ASPERGILLUS NIGER MEDIATED α-HYDROXYLATION OF CYCLIC KETONES

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ABSTRACT

ASPERGILLUS NIGER MEDIATED α-HYDROXYLATION OF CYCLIC KETONES

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Chiral α-hydroxy ketones are important structural units in many natural products, biologically active compounds and the hydroxyl group has frequently been used as a reagent directing group, such as for the selective elaboration of aldol products.

In this work, enzymatic synthesis of both enantiomers of the α-hydroxy ketones (2-hydroxy indanone, 2-hydroxy tetralone) using *Aspergillus niger* by selective α-oxidation of ketones (1-indanone, 1-tetralone) was studied. The α-oxidation of ketones was carried out by using whole cells of *Aspergillus niger* in different growth media

A. niger whole cell catalyzed reactions afforded (S)-configurated 2-hydroxy-1-tetralone with %87 e.e. in DMSO at pH 5.0. In addition to this,

while (S)-configurated 2-hydroxy-1-indanone with %33 e.e. in pH 8.0 (in DMSO) was synthesized, (R)-configurated-2-hyroxy-1-indanone with %32 e.e. in pH 7.0 (in DMSO) was synthesized.

Keywords: *Aspergillus niger*, α-hydroxy ketones, whole cell biocatalysis

ÖZ

ASPERGILLUS NIGER VASITASIYLA HALKALI KETONLARIN α-HİDROKSİLASYONU

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Aralık 2006, 80 Sayfa

Optikçe saf α-hidroksi ketonlar biyolojik olarak aktif olan bir çok doğal üründe bulunan önemli yapılardır ve hidroksi grup genellikle tekrar yönlendirme grubu olarak kullanır. Seçici aldol reaksiyonları bunlara örnek olarak verilebilir.

Bu çalışmada, ketonların (1-indanon, 1-tetralon) seçici oksiklenmesiyle α-hidroksi ketonların (2-hidroksi indanon , 2-hidroksi tetralon) her iki enantiomerininde sentezlenmesi için *Aspergillus niger* türünün kullanılması araştırılmıştır.

Ketonların indirgenmesi *Aspergillus niger* hücrelerinin değişik ortamlarda çoğaltılmasıyla gerçekleştirilmiştir.

A.niger'ın katalizör olarak kullanıldığı bütün hücre reaksiyonlarında (S)-2-hidroksi-1-tetralone, pH 5.0'te ve DMSO çözücülüğünde %87 e.e. ile elde edilmiştir. Buna ek olarak, (S)-2-hidroksi-1-indanone %33 e.e. ile pH 8.0'de DMSO varlığında sentezlenirken, (R)-2-hidroksi-1-indanone %32 e.e. ile pH 7.0'de DMSO varlığında sentezlenmiştir.

Anahtar kelimeler: *Aspergillus niger*, α-hidroksi keton, biokatalizörler

To My Family

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INTRODUCTION

1.1 IMPORTANCE OF BIOCATALYSIS IN BIOTECHNOLOGY

Biotechnology is described as the application of scientific and engineering principles to the processing of materials by biological agents [1]. Biotechnology has proved capable of generating enormous wealth and influencing every significant sector of the economy. Biotechnology has already substantially affected healthcare, production and processing of food; agriculture and forestry, environmental protection, and production of materials and chemicals [1].

Compared to conventional production, sustainable processes and production systems should be more profitable because they would require less wasteful use of materials and energy, result in fewer emissions of greenhouse gasses and other pollutants, and enable greater and more efficient use of renewable resources to lessen dependence on non-renewable resources [1]. Sustainability demands products that are not only perform well but, compared to their conventional counterparts, are more durable, less toxic, easily recyclable, and biodegradable at the end of their useful life. Such products would be derived as much as possible from renewable resources and contribute minimally to net generation of green house gasses [1].

Biotechnology is versatile and has been assessed a key technology for a sustainable chemical industry. Biotechnology provides entirely novel opportunities for sustainable production of existing and new products and services. Biocatalysts operates at lower temperatures, produces less toxic waste, fewer emissions and by-products compared to conventional chemical processes. In view of their selectivity, these biocatalysts reduce the need for purifying the product from by-products, thus reducing energy demand and environmental impact [1].

Biological production systems are inherently attractive because they use basic renewable resources of sunlight, water and carbon dioxide to produce a wide range of molecules using low-energy processes. Biotechnology has already been put to extensive use specially in the manufacture of biopharmaceuticals. Biotechnology has greatly impacted in production for customers from healthcare, medical diagnostics, environmental protection, agriculture, criminal investigation and food processing [1,11]. The biggest demand is in the pharmaceuticals industry, in which enantiomericaly pure substances or intermediates are used to manufacture active ingredients. This market places high demands on purity, because oversight authorities like FDA or the Federal Institute for Drug and Medical devices (BfArM) requires either the S or R enantiomer variants or else pharmacological verification and clinical testing for each enantiomer of a racemate [11]. This is why enantioselective synthesis methods are becoming increasingly important [11].

The global chemical industry has contributed immensely to

achieving the present quality of life, but is under increasing pressure to change current working practices in favor of greener alternatives [1].

Biotechnology uses the power of life to enable effective, rapid, safe and environmentally acceptable production of goods and services.

The chemical industry has used traditional biotechnological processes (e.g. microbial production of enzymes, antibiotics, amino acids, ethanol, vitamins, enzyme catalysis) for many years. In addition, traditional biotechnology is widely used in producing fermented foods and treating waste [1].

1.2 BIOCATALYSIS IN ORGANIC CHEMISTRY

Biocatalysts can be defined as the application of a biocatalyst to achieve a desired conversion under controlled conditions in a bioreactor. A biocatalyst can be an enzyme, an enzyme complex, a cell organelle or a whole cell. Over recent years there has been an exponential increase in the production of high-value specialty chemicals using either isolated enzymes, especially hydrolases, to catalyze single-step transformations or whole cells to catalyze multi-step reactions. This increase is motivated by the fact that enzymes, whether isolated or contained within whole cells, can catalyze a broad range of reactions, at times with high levels of chemo-selectivity, regioselectivity and stereoselectivity. In fact, biocatalysts in the form of isolated enzymes, or enzymes contained in whole cells, can often catalyze reactions of pH and temperatures and biocatalytic processes generate fewer residues than chemical processes

do, such that biocatalysis is often referred to as "green chemistry" [5].

The "green chemistry" concept was introduced in the early 1990s in the scientific community and soon adopted by mass-media as the new approach of chemistry in opposition to the pollute-and-then-clean up approach considered the common industrial practice [6].

"Sustainable chemistry" is the concept sometimes opposed and sometimes confused with "green chemistry". There is a key difference in the definition: while green chemistry indicates that a not risky and polluting chemical production process may exist, the sustainable chemistry concept links eco-efficiency, economic growth and quality of life in terms of a cost/benefit analysis [6].

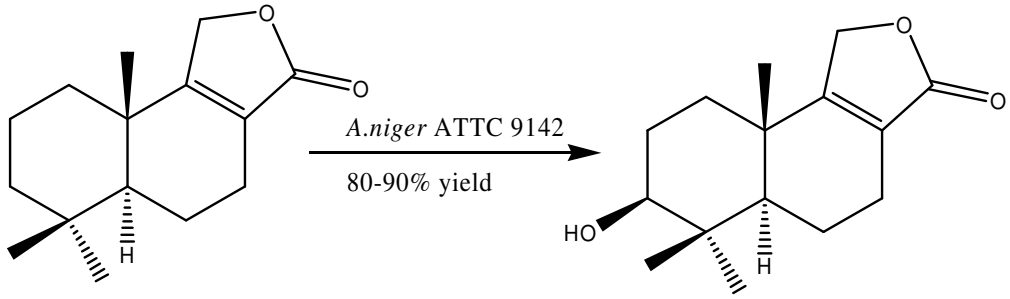
Biotransformation provides an alternative to the chemical synthetic methodology that is sometimes competitive, and thus represents a section of the tools available to the synthetic chemist [4]. Incorporation of biotransformation steps, using microorganisms and/or isolated enzymes, is increasingly being exploited both in industry and academic laboratories. The primary consideration for incorporation of a biotransformation in a synthetic sequence is the regio- and stereo-control that can be achieved using an enzyme-catalyzed reaction. Biotransformations are becoming accepted as a method for generating optically pure compounds and for developing efficient routes to target compounds [4].

Selectivity is an essential requirement in synthetic organic

chemistry, and there is little doubt that enzymes as highly selective catalysts have contributed heavily to meat this challenge. The regioselectivity of enzymes even on complex molecules, without any need of protecting groups is a fundamental strength of biocatalysis. This reduces the number of synthetic steps and thereby cuts occupation time of chemical reactors, which is an important factor of process economy in pharmaceutical and chemical production [7].

Briefly, microorganisms and biocatalysts are becoming increasingly important in the chemical industry. Among the many reasons for this are the growing demand for enantiomerically pure substances and the advantages of green biotechnology when it comes to selectivity, sustainability and environmental protection [11].

Furthermore, the features governing their regiospecificity and indeed it are possible to obtain biotransformation at center that are chemically un-reactive (Figure 1.1, 1 transformed to 2).



1 **2**

Figure 1.1 Example of a Fungal Biotransformation [40]

The dominant feature in a biotransformation is the topological

relation ship between the substrate and the active site of the enzyme. From a commercial point of view, biotransformation can be cheaper and more direct than their chemical analogues whitest the transformations proceed under conditions that normally regarded as "environmentally friendly"

1.3 ADVANTAGES AND DISADVANTAGES OF BIOCATALYSIS

Enzymes are very efficient catalysts; the rates of enzyme- mediated processes are accelerated compared with non-enzymatic reactions by a factor of 10^8-10^{10} [2,4,5,15].

They are environmentally acceptable; for example, unlike heavy metals, biocatalyst are environmentally friendly since they are completely degradable [2,5,15].

They act under mild conditions; enzymes act in a temperature range of 20 – 40 °C, and in a pH range of about 5-8. This minimizes problems of undesired side–reactions such as decomposition, isomerization, racemization and rearrangement, which often plague traditional methodology [2,4,5,15].

They are compatible with each other; enzymes generally function under the same or similar conditions. Therefore, several biocatalytic reactions can be carried out in a reaction cascade in one flask [2,15].

Enzyme-catalyzed processes exist for a wide range of reactions and

can often promote reactions at apparently non-activated sites in a substrate [2,4,5,15]. Indeed, it is possible to obtain biotransformations at center that are chemically unreactive [Figure1.1]

They display 3 major selectivity, such as (a) chemoselectivity: enzymes can act on a single type of functional group in the presence of other sensitive functional groups (b) regioselectivity and diastereoselectivity: they can distinguish between functional groups with different chemical environment. (c) enantioselectivity: they are chiral catalysts and their specificity can be exploited for selective and asymmetric conversions [2,4].

They can work outside an aqueous environment; some can operate in organic solvents [2,4,5,15,28].

The main disadvantages of enzymes in synthesis are that; enzymes are found in only one enantiomeric form and thus it is nearly impossible to invert their chiral induction on a reaction [2,4,7].

Enzymes are prone to product or substrate inhibition. Substrate inhibition can be overcome by keeping the substrate concentration at a low level through continuous addition. Product inhibition can be circumvented by gradual removal of product by physical means [2,4,7].

Enzymes display their highest catalytic activity in water, aqueous solvents. However, water is usually the least suitable solvent for most organic reactions, due to its high boiling point and high heat of

vaporization [2,4,7].

Enzymes require narrow operation conditions: the advantage of working under mild reaction conditions can sometimes turn into disadvantage. Elevated temperatures and extreme in pH, or high salt concentrations all lead to deactivation of enzymes [2,8].

Although microbes are extremely good in presenting us with an amazing array of valuable products, they usually produce them only in amounts that they need for their own benefit; thus they tend not to overproduce their metabolites [16].

Briefly, microorganisms are important to us for many reasons, but one of the principal ones is that they produce things of value to us. Most natural products are so complex and contain so many centers of asymmetry that they probably will never be made commercially by chemical synthesis [16].

1.4 CHIRALITY AND THE NEED FOR ENANTIOPURE COMPOUNDS

A chiral molecule is one that is not superimposable on its mirror image; it has the property of rotating the plane of polarization of plane-polarized monochromatic light that is passed through it. This phenomenon is called optical activity.

Carbon atoms carrying four different substituents possess a unique property. The substituents can be arranged in two alternative ways to bring about two forms of the molecule with the same constitution. In

Figure 1.2 two molecules of the same constitution [CHABC] are depicted so that in each case the smallest substituent, hydrogen, lies behind the plane of the paper. The substituent S is drown in each case in the plane, pointing up and the other two substituents occupy positions either on the left or right of the central carbon atom.

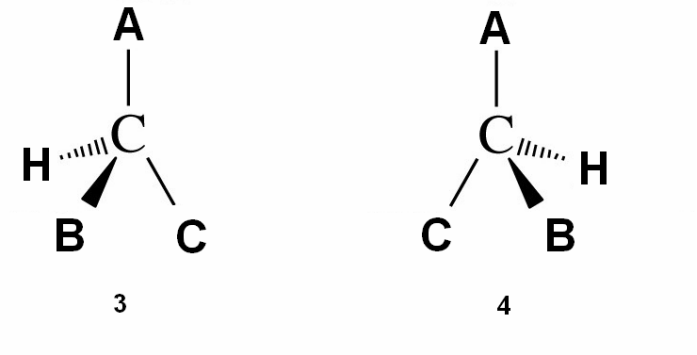


Figure 1.2 Chiral Molecules

Looking along the bond from the central carbon atom towards the hydrogen at the back, one finds that the two molecules differ in the way the remaining three substituents are arranged in space. In 3, the substituents A, B, and C follows a clockwise rotation, whereas in 4, the rotation is counter-clockwise.

The two forms of the molecule are related as hands to each other, being non-superimposable mirror images of each other. They are called "chiral" and the central carbon atom is known as the "chiral" or "stereogenic centre".

If the molecule contains more than one chiral center, there emerges the possibility of another form of stereoisomerism. Stereoisomeric molecules which cannot be superimposed by any symmetry operations

are called diastereomers.

If the compound contains two chiral atoms, it may exist in four stereoisomeric forms. Since the configuration at each chiral carbon may be either R or S, there are four stereochemical possibilities: RR, RS, SS, SR. the RR and SS stereoisomers are enantiomers. The RS and SR stereoisomers are also enantiomers. The RR stereoisomer is a diastereomer of both the RS and the SR stereoisomers. The SS stereoisomer is a diastereomers of both the RS and SR stereoisomers.

Chirality is a major phenomenon in nature. Molecular asymmetry in particular is playing a crucial role in science and technology. If the only difference between enantiomers was in their rotation of polarized light, the whole area of asymmetric synthesis would be consigned to little more than a scientific curiosity. That is not so because the world (nature) around us is chiral and most of the important building blocks which make up the biological macromolecules of living systems do so in one enantiomeric form only [L-form]. When, therefore a biological active chiral compound, such as a drug, interacts with a receptor site which is chiral, it should come as no surprise that the two enantiomers of the drug interact differently and may lead to different effects [11].

Enyzmes have gained most attention because of their stereoselectivity. Especially in pharmaceuticals the recent tendency to develop single stereoisomer drugs instead of racemates has helped tremendously to establish enzymes as tools in organic synthesis [7].

Chirality is a property which often determines the action and

behavior of molecules in rather unexpected ways. For example, lemons ad oranges both contain limonene, the different enantiomers giving rise to substle changes in the aroma properties of the fruits. Similarly, R- and S- carvone (which is a member of a family of chemicals called terpenoids) have different tastes: the former tasting of spearmint and the latter of caraway (Figure 1.3)

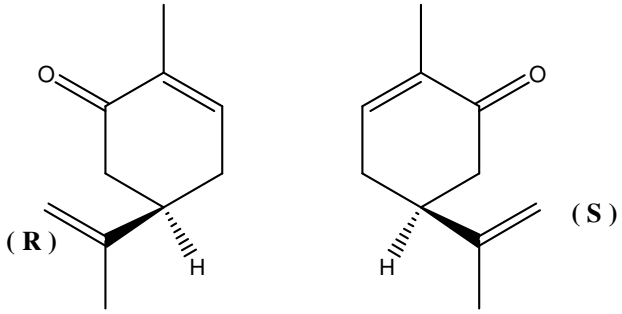


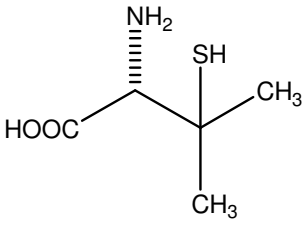
Figure 1.3 R- and S- carvone

As natural products and their derivatives and analogues find wide use in our everyday life, from medicine to food additives, it is undesirable that for the production of these compounds by synthetic means by need to secure them in enantiopure as "eutomer", where as its enantiomeric counterpart, possessing less or even undesired activities, is termed as the "distomer".

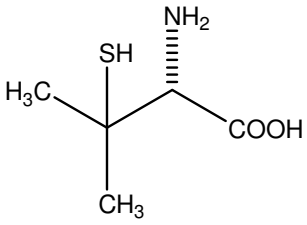
Probably, the most well-known and tragic example of a drug in which the distomer causes serious side-effect is "Thalidomide", which was administered as a racemate in 1960's. At that time, it was not known that the sedative effect resides in the (R)-enantiomer but the S-counterpart is highly teratogenic. [2]. A high incidence of fetal deaths and

malfunctions occurred due to its use by pregnant women [11]. Some representative examples of different biological affects are given in Figure 1.4. [2]

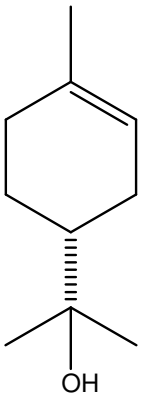
R-enantiomer **S-enantiomer**



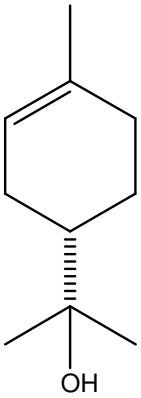
Penicillamine



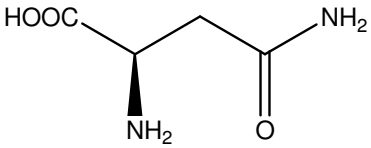
Toxic Antiarthritic



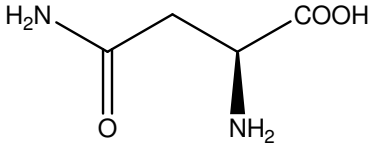
Terpene Alcohol



Liliac Smell Coldpipe Smell



Asparagine



Sweet Bitter

Figure 1.4 Examples of Different Biological Affects of Diastomers

As a consequence, this has led to an increased need for enantiopure compounds. Unfortunately, less than 10% of organic compounds crystallize as a conglomerate largely denying the possibility of separating enantiomers by simple crystallization techniques- such as by seeding a supersaturated solution of the racemate with crystals of one pure enantiomer. [2]

The principles of asymmetric synthesis make use of enantiomerically pure auxiliary reagents which are used in catalytic or sometimes in stoichiometric amounts. They are often expensive and cannot be recovered in many cases.

Considering the problems with the alternative ways of obtaining enantiomerically pure compounds, it is obvious that enzymatic methods represent a valuable addition to the existing toolbox available for the asymmetric synthesis of fine chemicals.

1.5 Synthesis of Chiral Compounds using Chemical and Biotechnological methods

Asymmetric synthesis defined as a synthesis in which an achiral unit in a molecule is converted to a chiral unit such that the possible stereoisomers are formed in unequal amounts.

There are two basic ways of asymmetric synthesis to form a chiral compound. The first is to create a chiral center. The second way is to start with an optically active compound and use a kind of synthesis that

does not affect the chiral center.

1.5.1 Asymmetric Synthesis with an Active Substrate (1st Generation Method)

If a new chiral center is created in a molecule that is optically active, the two diastereomers are not formed in equal amounts. The reason is that the direction of attack by reagent is determined by the groups already there. For certain additions to the carbon-oxygen double bound of ketones containing a asymmetric α-carbon, Crams rule predicts which diastreomer will predominate as seen in Figure 1.5.

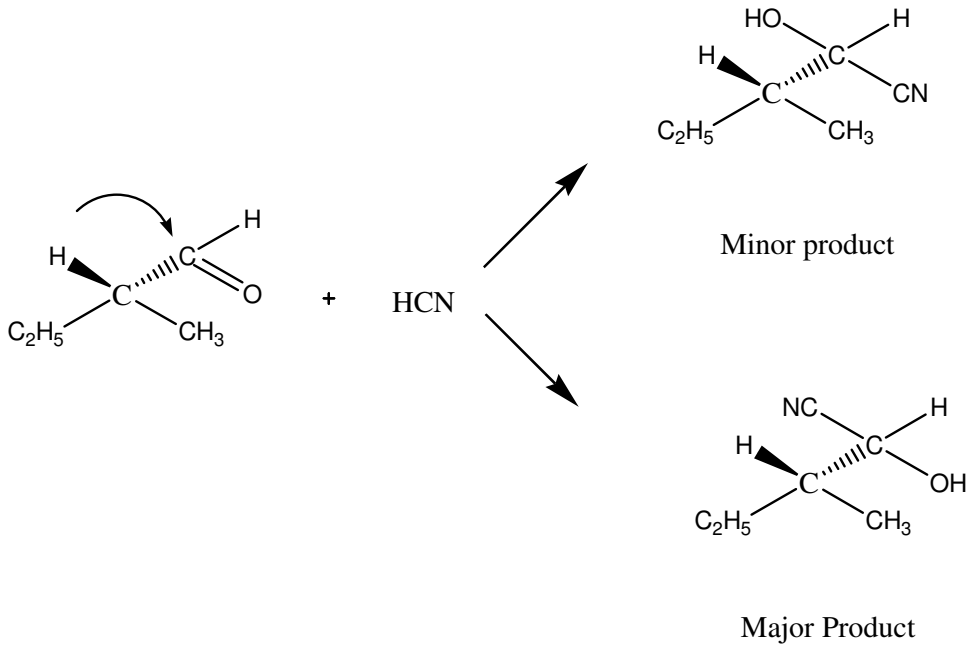


Figure 1.5 Prediction of Major Product with Crams Rule

The rule is that the incoming group (CN) preferentially attacks on the side of the plane containing the small group (H) [11,12].

1.5.2 Auxillary Controlled (2nd Generation) Method

Control is again achieved intramoleculary by a chiral group in the substrate. The difference is that the directing group in the substrate (the chiral auxiliary) is now deliberately attached to an achiral substrate in order to induce asymmetric synthesis (direct the reaction) and then removed again [11,12].

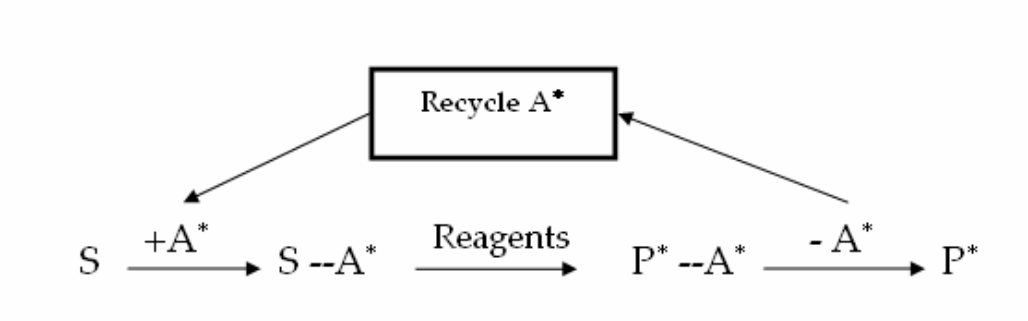


Figure 1.6 Auxillary Controlled Method to Produce Chiral Compound

An additional useful feature of this approach is that two possible products [$P_1^* - A^*$ and $P_2^* - A^*$] resulting from the alternative sides of attack on S are not enantiomers but diastereomers as a result of the presence of the additional stereogenic center of A^* [11].

This means that, the undesired diastereomer can be removed by chromatography or crystallization so that, after the removal of the auxiliary, the final product is obtained in very high e.e. [enantiomeric excess) [11].

1.5.3 Reagent Controlled (3rd Generation Method)

Although the 2nd method has proved very useful, the need for two extra steps to attach and then remove the chiral auxiliary is an unattractive feature. This can be avoided by using a reaction in which an achiral substrate is directly converted to the chiral product by the use of a chiral reagent.

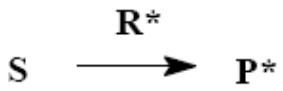


Figure 1.7 Reagent Controlled Method to Produce Chiral Compound

This is an attractive procedure but the range of reactions for which effective chiral reagents exists is somewhat limited at present [11,12].

1.5.4 Active Catalysts (4th Generation Method)

In each of the previously mentioned three classes, an enantiomerically pure compound has been required in stoichiometric amounts, although in some cases it could be recovered for reuse. The use of a chiral catalyst (chiral complex or enzyme) to direct the conversion of a chiral substrate directly to a chiral product with an achiral reagent (eg. Water, ammonia, etc.) [11].

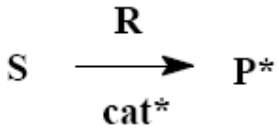


Figure 1.8 Active Catalysts Method to Produce Chiral Compound

This approach is the ideal solution to all problems of asymmetric synthesis. The major advantage is that only catalytic amounts of the chiral mediator are required, which provides obviously economic and practical irrelevant as so little is required [6].

In biotechnology, biocatalysis is commonly used in the production of chiral compounds. Often, only a single enantiomer of a chiral compound has therapeutic value. Typically, chemical syntheses of chiral compounds yield mixtures of enantiomers difficult and expensive to separate. In contrast, enzymatic processes often produce a single enantiomer in huge excess by eliminating the requirement for expensive separation processes.

1.6 Isolated Enzymes Versus Whole Cell Systems as Biocatalysts

In biocatalysis, enzymes can be used either as free enzymes or within cells (whole cell systems) [7]. The final decision as to whether one should use isolated, more or less purified, enzymes or whole microorganisms-either in a free or immobilized form- depends on many factors, such as

The type of reaction

Whether there are cofactors to be recycled or not, the scale in which the biotransformation has been performed.

The general pros and cons of the isolated enzymes and whole cells are listed in Table 1.1.

Table 1.1 Pros and Cons of Isolated Enzymes and Whole Cell Biocatalysts

	Form	Pros	Cons
Isolated Enzymes	Any	Simple apparatus, Simple work-up, better productivity due to higher concentration tolerance	Cofactor recycling necessary
	Dissolved in Water	High enzyme activity	Side reactions possible, lipophilic substrates insoluble, work-up requires extraction
	Suspended in Organic Solvent	Easy to perform, easy work-up, lipophilic substrates soluble, enzyme recovery easy	Reduced activity
	Immobilize	Enzyme recovery easy	Loss of activity during immobilization
Whole Cells	Any	☑No cofactor recycling necessary for redox reactions since the cells will recycle co-factor with existing machinery	☒Expensive equipment, tedios work-up due to large volumes, low productivity due to lower concentration

Table 1.1 Pros and Cons of Isolated Enzymes and Whole Cell Biocatalysts (Continued)

	Form	Pros	Cons
Whole Cells	Any	(true even if cells aren't growing) [2, 13] ☑ "Biomass is cheap" ☑ Only limited by the number of organisms that can be cultured, while enzymes are limited by the number that are commercially available [10,13]	tolerance, low tolerance of organic solvents, side reactions likely due to uncontrolled metabolism [2,8] ☒ Processes are more complicated, since you must design for cell growth and substrate conversion ☒ much more likely to have unwanted by products since many other enzymes exist
	Growing Culture	Higher activities	Large biomass, more by-product, process control difficult [2]
	Resting Cells	Work-upeasier fewer by-product	Lower activities
	Immobilize cells	Cell reuse possible	Lower activities

A whole conglomeration of biochemistry, microbiology and biochemical engineering-biotechnology-has led to the development of routes to a lot of specialty chemicals (ranging from amino acids to penicillin), starting from cheap carbon sources (such as carbohydrates) and cocktails of salts, by using viable whole cells. Such syntheses requiring a multitude of biochemical steps are usually referred to as "fermentation" processes, since they constitute de novo synthesis in a biological sense [2].

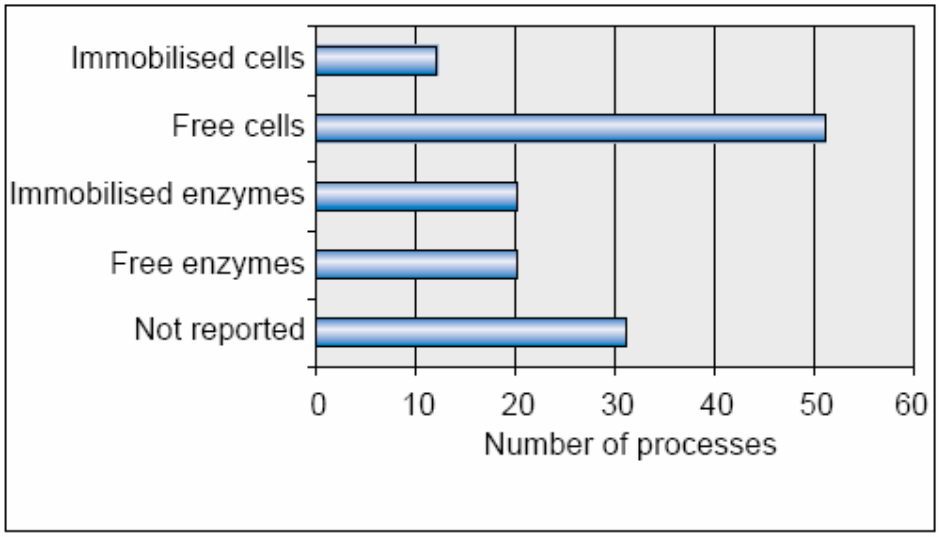


Figure 1.9 Use of Enzymes or Whole Cells in Industrial Biotransformations (Based on 134 processes) [3]

In general, whole cells are more popular than (partly) isolated enzymes [Figure 1.9]. When whole cells are used, immobilization is less common than when isolated enzymes are used.

Although the reaction control becomes, more complex on using fermenting micro organisms, the selectivities achieved are sometimes

significantly higher when compared to the use of isolated enzymes. This was shown by the successful resolution of a secondary alcohol via hydrolysis of its acetate by *Bacillus sp*. R-enantiomer was produced with e.e. 94% while S-enantiomer at little amounts, while other biocatalytic methods to obtain desired masked chiral hydrocyaldehyde failed. [2]

1.7 FUNGAL BIOCONVERSION REACTIONS

Filamentous fungi are found ubiquitously in the environment, inhabiting ecological niches, such as soil, living plants and organic waste material. The ability of fungi to rapidly adapt their metabolism to varying sources of carbon and nitrogen is an integrated aspect to their survival. This metabolic flexibility is achieved through the production of a large set of intra- and extracellular enzymes able to degrade complex biopolymers. In addition to the production a secretion of a number of enzymes, filamentous fungi can secrete a great diversity of primary and secondary metabolites (e.g. antibiotics) and perform many different complex conversions, such as the hydroxylation of complex polyaromatic hydrocarbons and steroid compounds.

Fungal bioconversion of organic compounds is by no means a new technique, but its use as a synthetic tool in organic chemistry is more recent, dating only from the 1950's. In that decade, the use of fungi to introduce hydroxy groups into the steroid skeleton was developed by the pharmaceutical industry as part of commercial synthesis of corticosteroids [9].

Since that time, the range of reactions that can be efficiently carried out by fungal bioconversion has been expanded enormously, and now includes examples of hydrolytic oxidation, condensation and reduction processes. Other studies have focused on substrate groups such as steroids, alkaloids, sulfides, environmental pollutants and bioactive compounds, single bioconversion reactions such as halogen metabolism and alcohol dehydrogenase activity.

The most frequently observed reactions of fungal bioconversion are in Figure 1.10 for two common substrate groups, steroids and aromatic compounds.

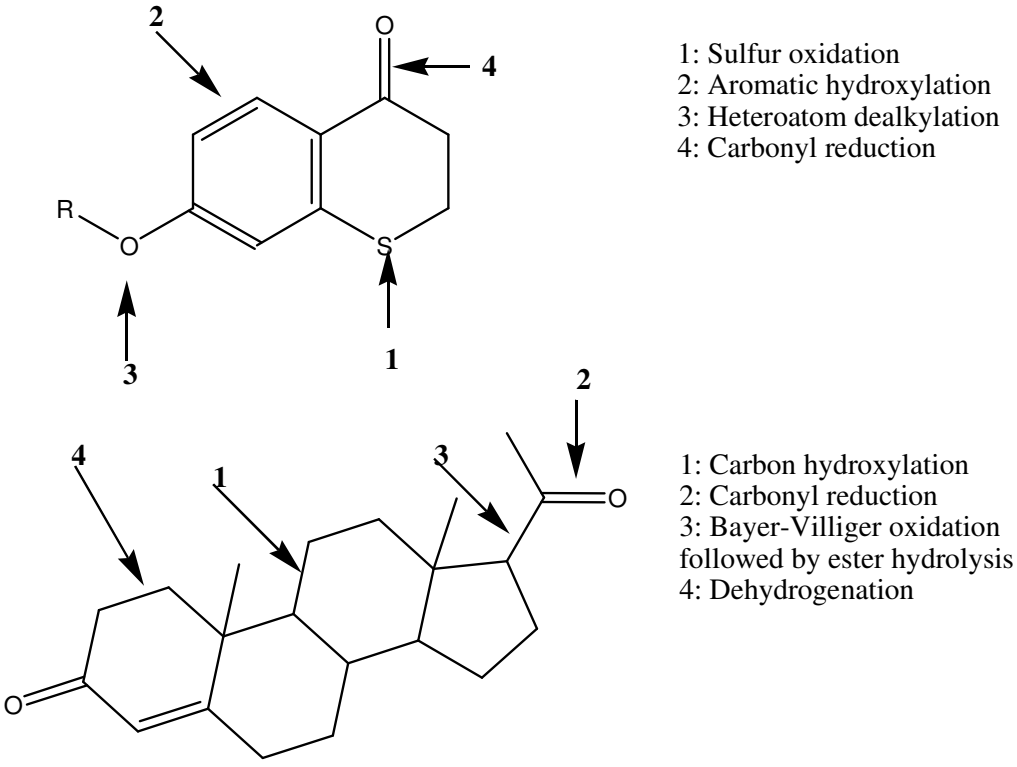


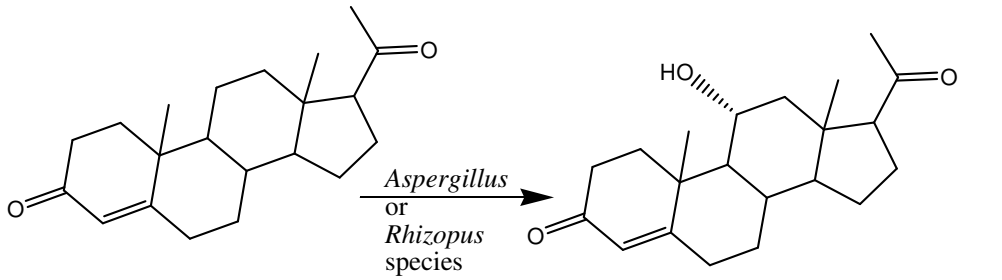
Figure 1.10 Common Reactions of Fungal Bioconversion

The selection of a particular reagent in synthetic chemistry is usually based on several criteria such as availability, cost, convenience of use, chemical yield, stereoselectivity, and ease of work-up and product purification, and it is by these criteria that fungal bioconversion as a method of doing organic chemistry must be judged. All other things being equal, two of these criteria are dominant, namely availability and stereoselection. In competition with an otherwise equivalent chemical processes, bioconversion is often superior on grounds of stereoselection; considering for example the methods available for oxidation of the alkaloid glucacine (3 in Figure 1.10] to dehyroglaucine. Chemical oxidants are non-stereoselective, but bioconversion using *Fusarium saloni* ATCC 12823 is specific for the S(+) enantiomer 3 of the substrate, permitting recovery of (R)-glaucine in quantitative yield [9]. Another example of fungus bioconversion is hydroxylation of progesterone at C-11α Conversion of 1 to 2 in Figure1.11.

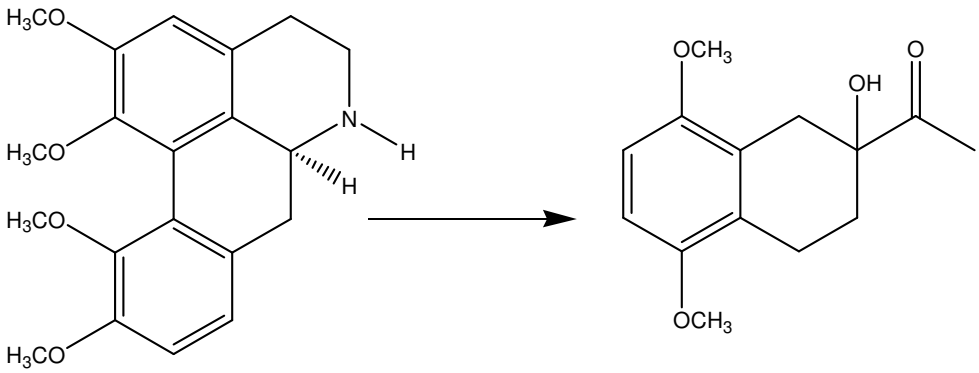
The important criterion of availability is, however, one that is often more difficult to meet, usually because the desired chemical transformation is one for which no equivalent body of literature, from primary to tertiary levels, one the choice of the appropriate chemical reagent for a desired synthetic conversion, where as the number of such methods applicable to given reaction is relatively small. When choosing a chemical reagent for ester hydrolysis, probably true to say that of the more than 15000 fungi available from world's culture collections, most are capable of ester hydrolysis by bioconversion.

For reason such as these, availability of correct microorganism is

often limited by the relative number of culture whose bioconversion potentials have been determined by through screening. It may be true to say that almost any desired carbon-carbon hydroxylation or stereospecific hydrolysis is possible by bioconversion, but the extend of screening that may be necessary to find the appropriate microorganism is daunting.



1 **2**



3 **4**

Figure 1.11 Typical Fungal Bioconversion Substrate and Products

Not all of these reactions occur with every substrate, but most common, the hydroxylation and Bayer-Villigers oxidation. Thus, although it is possible to distinguish, for example, steroid hydroxylation from Bayer-Villigers oxidation and dehydrogenation by selection of the appropriate fungus for bioconversion, it may not always be possible to select fungi that will hydroxylate a steroid ester without some degree of ester hydrolysis, or sulfoxidize a ketosulfide without reduction of carbonyl group.

The products are extensively utilized by pharmaceutical, fine chemical and food and agricultural industries. Some products of biotransformation showed in Figure 1.12.

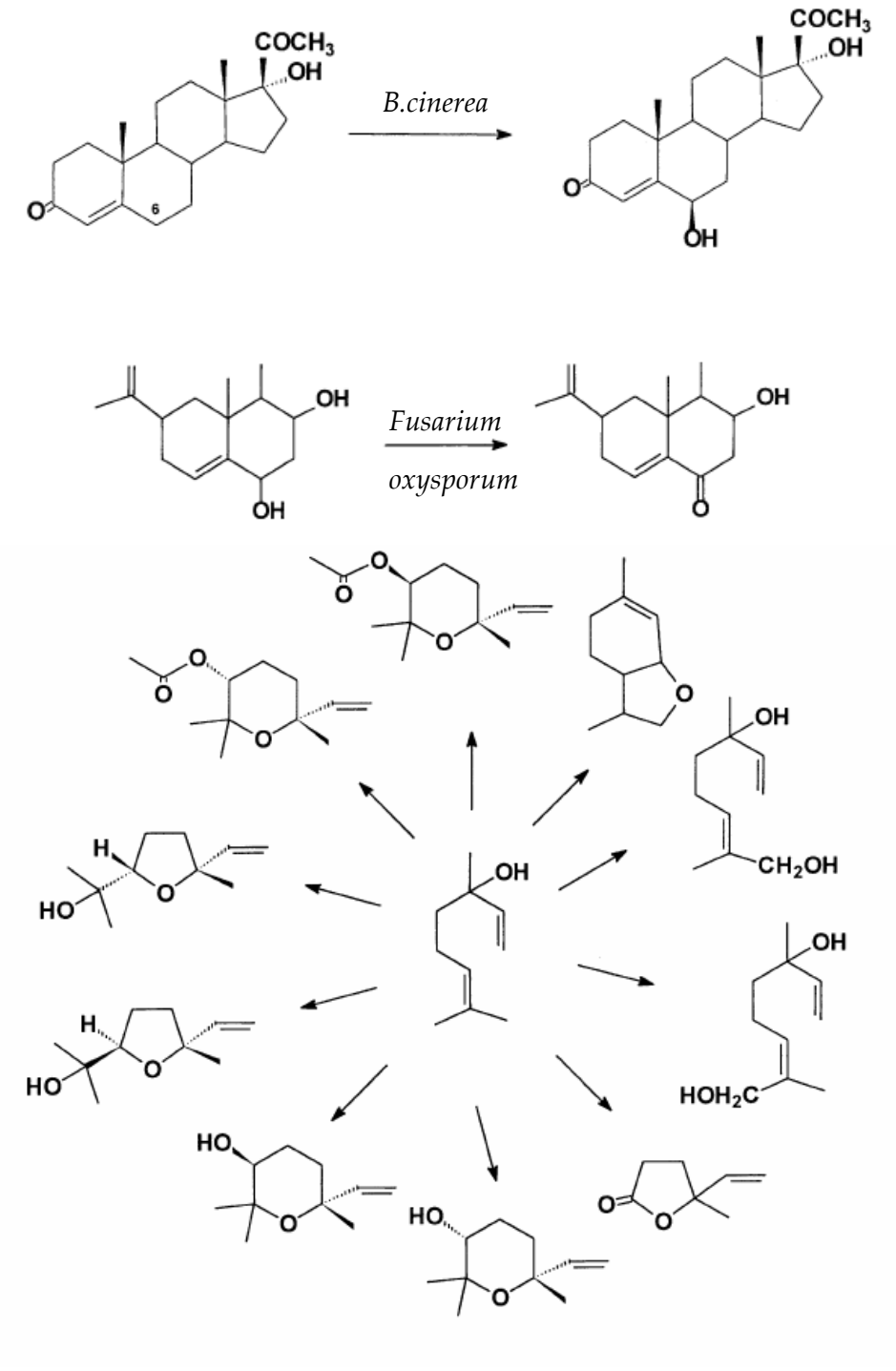


Figure 1.12 Some Products of Biotransformation

1.8 THE IMPORTANCE OF α-HYDROXY KETONES

Several functional groups display a high substrate induction. Most of them possess coordinating properties allowing a temporary binding of the incoming achiral reagent via, for example, Van-der Waals interactions. This pre-coordination of the reagent via the coordinating group (CG) in Figure 1.14 can be used to induce a high level for stereoselectivity, especially if the reacting functional group is located proximal to the coordinating group. Such a combination of directing functional group and a functionalizable achiral moiety can be regarded as 'privileged synthon' [17].

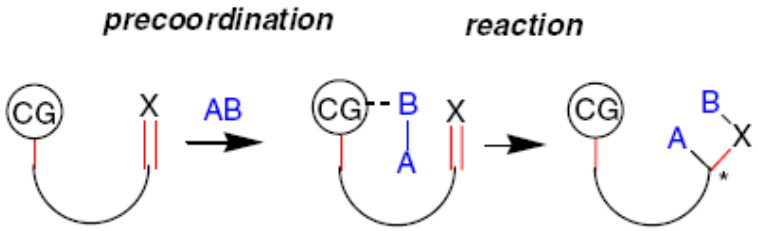


Figure 1.13 Mode of Operation in Privileged Synthon [17]

The hydroxyl group has frequently been used as a reagent directing group, for example, for the selective elaboration of alcohol products [17]

A structurally related motif that fulfils all the criteria of a privileged synthon is the α-hydroxy ketone (acyloin) [17]. The chiral ketones and chiral diols are valuable intermediates in synthesis of biologically active compounds. Several selective transformations of this

compound class have been developed and indicated that this motif is ideal for the creation of structural diversity and complexity of exemplified in Figure1.14.

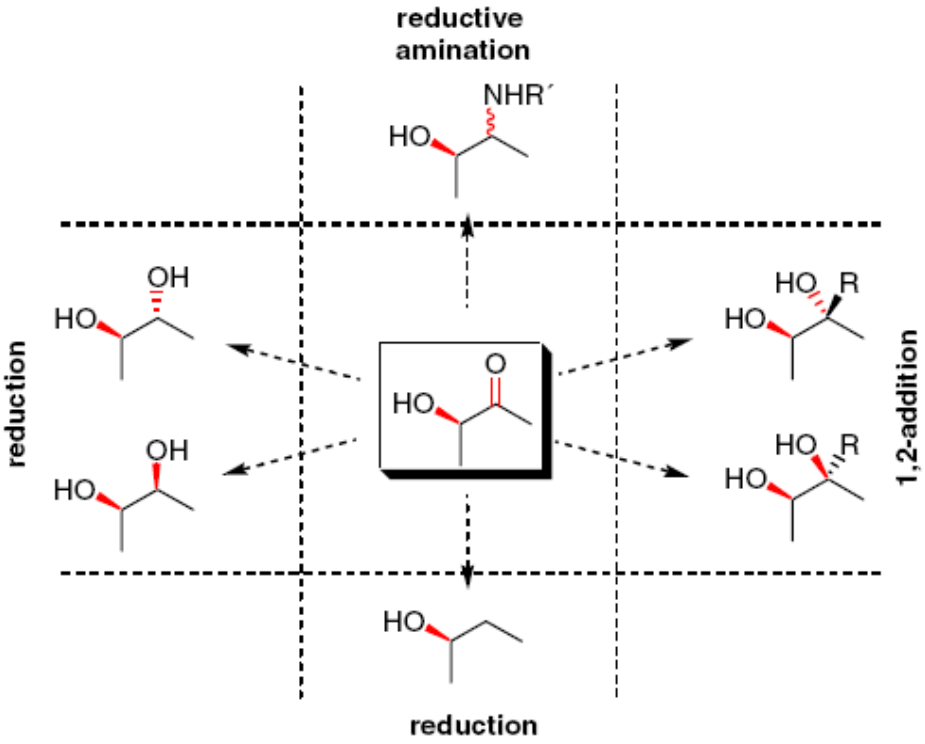


Figure 1.14 The Acyloin as a Privileged Synthon in Asymmetric Synthesis

Some example of usage area of α-hydroxy ketones are that; the condensation of α-hydroxy ketones with carbonate then crystallization give dihyrooxadiazione type molecules [11]. This type of molecules are useful blowing agent for polycarbonates. Also α-hydroxy ketones are important intermediates in the microbiological reduction of isopropilaminomethy-2-napthy ketones. [14].

1.9 METHODS FOR THE FORMATION OF α-HYDROXY KETONES

In literature, there are many methods given for the formation of α-hydroxy ketones. There are both chemical and biotechnological methods for the synthesis of α-hydroxy ketones.

1.9.1 Chemical Synthesis

Classic chemical methods have been supplemented by several heavy metal-containing oxidants such as MoO_5.Py.HMPA [19] and CrO_2Cl_2 [20], but these type agents are potentially contaminating so chemists are trying to minimize as much as possible the use of them.

1.9.1.1 Catalytic α-hydroxylation of ketones and ketohydroxylation of olefins.

Two main synthetic routes have been described in the past three years: catalytic α-hydroxylation of ketones and ketohydroxylation of olefins.

In catalytic α-hydroxylation of ketones, a carbonyl compound is transformed into an enolate or enamine with subsequent oxidation of the C-C double bound to provide access to α-hydroxylated products. [Path A, Figure 1.15]. Various oxidizing agents can be used for non-stereoselective C-O bond formation, with m-CPBA being the most prominent [33].

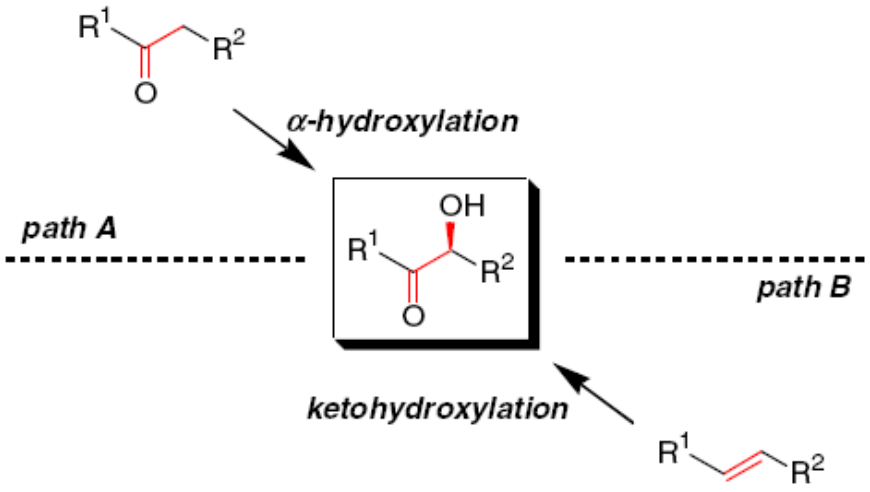


Figure 1.15 Oxygen Transfer Routes Towards α-Hydroxy Ketones [17]

The ketohydroxylation of olefins on the other side introduces three C-O bonds in one step [Path B, Figure 1.15], [33]. With regard to recent progress in the field of olefin metathesis, the ketohydroxylation seems to be a promising new alternative route towards α-hydroxy ketones complementing the acyloin synthesis developed so far. Some examples of ketohydroxylation are given in Figure 1.16 [17]

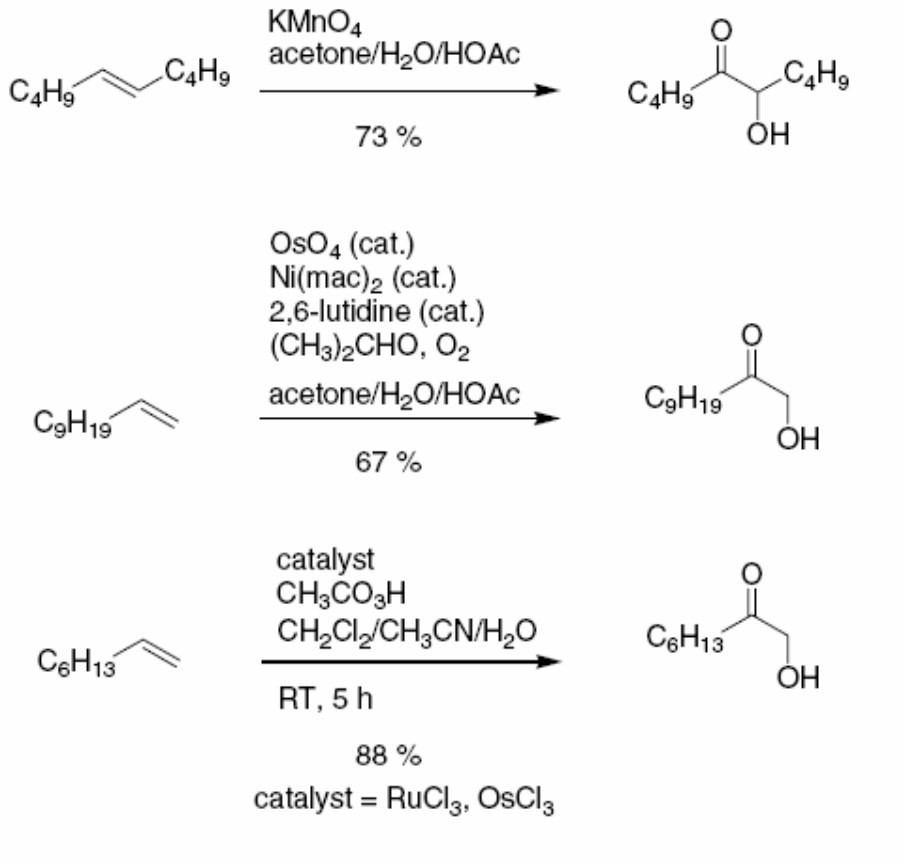


Figure 1.16 Ketohydroxylation of Olefins [17]

1.9.1.2 Direct Organocatalytic α-hydroxylation of Ketones

Another method to α-hydroxylation of ketones is organocatalytic α-hydroxylation of ketones. Organocatalysis is a rapidly developing area of research in organic chemistry. Most recently, amino acids and their derivatives were reported to catalyze α-oxidation reactions with nitrosobenzene as the oxygen source to give α-aminoxylated aldehydes and ketones[29]. Cordova and co-workers studied on 1,2-diols by enantioselective organocatalytic α-oxidation with molecular oxygen and before synthesising 1,2-diols they obtained α-hydroxylated of ketones

by direct catalytic enantioselective α-hydroxylation of aldehydes and ketones. (Figure 1.17-1.18). Enzymatic resolution could also been employed as a key step for their synthesis. However, most of these preparations require multiple manipulations, and no direct method from the corresponding aldehyde is available [30,31]. For these reasons, the development of new methodologies for the direct catalytic enantioselective α-hydroxylation of aldehydes has become an intriguing target in organic synthesis.

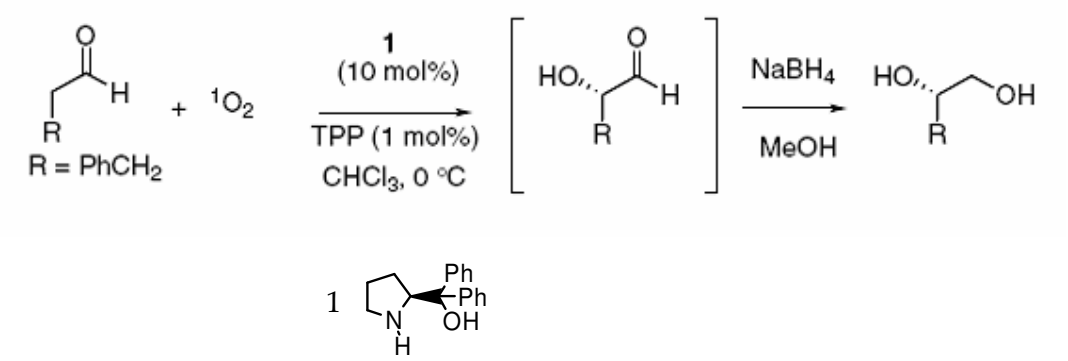


Figure 1.17 Direct Organocatalytic Enantioselective α-Oxidation of Aldehydes with Molecular Oxygen,

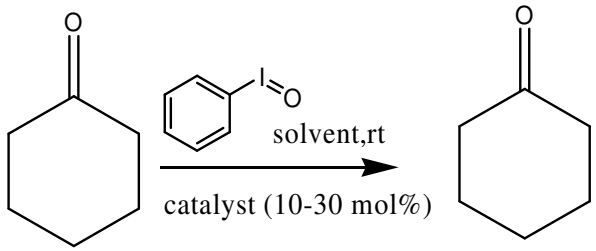


Figure 1.18 Direct Organocatalytic Asymmetric α-Oxidations with PhIO [31]

1.9.1.3 $Mn(OAc)_3$ mediated chemoenzymatic synthesis of α-hydroxy ketones

Beside these methods, literature methods gave unsatisfactory results to produce hydroxylated ketones. To overcome this problem, Demir and co-workers studied on $Mn(OAc)_3$ mediated acetoxylations to synthesize α-hydroxy ketones[32,35]. Manganese(III) acetate, bear many similarities with other one-electron oxidants like Co(III), Ce(IV) and some two-electron oxidants like Tl(III) and Pb(IV). However, lower reactivity and higher selectivities is observed with manganese (III) acetate compared with the other oxidizing agents. Demir and co-workers synthesized α-hydroxy ketones by chemoenzymatic synthesis [34] (Figure 1.19)

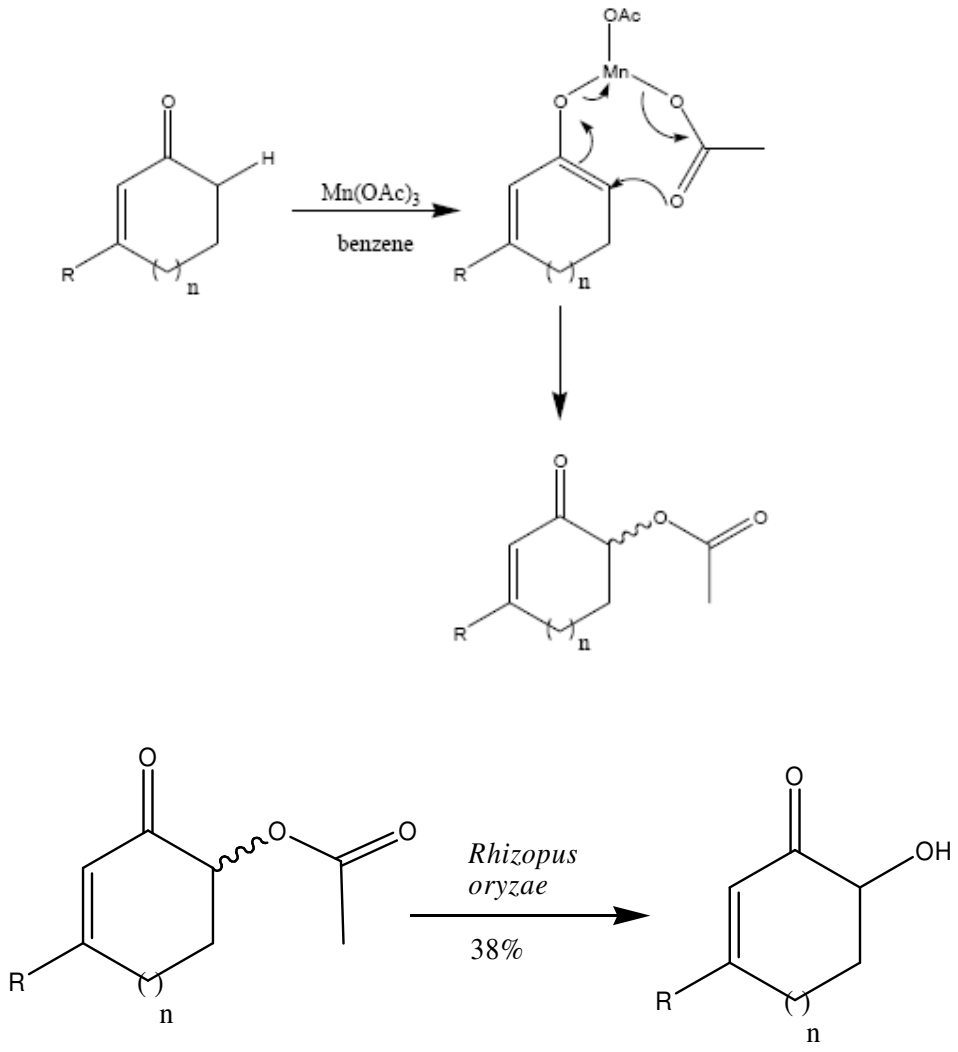


Figure 1.19 Chemoenzymatic Synthesis of α-Hydroxylation of Ketones

1.9.2 Biotechnological Sytnhesis

As an alternative way to the chemical methods, optically active α-hydroxy ketones can be prepared enzymatically by reduction of the α-

diketones with yeast (baker's yeast) as the biocatalyst [21]. However, this enzymatic method possesses the following disadvantages: further reduction of diketone to the vic-diol, formation of both regioisomeric α-hydroxy ketones and moderate chemical yields.

Esterases and lipases have a major application in the preparation of chiral molecules for synthesis. PLE (Pig liver esterase), PPL (Porcine pancreatic lipase), CCL (Candida cylindracea lipase), α-chymotrypsin, and PCL (Pseudomonascepacia lipase) are some commonly used enzyme systems for hydrolysis. Lipases have been widely used for the synthesis of optically active alcohols, carboxylic acids and esters via enantioselective esterification and transesterification in organic solvents. Although numerous α-hydroxy acids and esters have been resolved by lipases, reports on the kinetic resolution of structurally simple α-hydroxy ketones by these readily accessible enzymes are scarce. Recently, Gala et al. have described [22] the resolution of α-hydroxy aryl ketones (precursors of chiral azole antifungal reagents) by lipase catalyzed hydrolysis of the corresponding acetates in phosphate buffer; nevertheless, the irreversible transesterification route of this enzymatic reaction appears not to be known. Another report has been presented by Adam et al. that is the kinetic resolution of racemic α-hydroxy ketones by lipase-catalyzed irreversible transesterification with isopropenyl acetate in organic media (Figure 1.21) [23].

Biologically, the methods of preparation of optically active compounds are classified into two broad categories: optical resolution of racemic compounds and asymmetrization of prochiral compounds. In

both cases, biocatalysts are widely utilized. The most popular enzymatic approach to obtain the optically active compound is kinetic resolution, when the starting material is a racemic mixture. However, simple kinetic enzymatic resolutions are restricted to a maximum yield of 50% per enantiomer.

More useful is dynamic resolution which is the coupling of racemization with resolution. By dynamic resolution, it is essential that the starting material racemizes under the reaction conditions, while the product does not. The most straightforward is the synthesis of the target molecule in racemic form and its conversion to the optically active form. Demir and his co-worker studied on enzymatic deracemization reactions using the fungus *Rhizopus oryzae*. This deracemization reaction inverts the chirality of one enantiomer of a racemate to the other antipode, resulting in an optically active compound starting from a racemic mixture and it is entirely different from the above-mentioned dynamic resolution (Figure 1.20)[33].

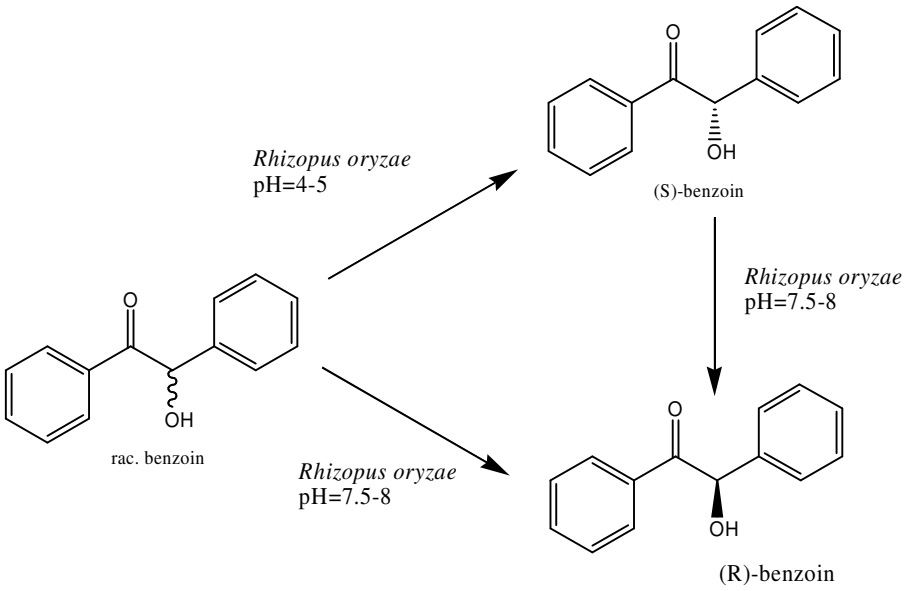


Figure 1.20 Deracemization of benzoin by *Rhizopus oryzae*

As mentioned in the chemical synthesis, as a result of Manganese (III) acetate – enzyme hydrolysis, enantiomerically pure α-hyroxy ketones could be obtained. The enantioselective synthesis of hydroxy ketones from ketones via Mn(OAc)3 mediated acetoxylation followed by enantioselective ester hydrolysis utilizing *Rhizopus oryzae* was presented by Demir and co-workers [24]. In an other study, they achieved the synthesis of α-hydroxy ketones by enzymatic kinetic resolution using a number of lipases [25-27,33].(Figure 1.2.)

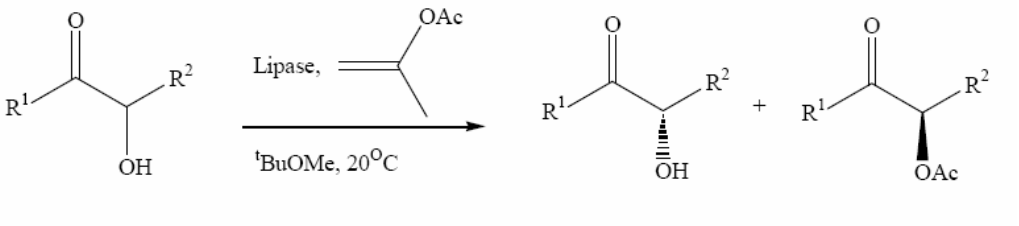


Figure 1.21 Kinetic Resolution of α-hydroxy ketones

1.10 ASPERGILLUS NIGER

Aspergillus species are highly aerobic and are found in almost all oxygen-rich environments, where they commonly grow as molds on the surface of a substrate, as a result of the high oxygen tension. Commonly, fungi are grown on carbon-rich substrates, mainly monosaccharides such as glucose. *Aspergillus*, however, can also secrete amylase enzymes, which allow it to use polysaccharides as a carbon source, for example starch. As a result of this, *Aspergillus* species are common contaminants of starchy foods, for example bread and potato, and grow in or on many plants and trees.

In addition to growth on carbon sources such as glucose, fructose, maltose, and starch, many species of *Aspergillus* demonstrate oligotrophy: they are capable of growing in nutrient-depleted environments, or environments in which there is a complete lack of key nutrients. *A. niger* is a prime example of this; it can be found growing on damp walls [18]

A. niger is a fungus and one of the most common species of the genus *Aspergillus*. It causes black mold on certain types of fruit and vegetables, and is a common contaminant of food. It is one of the common molds known to produce aflatoxin.

A. niger is cultured for the industrial production of some chemical compounds. Various strains of *A.niger* are used in the industrial preparation of citric acid (E330), gluconic acid (E574) and the enzymes glucoamylase and α-galactosidase, and have been assessed as acceptable

for daily intake by the world health organization. The fungus is also used by the biotechnology industry to produce biological macromolecules, especially as magnetic isotope-containing varieties for NMR analysis.

A niger colonies on potato dextrose agar at 25°C are initially white, quickly becoming black with conidial production [18].

1.11 AIM OF THE WORK

The aim of this study was to test the ability of *Aspergillus niger* to selective oxidation of ketones into α-hydroxy ketones, which are important key intermediates for the synthesis of bioactive compounds. In addition to this, conditions for the synthesis of both enantiomers of the product with high enantiomeric excess were analysed.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

The chemicals used in this study, glucose, peptone, yeast extract, KH_2PO_4, K_2HPO_4, $NaNO_3$, KCl, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$, starch, potato dextrose agar were purchased from Sigma & Aldrich The substrates 1-tetralone and 1-indanone were obtained from Merck. DMSO (Dimethyl Sulfoxide), ethanol and all other chemicals were of analytical grade and purchased from Sigma & Aldrich.

2.1.2 Microorganisms

Aspergillus niger strain was obtained from Tübitak-MAM research center.

2.2 METHODS

2.2.1 Microbial Cultivation

Aspergillus niger culture was inoculated onto potato dextrose agar (PDA prepared by adding 19 g potato dextrose agar in 1 L distilled

water) and incubated at 37 °C for 7-8 days until complete sporulation and stored at 4 °C for maximum 3 months.

Spores from the stock culture were inoculated into liquid precultures which were named as medium B, medium C and medium D. Composition of culture medium : [B]= glucose 30 g/L, peptone 10 g/L, (C/N=7.5) [C]= glucose 20 g/L, yeast extract 20 g/L, peptone 5 g/L, KH_2PO_4 2g/L, K_2HPO_4 2g/L, $NaNO_3$ 2g/L, KCl 0.5 g/L, $MgSO_4.7H_2O$ 0.5 g/L, $FeSO_4.7H_2O$ 0.02 g/L (C/N= 2.55), [D]= yeast extract 4g/L, starch 15 g/L, K_2HPO_4 1g/l, $MgSO_4.7H_2O$ 0.5 g/L (C/N= 16.67). [34].

The preculture was used for the vegetative growth of the spores before inoculation into main culture. After 24 hours incubation at 37 °C, preculture was transferred into the main cultures which contained the same medium as the precultures. Preculture volume was 4% of the main culture volume. All the cultures were incubated at 175 rpm in a shaker incubator at 37 °C.

2.2.2 General Procedures for Biotransformation

2.2.2.1 Biotransformation Procedure for Growing Cells

After 72 hours of incubation, the appropriate ketones (1-tetralone and 1-indanone), were added into the growth media and incubation was continued. The progress of the biotransformation was monitored by TLC and GC-MS analysis. Every day, 5 ml sample were taken and extracted to prepare TLC and GC-MS samples.

2.2.2.2 Biotransformation Procedure for Resting Cells

After 72 hours of incubation, the fungal cells were harvested by filtration. Biotransformation was performed by re-suspending the wet cells of *A. niger* in 40 ml phosphate buffer solutions (pH 7.0, 0.1 M) in a 100 ml Erlenmeyer flask. To these cell suspensions, ketones (200 µl 1-tetralone and 120 µl 1-indanone) were added and incubation was continued. The progress of the biotransformation was monitored by TLC and GC-MS analysis. Every day, 5 ml sample was taken and extracted to prepare TLC and Gc-MS samples.

2.2.3 Optimization of Growth Medium to Obtain Highest Enantiomer Excess (E.E) Values

Enantiomeric excess (e.e.) value is important to obtain enantiomerically pure compounds. To increase e.e. values of the product, different medium compositions (B, C, and D); different medium pH's (4, 4.5, 5, 5.5, 6, 7 and 8), and different organic solvents to dissolve substrates (DMSO and ethanol) were used.

2.2.4 Monitoring Product Formation by Thin Layer Chromatography (TLC)

All samples were loaded onto TLC silica plates purchased from Sigma-Aldrich at suitable size by capillary tubes. A small amount of mixture to be analyzed was loaded as a small spot (not more than a few millimeters) on a TLC plate sheet. If the sample was taken from the growth medium, a small amount of ethyl acetate was added into sample

and mixed to extract products to the organic phase. The TLC plates were 4 cm width and 5 cm height. The sheet is then placed in a TLC container containing a small amount of solvent which was the mobile phase. The mobile phase- the running solvent- was ethyl acetate: distilled hexane solvent system at different ratios (v:v) depending upon the type of substrate (1:3 for 1-tetralone, 1:2:0.01 methanol for 1-indanone).

After solvent migration was completed, thin layer plates were left to drying and were then monitored under 254 nm UV light. In the case where the products were not UV active, the plates were stained with a suitable dye (such as anisaldehyde).

2.2.5 Extraction Procedures

When product formation was detected by UV light or by staining, the product was extracted by ethyl acetate from the reaction mixture. The extract was dried over anhydrous $MgSO_4$ and ethyl acetate removed by rotary evaporator to obtain product. The products were dissolved in chloroform and GC-MS and HPLC were applied to the organic solvent containing the product.

2.2.6 High Performance Liquid Chromatography (HPLC) Analysis of Products

HPLC from Thermo Separation with P2000 vacuum pump and UV detector and Agillent 1100 series with G1315B DAD, G1313A ALS, G1311A QuatPump and G1379A Degasser were used to analyze enantiomeric excesses of the organic products. Chromquest was the

controller program for retention time, peak width and peak area. Detection was performed at 254 nm. OD and OB-H chiral columns were used to analyze all fractions and the solvent system consisted of 10% isopropanol and 90% hexane (HPLC grade).

2.2.7 Gas Chromatography- Mass spectrophotometer (GC-MS) Analysis of Products

GC-MS analysis was performed by Agillent 6890N Series GC and the column used was HP-5ms with the specifications; (5%-Phenyl)-methylpolysiloxane, Non-polar, Very low bleed characteristics, ideal for GC/MS, Excellent inertness for active compounds including acidic and basic compounds, Improved signal-to-noise ratio for better sensitivity and mass spectral integrity, Bonded and cross-linked; solvent rinsable and Equivalent to USP Phase G27.

2.2.8 Nuclear Magnetic Resonance (NMR) Spectra Analysis of Products.

In this study, all compounds were identified by Carbon (^{13}C-NMR) and Proton (^{1}H-NMR) NMR spectra (BRUKER DPX 400 MHz) by using tetramethylsilane (TMS) as an internal standard and deutereo chloroform as solvent.

2.2.9 Purification of Product by Flash Column Chromatograhy

Flash column chromatography was done for purifying the product by using silicagel 60 (mesh size 40-60 μm). The solvent system used in flash column chromatography was same with the system used in TLC

analysis

2.2.10 General Procedure for Synthesis of Products

2.2.10.1 Synthesis of 2-hydroxy-1-Tetralone

Commercially available 1-Tetralone (200 μl) were dissolved in 600 μl DMSO for studies of enantiomeric excess. Additionally, the effect of pH's on e.e % was investigated by changing medium pH's (4, 4.5, 5, 5.5, 6, 7, 8). Medium pH was adjusted by using 1 N NaOH and 1 M HCl.

The product was isolated as a yellow liquid after flash column chromatography (Ethyl acetate/Hexane; 1:3)

For (R)-isomer, t_R= 22,43 min, for (S)-isomer t_R= 23.42 min

1H-NMR (400 MHz, CdCl3+CCl4)

δ (ppm) : 2.02 (m, 3-H_{ax})

2.52 (m, 3-H_{eq})

3.10 (m, 2H, 4-H)

3,81 (br s, 2-OH)

4.40 (dd, J=13.5, 5.4 Hz, 2-H)

7.26-7.39 (m, 6,8-H)

7,54 (m, 7-H)

8,15 (dd, J=7.7, 1.3 Hz, 5-H)

13 C-NMR (100 MHZ, CDCl3+CCl4)

δ (ppm): 32.2

35.15

68.1

127.5

127.7

128.9

131.9

134.6

146.2

197.9

MS: 162,144,134,115,105,77

2.2.10.2 Synthesis of 2-hydroxy-1-Indanone and Indane-1,2-diol

Commercially available 1-Indanone (120 µl) were dissolved in 480µl DMSO and also 480µl ethanol for studies of enantiomeric excess. Additionally, the effect of pH's on e.e % was investigated by changing medium pH's (4, 4.5, 5, 5.5, 6, 7, 8).

The product was isolated as a yellow liquid after flash column chromatography (Ethyl acetate/Hexane/metanol; 1:2:0.02)

For (R)-isomer, t_R= 23,260 min, for (S)-isomer t_R= 26,062 min

1H-NMR (400 MHz, CdCl3+CCl4)

δ (ppm) : 3.02 (dd, J=5.0, 16.5 Hz,1H)

3.30 (s, 1H)

3.58 (dd, J=8.0, 16.5 Hz, 1H)

4.56 (dd, J=5.0, 8.0 Hz, 1H)

7.37-7.47 (m-2H)

7.61-7.67 (m, 1H)

7.77 (d, J=8.0 Hz, 1H)

13 C-NMR (100 MHZ, CDCl3+CCl4)

δ (ppm): 35.3

74.2

124.4

126.9

128.0

134.0

135.9

151.0

206.7

MS: 148, 130,119,102,91,77,51

CHAPTER 3

RESULTS & DISCUSSION:

3.1 PERSPECTIVE OF THE WORK

α-hydroxy ketones are important synthetic building blocks, particularly in their enantiopure form due to reactive functional groups which can be transformed to other functional groups. As mentioned before, many biologically active compounds contain these structures.

Patonay and Krawczyk studied on the organic synthesis of α-hydroxy-ketones[36,37]. During these studies (R,R)-(-)N-N′-Bis(3,5-di-tert-butylsali cylidane)-1,2-cyclohexane diamino manganese(III) chloride (Jacobsen's complex) was used as the metal-catalyst to afford optically active α-hydroxy ketones from prochiral substrates.

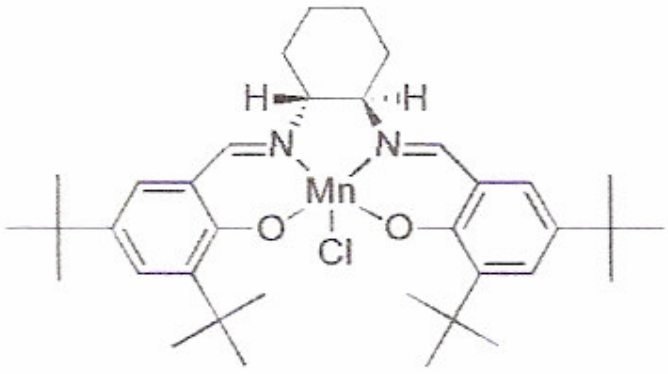
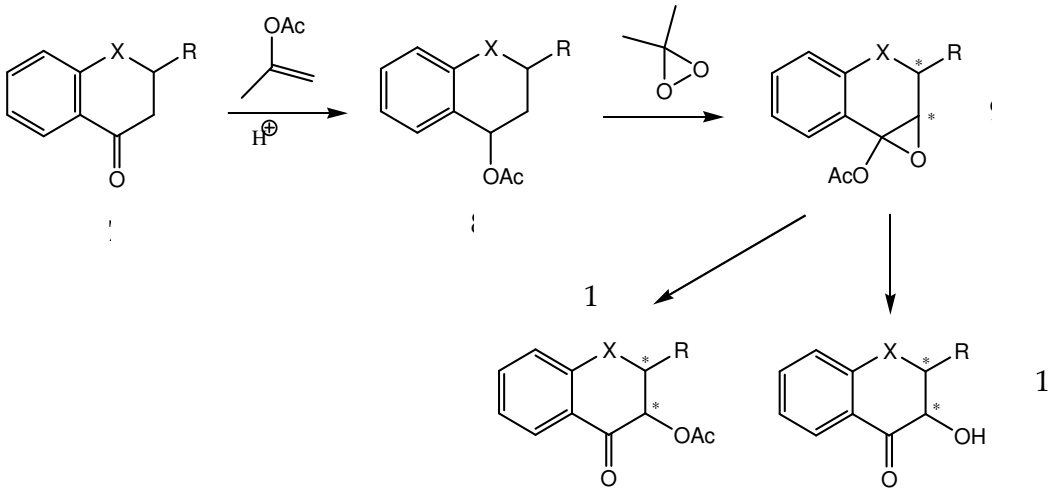


Figure 3.1 Jacobsen's complex

In the work of Patonay, firstly the starting material enolacetates

were synthesized by the classical acid-catalyzed trans esterification of the corresponding ketones and isopropenylacetate [37]. Accordinglu mixtures of α-hydroxy ketones and α-acetoxy ketones were obtained in moderate yields and low enantiomeric excesses (15-57% and 34-48% respectively), when enolacetates were allowed to react with dimethyl-dioxirane (DMD) as oxidant, without any chiral catalyst.



7-11	a	B	c	*d*
X	Bond	CH_2	O	*O*
R	H	H	H	Ph

Ph: phenyl group

Figure 3.2 Synthesis of 1-tetralone and 1-indanone chemically

Instead of DMD, when they use (salen)Mn(III) Jacobsen's complex was used as the catalyst, the yields and enantiomeric excesses were increased up to 96% [38].

Additionally, Demir and coworkers have reported the Manganese

(III) acetetate [$Mn(OAc)_3$] mediated acetoxylation of enones and aromatic ketones, enzymes and fungus mediated resolution of acetoxy enones to obtain optically pure α-hydroxy ketones as shown in Figure 2.3 [32].

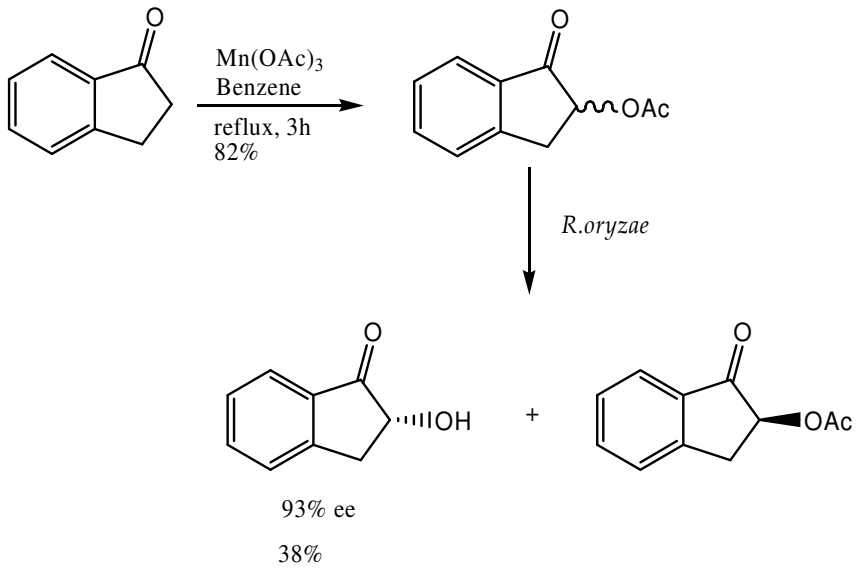


Figure 3.3 Mn(Oac)3 mediated Acetoxylation of 1-indanone and formation of 2-hydroxy-1-indanone by bioconversion

Based on preliminary information available from previous studies, we were able to develop an effective route for the synthesis of 2-hydroxy-1-tetralone, 2-hyroxy-2-indanone and indane-1,2-diol by using the whole cell biocatalysis (Figure 3.4).

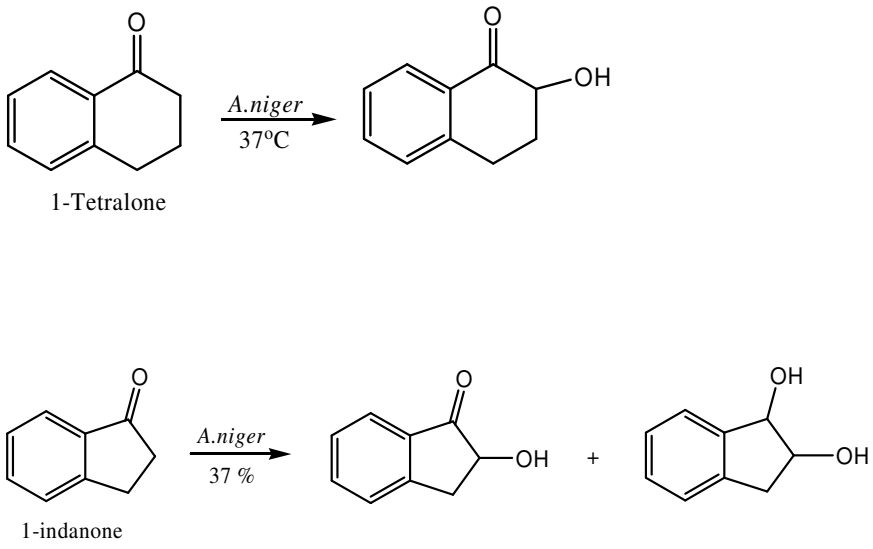


Figure 3.4 Synthesis of hydroxy ketones by *A.niger*

3.2 SYNTHESIS OF 2-HYDOXY-1-TETRALONE

The enantioselective hydroxylation of 1-tetralone was performed using *Aspergillus niger*. The bioconversion was performed at different conditions (different culture media and different pH's).

3.2.1 Effect of Medium Composition on Bioconversion and Enantiomeric Excess

In order to evaluate *Aspergillus niger* for the biotransformation of 1-tetralone, three culture media (B; C; D) were selected on described in Section 3.2.1. These media are the most preferred defined media formulations for the cultivation of this microorganism. After fungi were

grown in all three culture media for 3 days, 1-tetralone (200μL in 600μL DMSO) was added into each growth medium (B, C, D) and incubation was continued in the presence of 1-tetralone. The bioconversion was monitored via TLC by taking daily samples from the growth. After 48 hours, it was observed that bioconversion started in medium B and D mediums. After 12 days, conversion of the substrate in these media reached to 30-40%. There was little product formation in medium C. Reaction was terminated by adding organic solvent (Ethyl acetate or Dichloromethane) and the medium was extracted with ethyl acetate. 2-hydroxy-1-tetralone was obtained after separation via flash column chromatography. The product formation was justified by GC-MS and NMR analyses. (Figure A.1-A.2 and Figure A.5-A.6). The α-value for 2-hydroxy-1-tetralone isolated from medium B was $[\alpha]_D^{20}$= -17.51 (c=0.0026 ,$CHCl_3$). According to the α-value, the absolute configuration of the major enantiomer is found as S(-).

Since, for the *A. niger* mediated bioconversion of 1-tetralone, medium B and D gave higher conversion as compared to medium C, for further reactions, medium B and D were used.

3.2.2 Effect of pH on Enantioselectivity

The effect of pH on the enantioselectivity was studied with medium B and medium D at pH 5-8.

A. niger was cultivated in media B and D by adjusting the initial pH values of the growth media to 5, 6, 7 and 8. Biotransformation was monitored by TLC and after 12 days, reactions were terminated. The

products were again separated using flash column chromatography (1:3, ethylacetate: hexane), and enantiomeric excess values of the product were determined by HPLC analysis using a chiral column (DIACEL OD: hexane/2-propanol : 90:10, 0,5 ml/min, 254nm UV detection).

Table 3.1 Effect of pH on enantiomeric excess of 2-hydroxy-1-tetralone

pH	E.E.	
	Medium B	Medium D
5	87 % S(-)	N.D.
6	N.D.	22 % S(-)
7	75 % S(-)	N.D.
8	81 % S(-)	47 % S(-)
Unadjusted pH	52 % S(-)	N.D.

According to HPLC data, the medium which gave the highest ee value was chosen for further studies. Accordingly, the highest ee value was at pH 5, and suggested that the e.e values could be further increased by decreasing the pH. Therefore, further pH screening was done in medium B to obtain optimum conditions for the whole cell biocatalysis of 1-tetralone at pH's 4, 4.5, 5, 5.5 and 6. The same procedure was followed for the cultivation of *A. niger* and biotransformation of 1-tetralone. The results of HPLC analysis showed that the best ee value was obtained in medium B at pH 5.0. (Figure A.12)

According to the results, at pH 4, there is a sharp decrease in ee

value (Table 2.2). The reason for this low ee value could be the result of a change in the 3-D structure of the active site of the enzyme or enzymes which are responsible from bioconversion of 1-tetralone. Since the enantioselectivity comes from the selectivity of the enzyme, and the selectivity of the enzyme is a result of its three-dimensional structure (i.e., its folding and the active site), when the enzyme three dimensional structure change with varying pH, the enantioselectivity value will also change.

Table 3.2 Effect of acidic pH on enantiomeric excess of 2-hydroxy-1-tetralone in Medium B

pH	E.E
	Medium B
4	6 % S(-)
4.5	79 % S(-)
5	85 % S(-)
5.5	82 % S(-)
6	81 % S(-)

To check whether the reaction was a microorganism-mediated bioconversion or not, a control experiment was carried out. For the control experiment, the substrate was added to medium without inoculating microorganisms. All the reaction parameters (reaction time, temperature, pH value, etc.) were kept constant and product formation was monitored for 12 days. Product formation was not observed in this control experiment. Therefore, it was concluded that the conversion of 1-

teralone was catalysed by *A.niger*. Enzymes responsible from bioconversion could be either intracellular or extracellular monooxygenases and hydroxylases.

The biomass was measured day by day and the results showed that up to 9th day, biomass was increasing gradually and after 9th day, it stayed nearly constant. The reason of this cessation in growing could be that microorganism was in stationary or dead phase. Furthermore, the biotransformation rate was not decreaded, on the contrary increased after the 9th day. Therefore we thought that the enzyme used for biotransformation was secreted the medium during growth phase, while it was diffused to growth media during dead phase via cell lysis.

The enzymes was teking role in biotransformation could be monooxygenases and hydroxylases. Since there was oxygen insertion to used substrates. Monooxygenases use molecular oxygen to insert one oxygen atom into a substrate while the second oxygen atom is reduced with electrons from NADH or NADPH yielding water. This relatively complex reaction often requires a metal center, and electron transfer from the reduced cofactors usually requires additional proteins[40]. Hydroxylases introduces one or more hydroxyl groups (-OH) into a compound (or radical) thereby oxidising it. In biochemistry, hydroxylation reactions are often facilitated by enzymes called hydroxylases.

3.3 SYNTHESIS OF 2-HYDROXY-1-INDANONE AND INDANE-1,2-DIOL

3.3.1 Effect of Medium Composition on Bioconversion

As in the synthesis of 2-hydroxy-1-tetralone (1), the three different defined culture media (B,C,D) were selected for the *A.niger*-mediated bioconversion of 1-indanone.

After *A.niger* was grown in the selected media for 3 days, 1-indanone was added into the growth media. The bioconversion was monitored via TLC by taking daily samples from the growth media inoculated with 1-indanone. It was observed that the bioconversion started after 72 h in medium B and D. In medium C, very slow bioconversion took place. While 35-40% of the substrate was converted in medium B and D at 4^{th} day, only 5-10% conversion of the substrate was observed in medium C. Therefore it was decided to continue with medium B and D. Reaction was terminated by adding organic solvent (ethyl acetate or dichloromethane) and the medium was extracted with distilled ethyl acetate. The ethyl acetate was evaporated in vacuum. 2-hydroxy-1-indanone was obtained after separation via flash column chromatography. The product formation was justified by GC-MS and NMR analyses (Figure A.3-A.4 and Figure A.7-A.10).

According to GC-MS analysis it was observed that there are three products formed in the growth media, one has a molecular weight of 148 and the other two have 150. However these products (MW=150) could not be observed via TLC analysis as they were not UV active, they could

only be detected by staining with anisaldehyde. GC analyses showed that those products, which has molecular weight of 150, were diastereomers of indane-1,2-diol (Figure A.9-A.10). The product with molecular weight 148 is 2-hydroxy-1-indanone. The α-value for 2-hydroxy-1-indanone isolated from medium D was $[\alpha]_D^{20}$ = + 9.73 (c=0.002, $CHCl_3$).

Indane-1,2-diol is an important structural unit for the synthesis of more complex compounds with important biological activity. Recently this compound was used to synthesize optically active *cis*-1-amino-2-indanol through oxime formation and diastereoselective hydrogenation. *Cis*-1-amino-2-indanol is a key precursor of the chiral ligands, and the chiral auxiliaries for asymmetric synthesis and an important intermediate to a leading HIV protease inhibitor Crixivan®. Here, it was aimed to synthesize indane-1,2-diol by microbial biotransformation, since the organic synthesis of optically active indane-1,2-diol is rather laborious and the reaction is carried out using organic solvents that are not preferred because of environmental considerations. It is synthesized in five steps via an intramolecular Friedel-Crafts reaction of 2-acetoxy-3-phenylpropanoyl chloride[32]. Additionaly, indane-1,2-diol can be synthesized by the oxidation of 1-indanone with $Mn(OAc)_3$.[32].

For the synthesis mechanism of Indane-1,2-diols and 2-hydoxy-1-indanone, we can put forward different ideas. Firstly, they can be formed independently from each other by several enzymes. Those enzymes can be hydroxylases and monooxygenases. Another and more possible approach is that one product is synthesized from the other:

indane-1,2-diols are the minor products and they can be transformed from 2-hydroxy-1-indanone or the reverse is true: 2-hyrdoxy-1-indanone is synthesized from indane-1,2-diols. The study done by Taylor[41] showed that optically active 2-hydroxy-1-indanone, can be synthesized from *cis*- and *trans*- indane-1,2-diol by microbial stereoselective oxidation [39,41], (Figure 3.5)

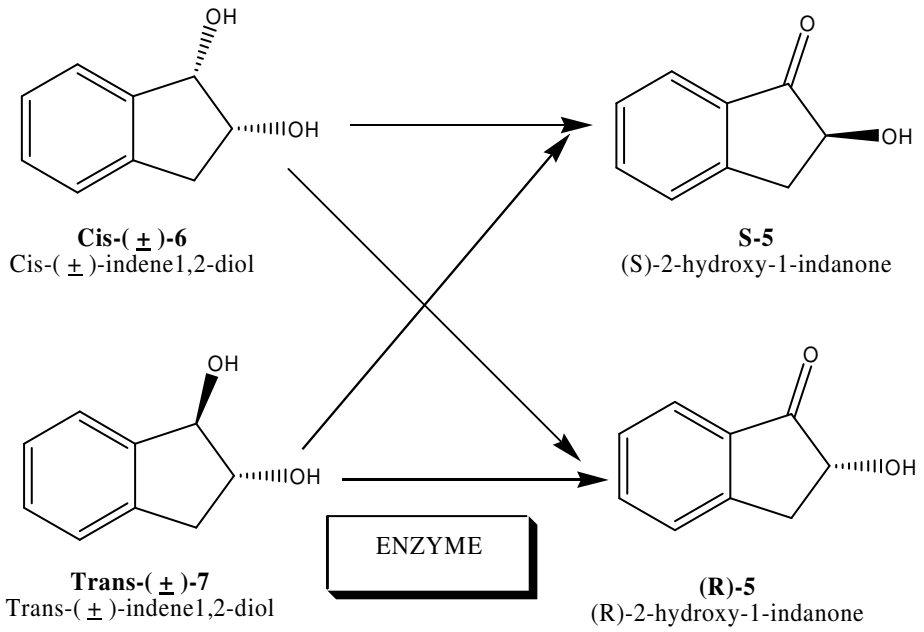


Figure 3.5 Microbial selective oxidation of cis and trans indane-1,2-diol into 2-hydroxy-1-indanone

Since the enantiopure synthesis of hydroxy ketones are important, studies were continued to further increase the ee value of 2-hydroxy-1-indanone. In our cases, first α-hydroxylation of indanone followed by reduction of ketone may occur.

3.3.2 Effects of Growing and Resting Cell on Bioconversion Rate

The growing cell and resting cell studies were done to investigate their effect on enantioselectivity and the efficiency of biotransformation. The same procedure was applied as in previous chapters. 1-indanone was either added to the growth media of *A.niger* (growing cell) or the substrate was added to the phosphate buffer containing resting cells. In order to obtain resting cells, the microorganism was firstly grown for 3 days in the growth medium, than cells were filtered and washed with sterile distilled medium to get rid of the residues, lastly wet cells were re-suspended in sodium phosphate buffer (0.1 M, pH 7.0) and 1-indanone was added. Bioconversion was monitored via TLC by taking daily samples from the reaction media. Results showed that bioconversion was very slow in the resting cells as compared to growing cells.

The reason of a slow conversion in resting cells could be due to the absence of metabolic reactions responsible for the biotransformation of 1-indanone. Therefore, growing cells of *A.niger* were used for further studies.

3.3.3 Effect of pH on Enantioselectivity

Medium B and D were prepared by adjusting their initial pH's to 5, 6, 7 and 8. The conversions were monitored via TLC for 14 days. The reactions were stopped and the products were separated using flash column chromatography (ethyl acetate:hexane:methanol = 1 : 2 : 0.01). The

enantiomeric excess (ee value) were determined by HPLC analysis using a chiral column (DIACEL OB-H, hexane/2-propanol, 90:10, 0.5 ml/min, 254 nm).

Table 3.3 Effect of pH on enantiomeric excess

pH	E.E.	
	Medium B	Medium D
pH.5	8 % S(+)	24 % S(+).
pH.6	N.D.	18 % S(+).
pH.7	9 % S(+)	32 % R(-).
pH.8	27 % S(+)	33 % S(+)
Unadjusted pH	8 % S(+)	N.D.

N.D.: Not Determined

Since medium D gave higher ee values than medium B, further studies were conducted to increase ee value with medium D.

According to the ee values, the product (2-hydroxy-1-indanone) was a racemate. This could be the result of the deracemization of the product after purification. In the literature, the studies which were done with chiral 2-hyroxy-1-indanone, showed that the racemization of the product was considerably easy even at weak alkaline conditions and the ee of optically pure compound was decreased to half of its initial value after 4 hour incubation at pH 8.0. In the same study, best results were obtained at around neutral pH.

In our experiment, very long incubation times (14 days) were

needed to reach nearly %35-40 conversion. Therefore, low ee values might be a result of deracemization of the product due to long incubation time in our case. Interestingly, reverse selectivity is obtained by pH 7 with medium D.

3.3.4 Effect of Organic Solvent Used to Dissolve Substrate on Enantiomeric Excess

Since enzymes exhibit striking new properties in organic solvents [38], substrate (1-indanone) was dissolved in DMSO and ethanol to investigate their effects on the % e.e value. For this purpose, first, medium D at pH 4, 5, 6, 7 and 8 were prepared and 120 mg 1-indanone in 480 μl DMSO was added after the 3rd day of growth of *A.niger*. Second, medium D at pH 4, 7 and 8 were prepared and 120 mg 1-indanone in 480 μl ethanol were added after the 3rd day of growth. Since the best pH range for the growth of *A.niger* is 4-8, we choose this range was selected for the reactions.

For the cultivation of *A.niger*, the same procedure was followed and after 14 days, reaction was terminated by adding ethylacetate. After separation of the organic phase from water, the ee values were determined by HPLC. (Table 2.3). HPLC analysis showed that the highest ee value was obtained at pH. 8 in DMSO (Figure A.11) and pH 4 in ETOH.

Table 3.4 Effect of organic solvent on enantiomeric excess

pH	E.E.	
	Medium D/DMSO	Medium D/Ethanol
4	5 % S(+)	18 % S(+)
5	12 % S(+)	racemic
6	Racemic	17 % S(+)
7	11 % S(+)	9 % R(-).
8	18 % S(+)	racemic

CHAPTER 4

CONCLUSION

In this study, in order to make bioconversion of target compounds, *Aspergillus niger* species cultivated in different media and the best media which gives the higher conversion rates and enantiomeric excess were chosen for further studies. These media were B for 1-tetralone biotransformation and D for 1-indanone biotransformation. As a result of biotransformation, the enantiomerically enriched form of 2-hydroxy-1-tetralone and 2-hydroxy-1-indanone, biologically active starting materials, was synthesized via biocatalytic hydroxylation of ketones with high stereoselectivity in 2-hydroxy-1-tetralone. According to the results, whole cell catalyzed reactions afforded (S)-configurated 2-hydroxy-1-tetralone with % 87 ee in pH 5, DMSO. In addition to this, (S)-configurated 2-hydroxy-1-indanone with %33 ee in pH 8, DMSO and (R)-configurated 2-hydroxy-1-indanone with %32 ee in pH 7, DMSO were synthesized. Also, in the synthesis of hydroxy indanone, indane-1,2-diols were produced as minor product.

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APPENDIX

H-NMR, C-NMR, GC-MS an HPLC results of 2-hydroxy-1-tetralone and 2-hydroxy-1-indanol

GC-MS results of indane-1,2-diol diastereomers

In this study GC-MS analysis were used for identification of volatile and semivolatile organic compounds in complex mixtures, determination of molecular weights and (sometimes) elemental compositions of unknown organic compounds in complex mixtures.

Nuclear magnetic resonance (NMR) spectrometry was provide detailed information on the exact molecular conformation.

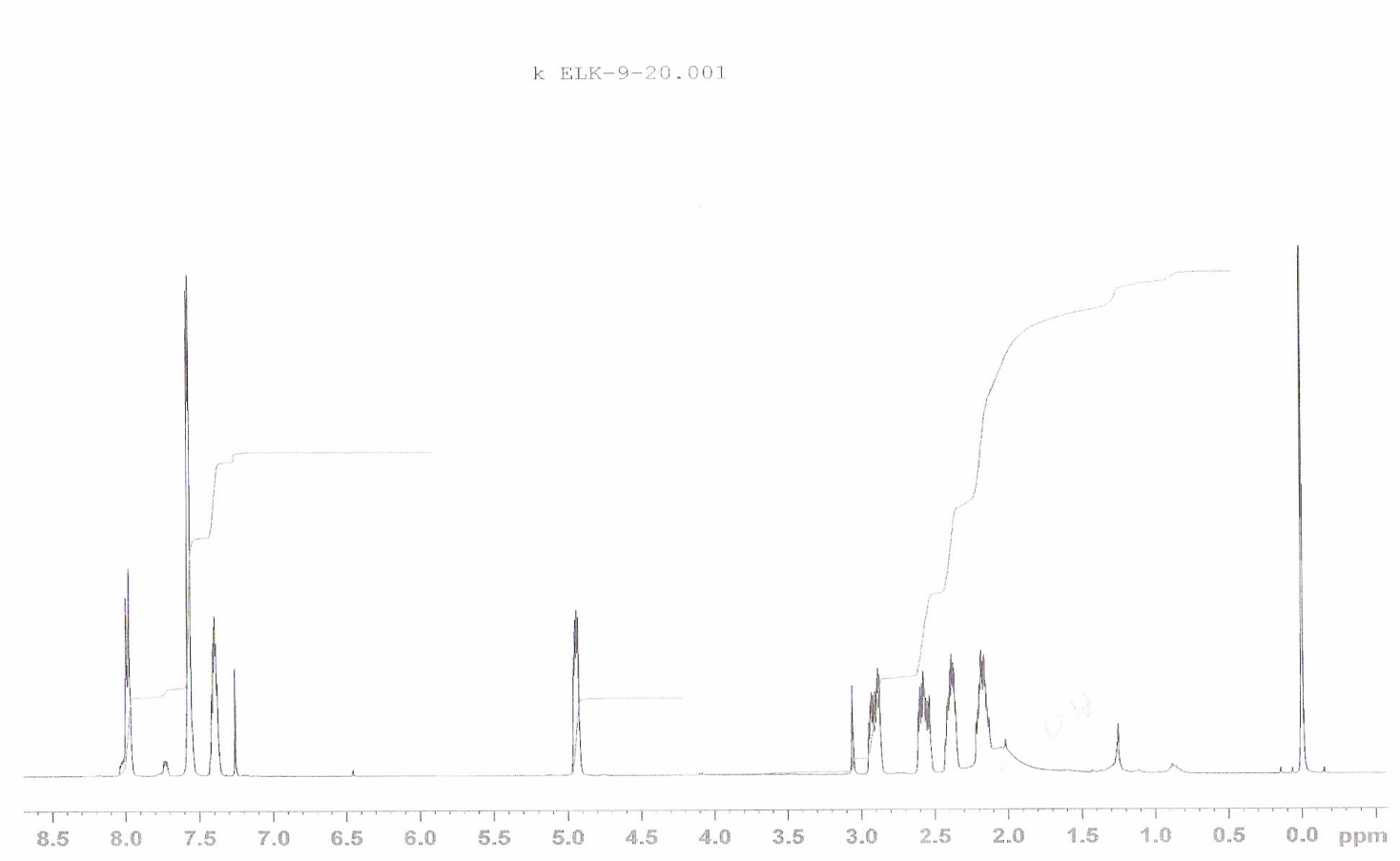

Figure A.1 H-NMR analysis results of 2-hydroxy-1-tetralone

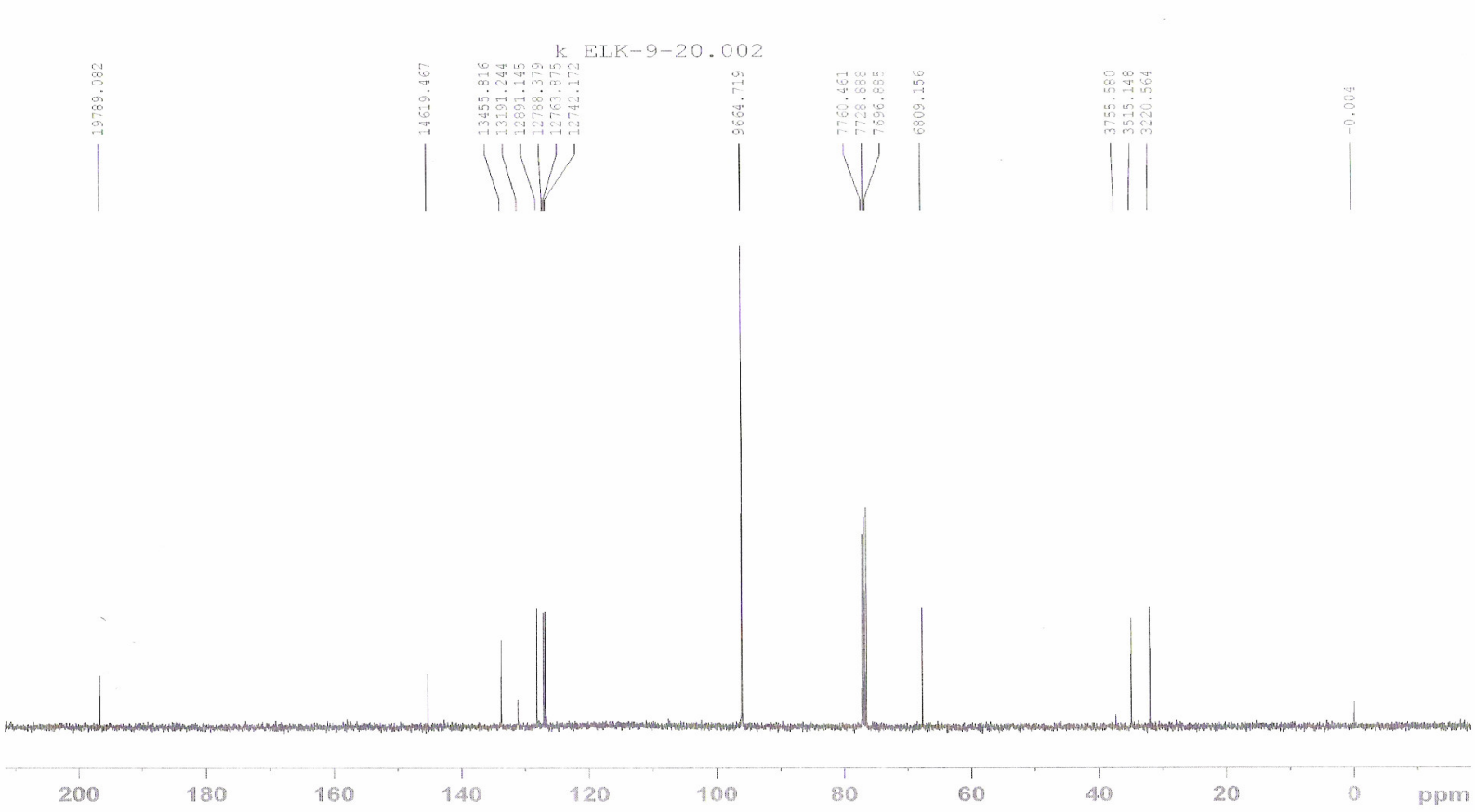

Figure A.2 C -NMR analysis results of 2-hydroxy-1-tetralone

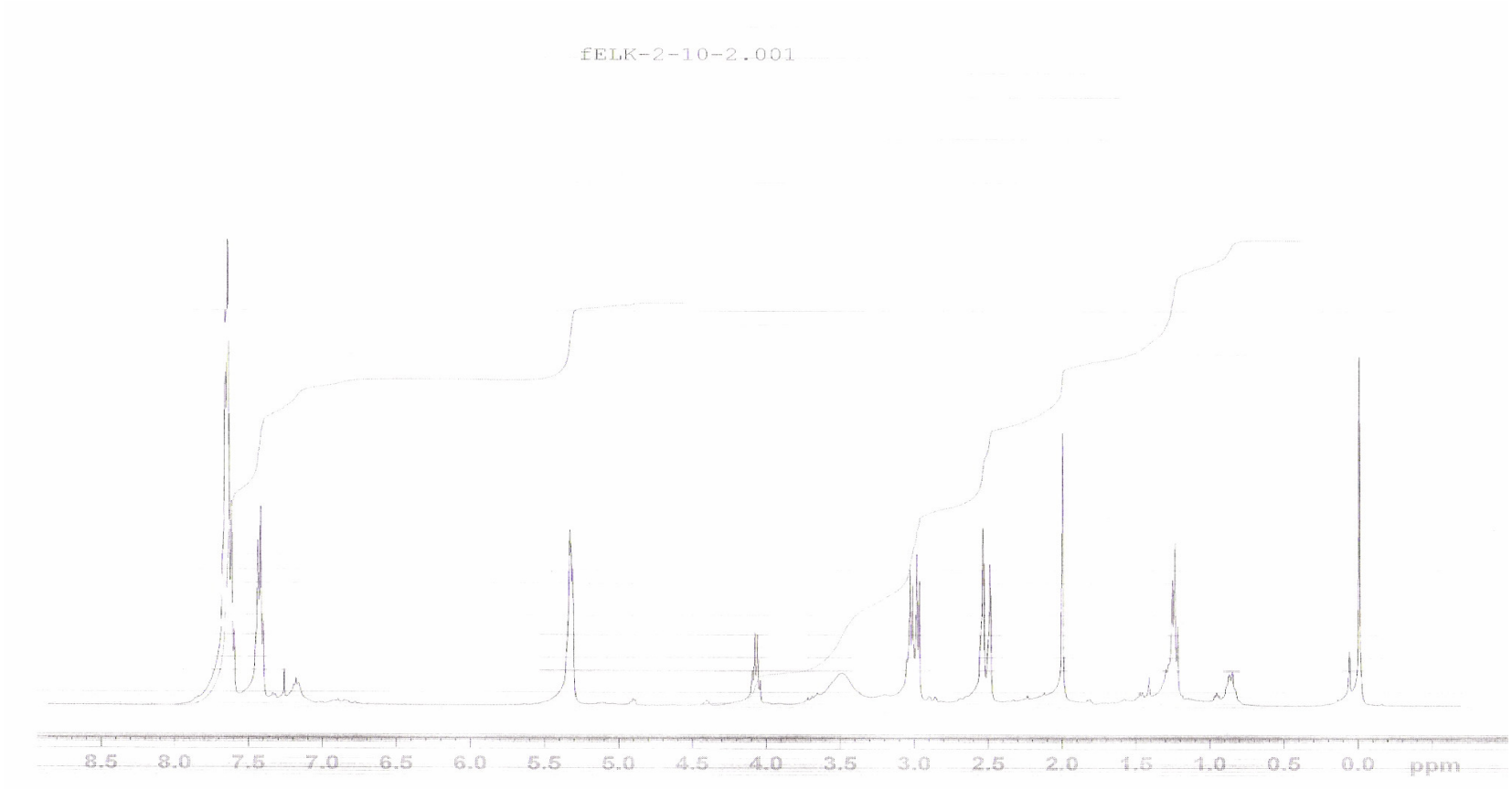

Figure A.3 H-NMR analysis results of 2-hydroxy-1-indanol

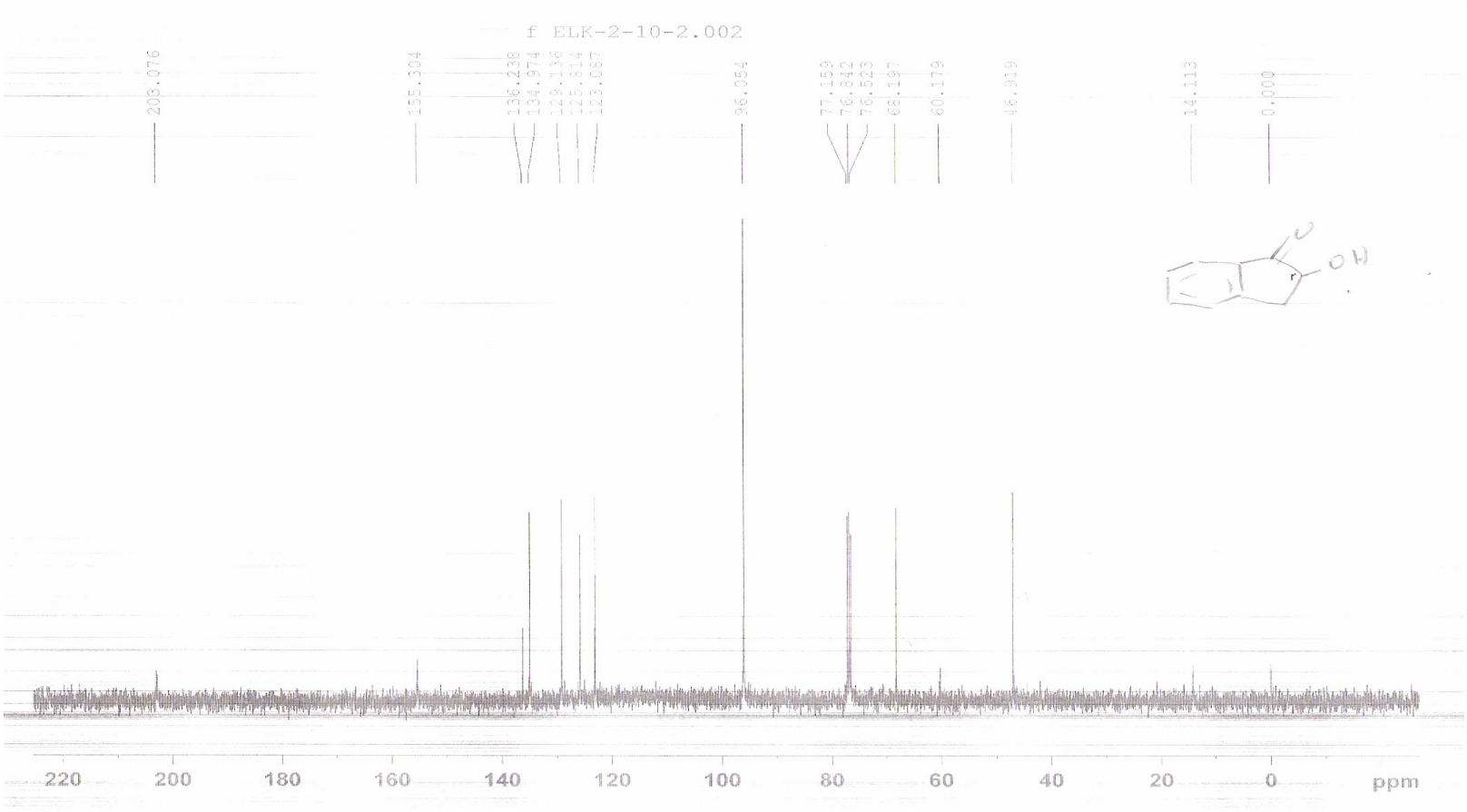

Figure A.4 C-NMR analysis results of 2-hyroxy-1-indanol

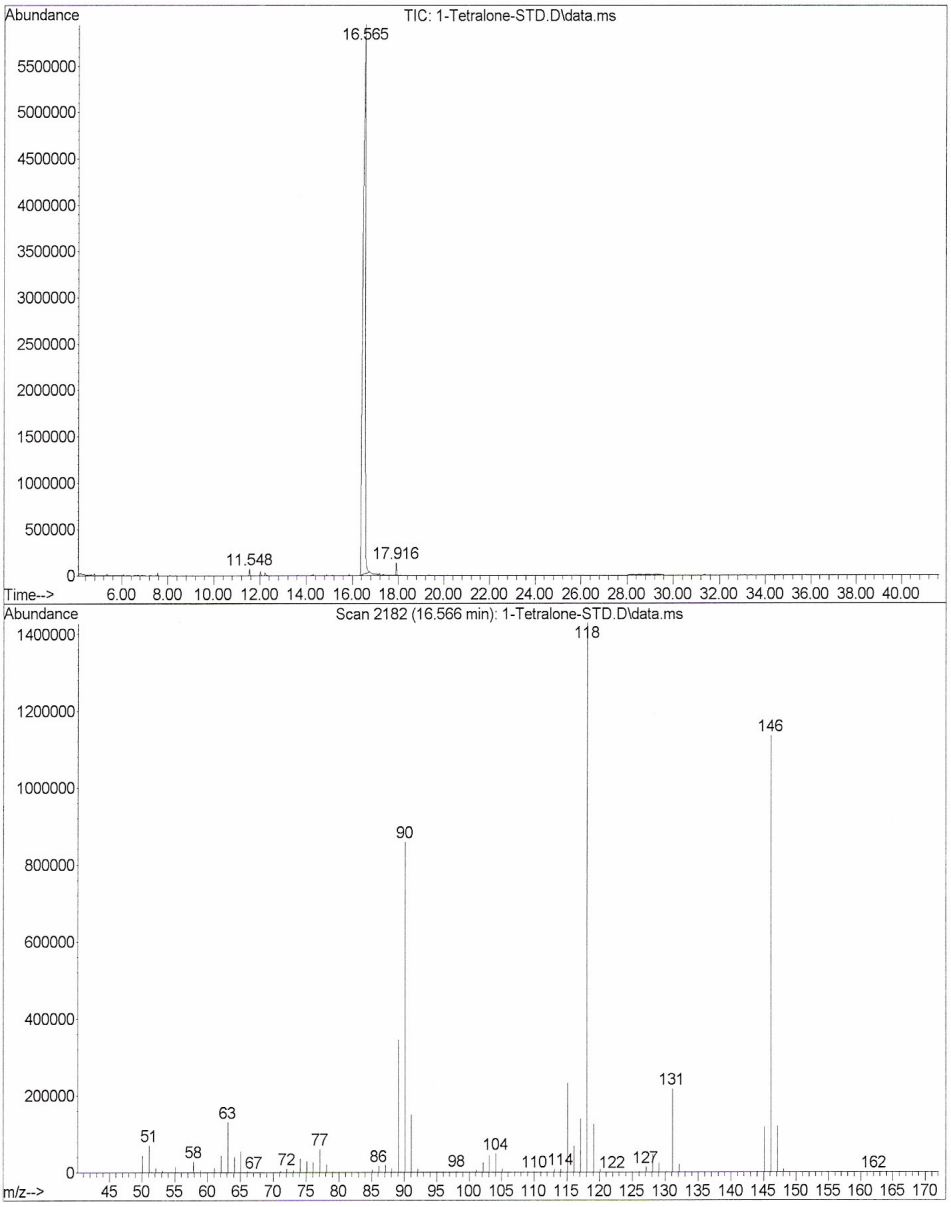

Figure A.5 GC-MS analysis of 1-Tetralone standard

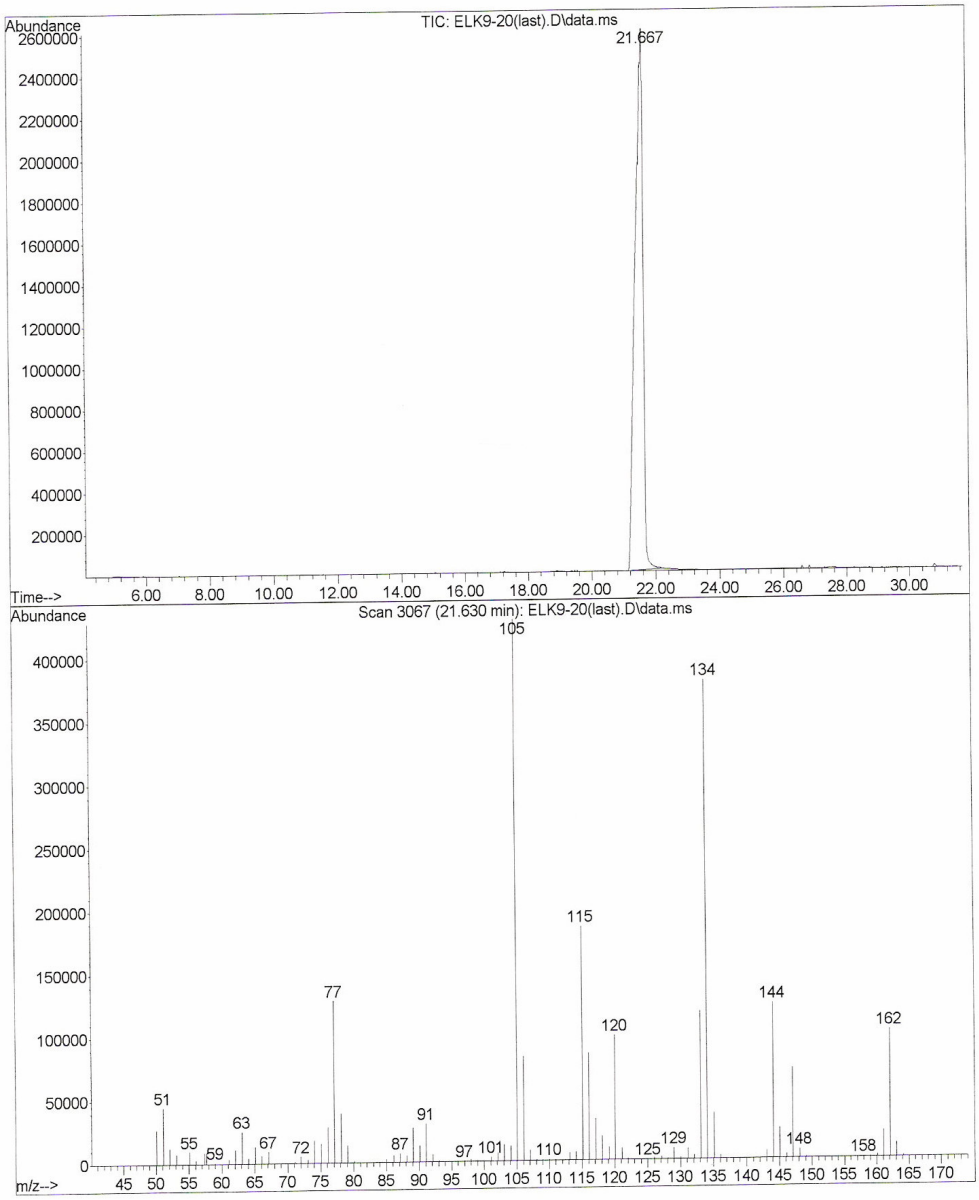

Figure A.6 GC-MS analysis results of 2-hydroxy-1-tetralone

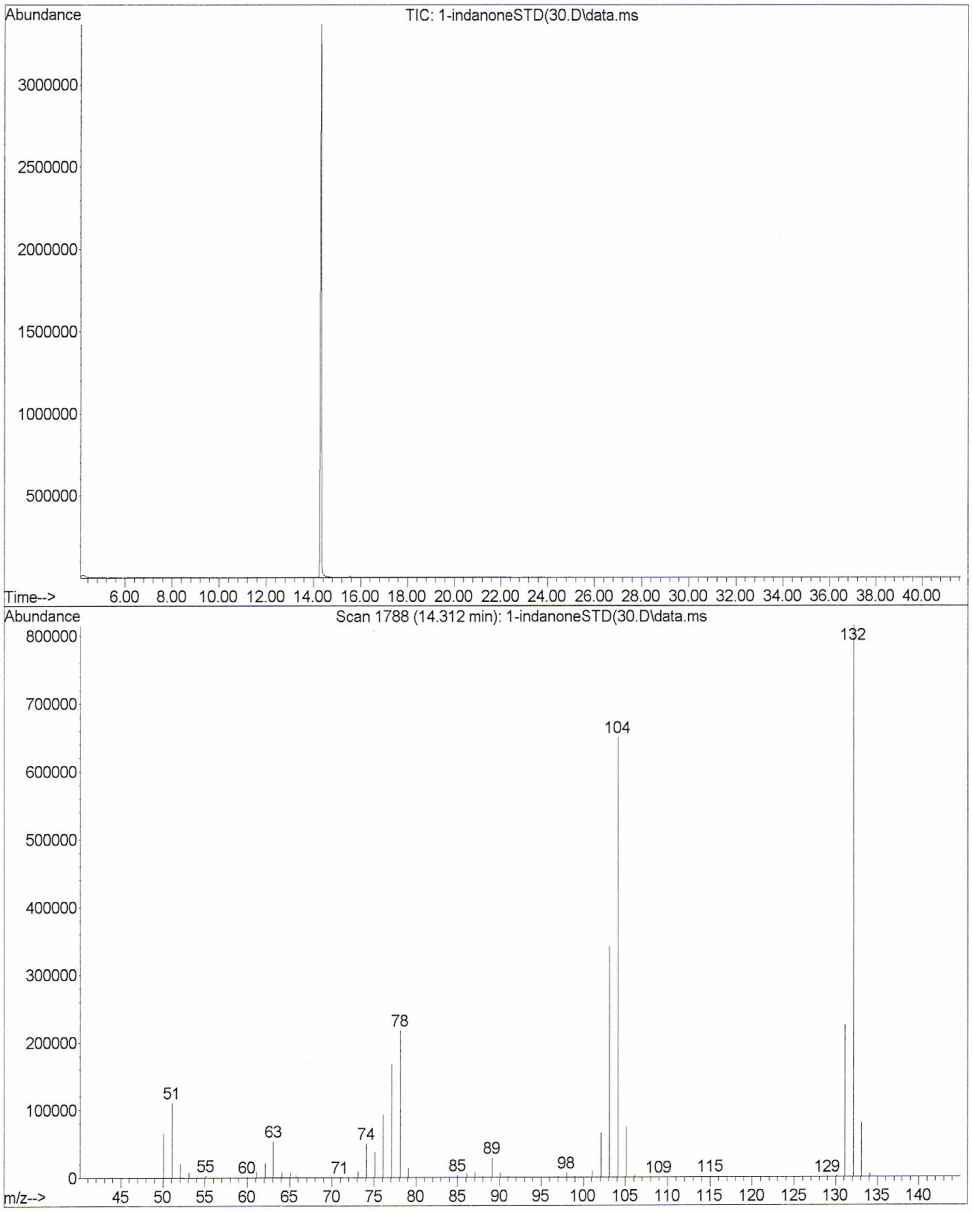

Figure A.7 GC-MS analysis of 1-indanone Standard

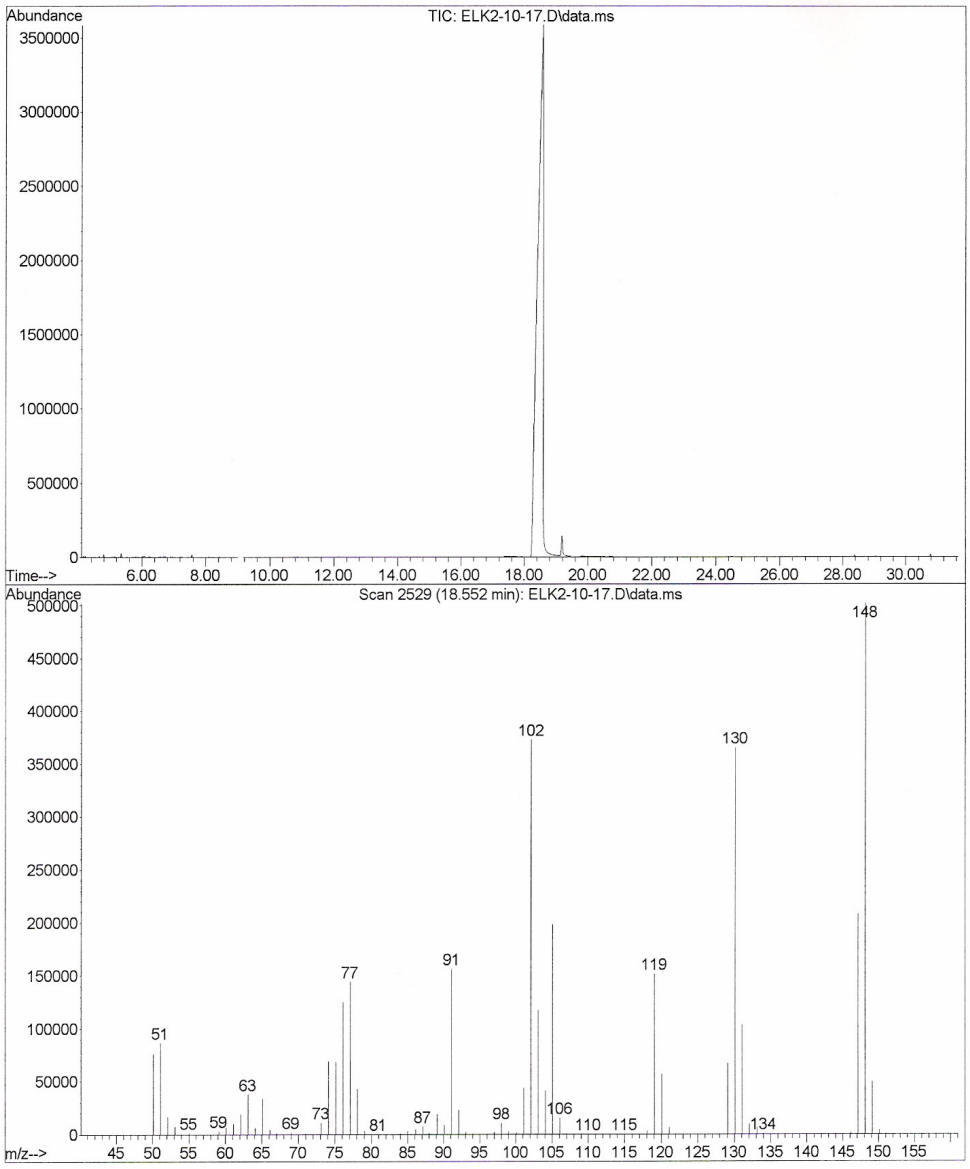

Figure A.8 GC-MS analysis results of 2-hydroxy-1-indanone

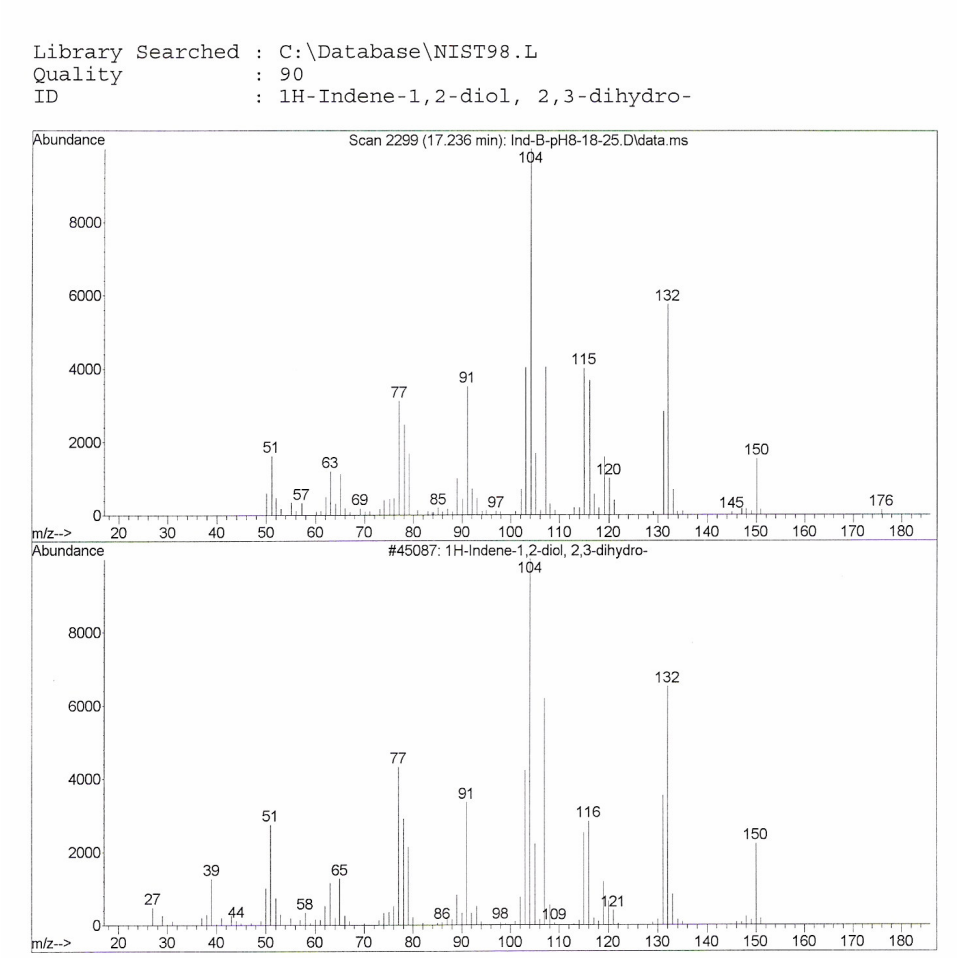

Figure A.9 GC-MS analysis of indane-1,2-diol diastereomers

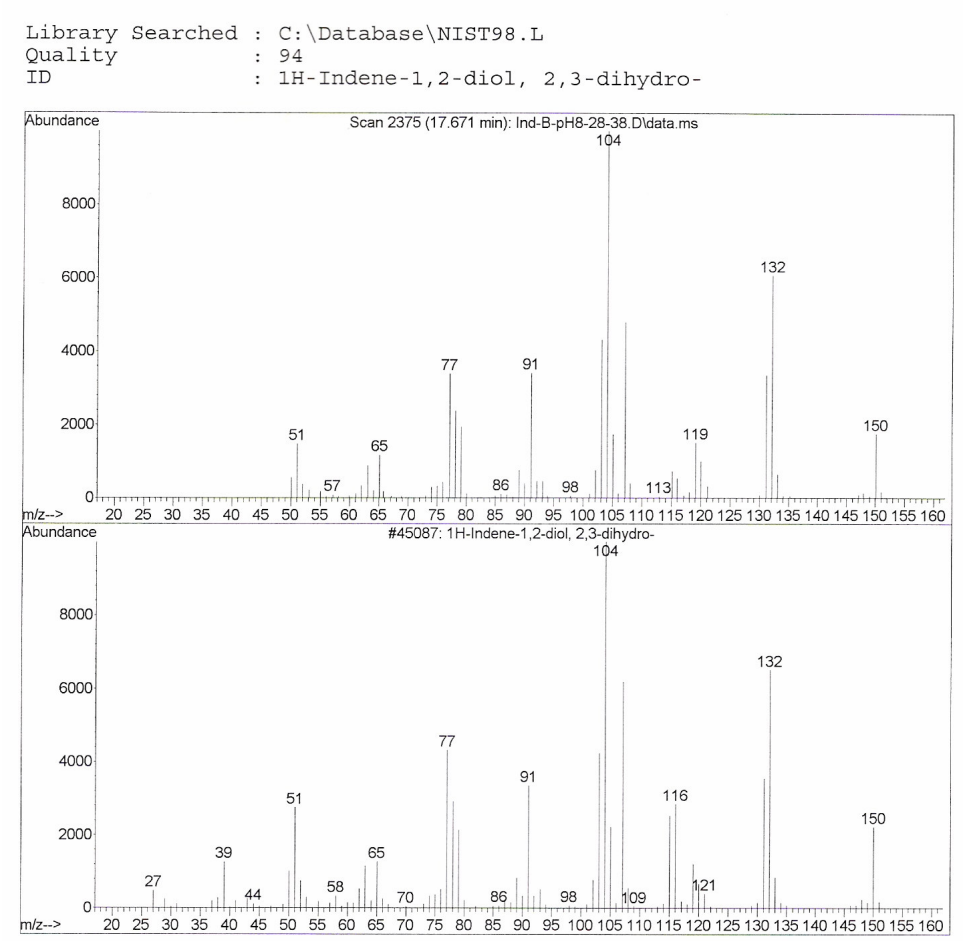

Figure A.10 GC-MS analysis of indane-1,2-diol diastereomers

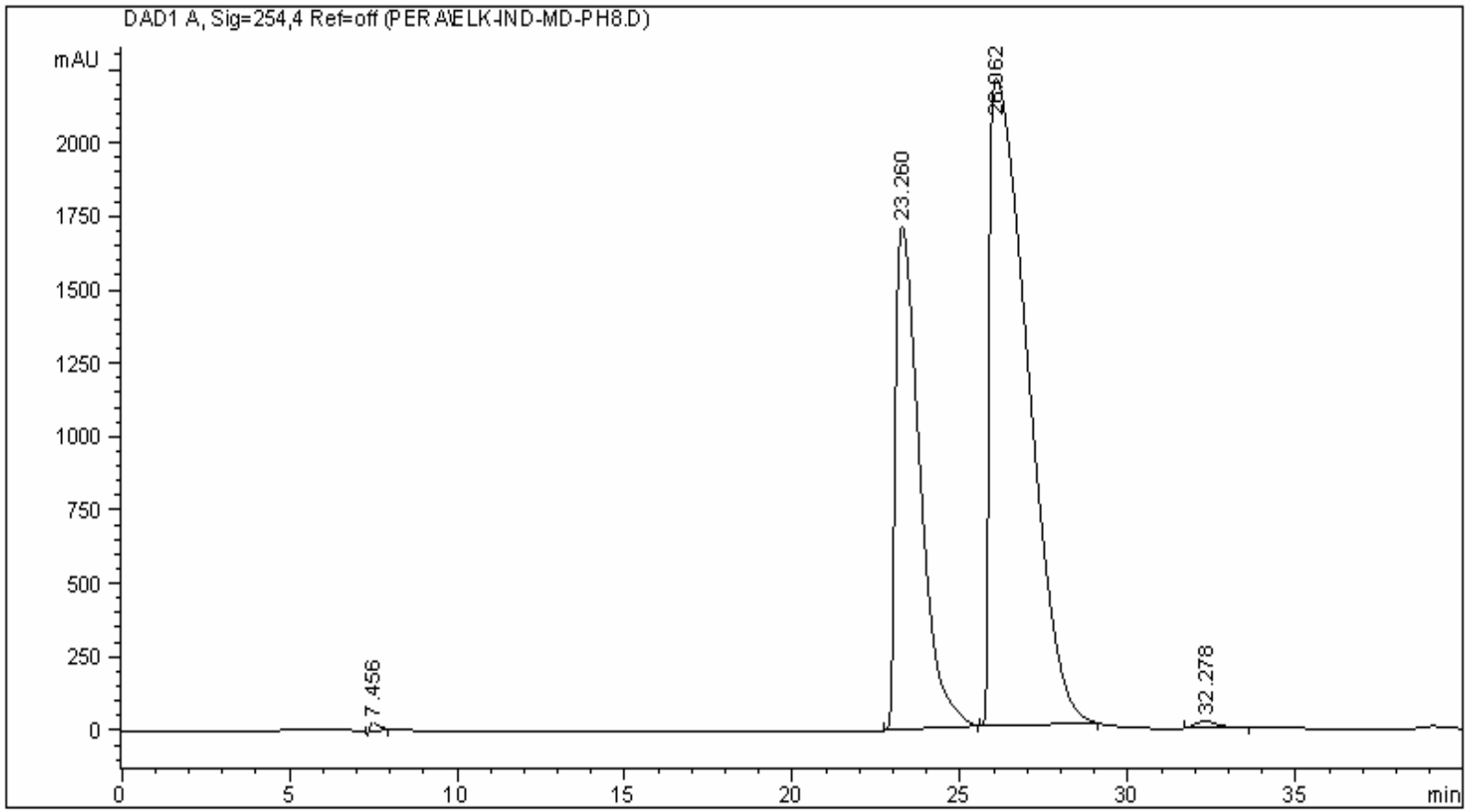

Figure A.11 HPLC analysis of 2-hydroxy-1-indanone purified from medium D, pH.8, DMSO

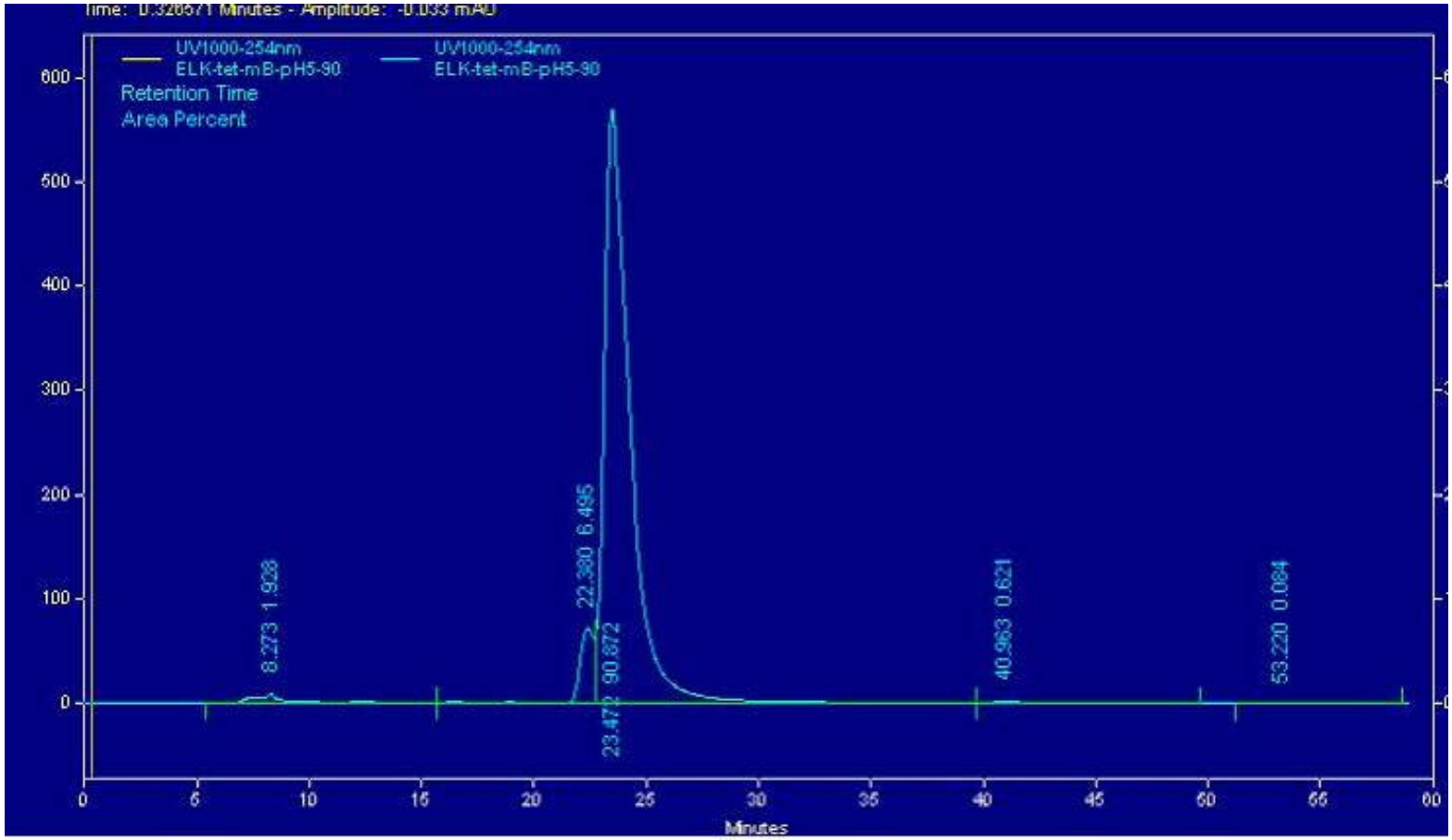

Figure A.12 HPLC analysis of 2-hydroxy-1-tetralone purified from medium B, pH.5, DMSO