuda, N.; Kanai, M. *J. Am. Chem. Soc.*, **2006**, *128*, 6312.

58. Cong, X.; Yao, Z-J. *J. Org. Chem.*, **2006**, *71*, 5365.

59. Shibasaki, M.; Fukuta, Y.; Mita, T.; Fukuda, N.; Kanai, M; Roca, F.X. *Org. Lett.*, **2007**, *9*, 259.

60. Shibasaki, M.; Kanai, M.; Yamatsugu, K.; Kamijo, S.; Suto, Y. *Tetrahedron Lett.*, **2007**, *48*, 1403.

61. Fang, J-M.; Shie, J-J.; Wang, S-Y.; Tsai, K-C.; E.Cheng, Y-S.; Yang, A-S.; Hsiao, S-C.; Su, C-Y.; Wong, C-H. *J. Am. Chem. Soc.*, **2007**, *129*, 11892.

62. Kann, N.; Bromfield, K.M.; Graden, H.; Hagberg, D.P.; Olsson, T. *Chem. Commun.*, **2007**, *30*, 3183.

63. Ghanem, A. *Tetrahedron*, **2007**, *63*, 1721.

64. Kim, C.U.; Lew, W.; Williams, M.A.; Liu, H.; Zhang, L.; Swaminathan, S.; Bischofberger, N.; Chen, M.S.; Mendel, D.B.; Tai, C.Y.; Laver, W.G.; Stevens, R.C. *J. Am. Chem. Soc.*, **1997**, *119*, 681.

65. Dreitlein, W.B.; Maratos, J.; Brocavich, J. *Clin.l Therapeutics*, **2001**, *23*, 327.

66. Agosta, W.C.; Lowrance, W.W.Jr. *J. Org. Chem.*, **1970**, *35*, 3851.

67. Demir, A.S.; Jeganathan, A. *Synthesis*, **1991**, 235.

68. Demir, A.S.; Reis, Ö.; Igdir, A.C. *Tetrahedron*, **2004**, *60*, 3427.

69. William, G.J.; Hunter, N.R. *Can. J. Chem.*, **1976**, 54, 3830.

70. Corey, E.J.; Kang, M. *J. Am.Chem. Soc.*, **1984**, *106*, 5384.

71. Pannuri, S.; DiSanto, R.; Kamat, S. *Kirk-Othmer Encyclopedia of Chemical Technology*, **2007**, John Wiley&Sons, Inc.

72. Floresca, R.; Kurihara, M.; Watt, D.S.; Demir, A.S. *J. Org. Chem.*, **1993**, *58*, 2196.

73. Chen, C.-S.; Fujimoto, Y.; Girdauskas, G.; Sih, C.J. *J. Am.Chem. Soc.*, **1982**, *104*, 7294.

74. Wong, J.W.; Burns, M.P. *Tetrahedron: Asymmetry*, **1999**, *10*, 4295.

75. Larroche, C.; Cappaert, L. *Biocatal. and Biotransform.*, **2004**, *22*, 291.

76. Berger, R.G.; Nanzad, T.; Kaspera, R.; Krings, U. *Appl. Microbiol. Biotechnol.*, **2005**, *67*, 477.

77. Fonken, G.S.; Herr, M.E.; Murray, H.C.; Reineke, L.M. *J. Am. Chem. Soc.*, **1967**, *89*, 672.