# BENZALDEHYDE LYASE FROM*PSEUDOMONAS FLUORESCENS* BIOVAR I MEDIATED BIOTRANSFORMATION FOR THE SYNTHESIS OF CHIRAL ALPHA HYDROXY KETONES

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**BENZALDEHYDE LYASE FROM *PSEUDOMONAS FLUORESCENS* BIOVAR I MEDIATED BIOTRANSFORMATION FOR THE SYNTHESIS OF CHIRAL ALPHA HYDROXY KETONES**

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## ABSTRACT

### BENZALDEHYDE LYASE FROM PSEUDOMONAS FLUORESCENS BIOVAR I MEDIATED BIOTRANSFORMATION FOR THE SYNTHESIS OF CHIRAL ALPHA HYDROXY KETONES

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Optically active α-hydroxy ketones are important subunits of many biologically active compounds and indispensable synthons for asymmetric synthesis. Benzaldehyde Lyase from *Pseudomonas fluorescens* Biovar I is a novel ThDP-dependent enzyme that catalyzes the synthesis of benzoin type chiral α-hydroxy ketones starting from both benzaldehyde and racemic benzoin derivatives. Benzaldehyde Lyase is the first example of enzymes in the literature which leads to a chemical resolution of enantiomers of benzoin derivatives through a C-C bond cleavage reaction.

Chiral 2-hydroxypropiophenone derivatives are formed by benzaldehyde lyase (BAL), catalyzing C-C bond formation after a selective C-C bond cleavage of a benzoin derivative accepted as a substrate. The enzyme uses only the (R)-benzoin derivatives as substrate for the formation of (R)-HPP derivatives and it is highly stereoselective. Thus, in the presence of the acetaldehyde as the acceptor aldehyde, the C-C bond cleavage of the benzoin molecule followed by the carboligation of the acetaldehyde to yield chiral 2-hydroxy propiophenone derivatives.

Given the racemic benzoin to the enzyme as the substrate in the presence of acetaldehyde, both the racemic resolution of the substrate, revealing the unreacted (S)-Benzoin and the formation of the corresponding R-HPP occur.

**Keywords:** benzoin; biocatalyst; hydroxy ketones; hydroxy propiophenones; racemate resolution; thiamine diphosphate.

# ÖZ

## *PSEUDOMONAS FLUORESCENS* BIOVAR I'DEN BENZALDEHİT LİYAZ ENZİMİ İLE KİRAL α-HİDROKSİ KETONLARIN SENTEZİ

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Ocak 2010, 60 sayfa

Optikçe aktif α-hydroksi ketonlar biyolojik aktivite gösteren bir çok bileşiğin önemli yapı taşları olmakla birlikte asimetrik sentez için vazgeçilmez başlangıç maddeleridirler. *Pseudomonas fluorescens* Biovar I bakterisinden elde edilen Benzaldehit liyaz enzimi, benzoin tipi kiral α-hydroksi ketonları hem benzaldehit hem de benzoin türevlerinden başlayarak sentezleyebilen ve tiyamin bifosfatı ko-enzim olarak kullanan yeni bir enzimdir. Benzaldehit Liyaz, literatürdeki enzimler arasında benzoin türevi enansiyomerlerinin kimyasal rezolüsyonunu C-C bağı kırma tepkimesi esnasında gerçekleştiren ilk enzimdir.

Kabul ettiği benzoin türevlerini, seçici bir C-C bağı kırma tepkimesini takiben benzaldehit liyaz enzimi, C-C bağı oluşmasını katalize ederek kiral 2-hidroksi propiyofenon türevleri sentezler. Enzim (R)-HPP türevlerini oluşturma aşamasında sadece (R)-benzoin türevlerini substrat olarak kabul eder ve bu yönden çok stereoseçicidir. Böylece, asetaldehit gibi alıcı bir aldehitinin ortamda bulunmasıyla, benzoin molekülünün C-C bağı kırılmasına uğratımı, asetaldehit ile substratın karboligasyonu ile kiral 2-hidroksi propiyofenonlar verir.

Enzime asetladehit varlığında rasemik benzoin verildiği takdirde, substratin rasemat ayrımı, reaksiyona girmeyen (S)-benzoinin elde edilmesi ve (R)-HPP türevinin olustuğu görülür.

**Anahtar Kelimeler:** benzoin; biyokatalizör; hidroksi keton; hidroksi propiyofenon; rasemat ayrımı; tiyamin bifosfat.

*To Sertaç, my father & my mother*

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| **YPDC** | Yeast Pyruvate Decarboxylase |
| **BFD** | Benzoylformate Decarboxylase |
| **BAL** | Benzaldehyde Lyase |
| **HPP** | Hydroxypropiophenone |
| **ThDP** | Thiamine diphosphate |
| **DMSO** | Dimethyl Sulfoxide |
| **HPLC** | High Performance Liquid Chromatography |
| **GC** | Gas Chromatography |
| **Rt** | Retention Time |

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# CHAPTER I

## INTRODUCTION

The construction of organic compounds containing one or more chiral centers utilizing chiral starting materials is certainly one of the most exciting and spectacular chapters of the organic chemistry. [1] While the abilities of the enzymes to act as specified and chiral catalysts have been recognized for many years, particularly by the pharmaceutical industry, it is only now that these biochemical procedures are becoming accepted as routine procedures in organic synthesis. However, if the enzymes are used for the transformation of non-natural organic compounds, the following prejudices are, generally, encountered: [2]

**i. "Enzymes are sensitive"**

This is certainly true for most enzymes, if one thinks of boiling them in water, but that also holds for organic reagents. If certain precautions are met, enzymes can be remarkably stable. [2]

**ii. " Enzymes are expensive"**

Some are, but others can be very cheap if they are produced on a reasonable scale. Considering the higher catalytic power of enzymes compared to chemical catalysts, the

overall efficiency of a process may be better even if a rather expensive enzyme is required. Moreover, enzymes can be re-used, if they are immobilized. [2]

### iii. "Enzymes are only active on their natural substrates"

This statement is certainly true for some enzymes, but it is definitely false for the majority of them. The fact, that nature has developed its own peculiar catalysts $3x10^9$ years does not necessarily imply that they are designed to work only on their natural target molecules. As a matter of fact, many enzymes are capable of accepting non-natural substrates of an unrelated structural type and can convert them. It seems to be a general trend, that the more complex the enzyme's mechanism, the narrower the limit for the acceptability of 'foreign' substrates. It is a remarkable paradox that many enzymes display high specificities for a specific type of reaction while accepting a wide variety of substrate structures. [2]

### iv. "Enzymes work only in their natural environment"

It is generally true that enzymes display their highest catalytic power in water, which in turn represents something of a nightmare for the organic chemist if it is the solvent of choice. Only a few years ago, some noteworthy rules for conducting biotransformations in organic media were delineated. Although the activity is usually lower in such an environment, many other advantages can be accrued thus making processes more effective by using biocatalysts in organic solvents. [2]

There are many advantages of biocatalysts:

**i. " Enzymes are efficient catalysts"**

Typically the rates of enzyme-mediated processes are accelerated, compared to those of the corresponding non-enzymatic reactions, by a factor of $10^8$-$10^{10}$. Generally, chemical catalysts are employed in concentrations of a mole fraction of 0.1-1 %, whereas most enzymatic reactions can be performed at reasonable rates with a mole fraction of $10^{-3}$-$10^{-4}$ %, which clearly makes them more effective by some orders of magnitude.

**ii. " Enzymes are environmentally acceptable"**

Unlike heavy metals, for instance, biocatalysts are completely degraded in the environment.

**iii. "Enzymes act under mild conditions"**

Enzymes act in a pH range of about 5-8, typically around 7, and in a temperature range of 20-40°C, preferably at around 30°C. This minimizes problems of undesired side-reactions such as decomposition, isomerization, racemization and rearrangement, which often plague traditional methodology.

**iv. "Enzymes are not bound to their natural role"**

They exhibit a high substrate tolerance by accepting a large variety of manmade unnatural substances and often they are not required to work in water. If advantageous for a process, the aqueous medium can sometimes be replaced by an organic solvent. [2]

### v. "Enzymes can catalyze a broad spectrum of reactions."

Like all catalysts, enzymes only accelerate a reaction, but they have no impact on the position of the thermodynamic equilibrium of the reaction. Thus, in principle, some enzyme-catalyzed reactions can be run in both directions.

There is an enzyme-catalyzed process equivalent to almost every type of organic reaction: for example

- Hydrolysis-synthesis of esters, amides, lactones, lactams, ethers, acid anhydrides, epoxides and nitriles.

- Oxidation-reduction of alkanes, alkenes, aromatics, alcohols, aldehydes and ketones, sulfides and sulfoxides

- Addition-elimination of water, ammonia and hydrogen cyanide

- Halogenation and dehalogenation, alkylation and dealkylation, isomerization, acyloin- and aldol condensation. Even Michael additions are reported.

Enzymes display three types of selectivities: [2]

### i. Chemoselectivity

Since the purpose of an enzyme is to act on a single type of functional group, other sensitive functionalities which would normally react to a certain extent under chemical catalysis, survive.

### ii. Regioselectivity and Diastereoselectivity

Due to their complex three dimensional structure, enzymes may distinguish between functional groups which are chemically situated in different regions of the same substrate molecule.

### iii. Enantioselectivity

Almost all enzymes are made of L-amino acids and thus are chiral catalysts. As a consequence, any type of chirality present in the substrate molecule is recognized upon the formation of the enzyme-substrate complex. Thus a prochiral substrate may be transformed into an optically active product and both enantiomers of a racemic substrate may react at different rates, affording a kinetic resolution.

These latter properties collectively constitute the specificity of an enzyme and represent its most important feature for selective and asymmetric exploitation.

## 1.1. Chirality - Activity Relationship

The dramatic variation in biological activity that enantiomers of a chiral compound can display is of key importance to the pharmaceutical, agrochemical and food industries.[3] The presence of the ‘wrong’ stereoisomer in a preparation of a chiral pharmaceutical compound can have severe adverse effects on patients, or in the case of a agrochemical, may cause an additional environmental load. To obtain approval for new chiral drugs, pharmaceutical companies are required to determine the effects of all stereoisomers

present, and any stereoisomers without the requisite function are considered as impurities. [3] The desirable reasons for producing optically pure materials include the following: [4]

**i. Biological activity often associated with only one enantiomer.**

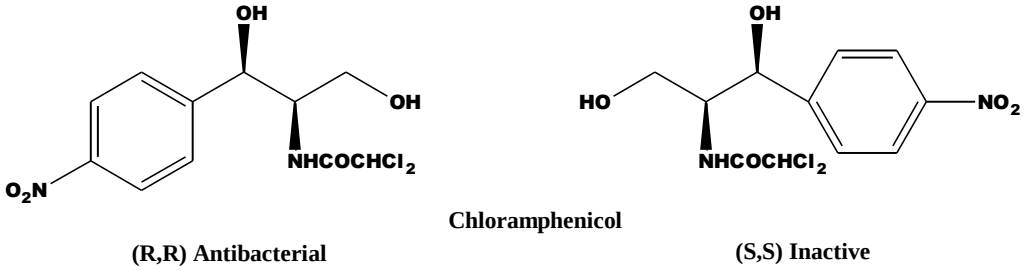



**Figure 1.** Different properties of the enantiomers of Chloramphenicol [4]

**ii. Enantiomers may exhibit very different types of activity, both of which may be beneficial or one may be beneficial and the other undesirable.**

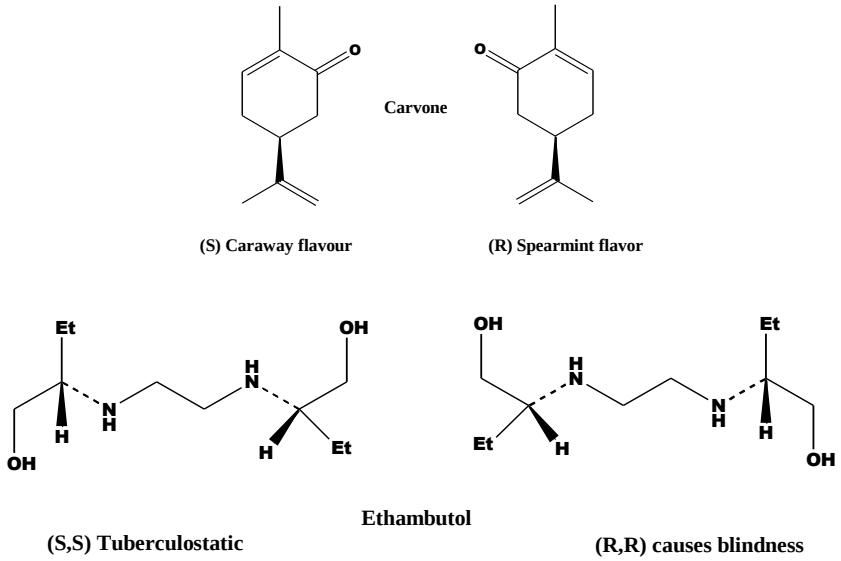



**Figure 2.** Different properties of the enantiomers of Carvone and Ethambutol [4]

iii. **The unwanted isomer is at best 'isomeric ballast' gratuitously applied to the environment.**

iv. **The optically pure compound may be more than twice as active as the racemate because of antagonism**

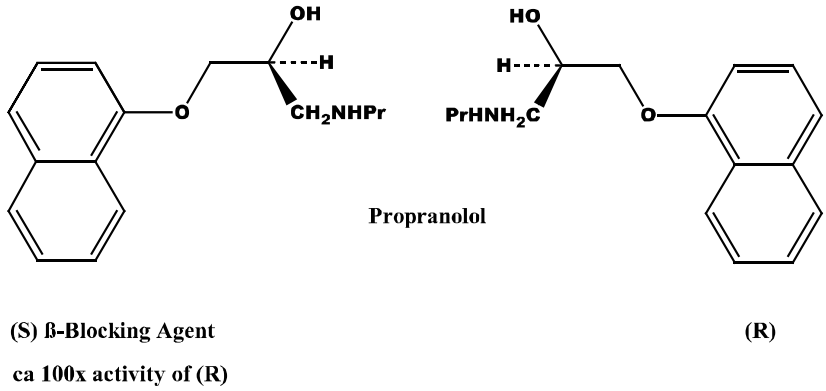



**Figure 3.** Different biological activities of the enantiomers of Propanolol

v. **Registration considerations;** production of the material as the required enantiomer is now a question of law in certain countries, the unwanted enantiomer being considered as an impurity.

vi. Where the switch from racemate to enantiomer is feasible, there is the opportunity effectively to double the capacity of an industrial process; alternatively, where the optically active component of the synthesis is the most costly, **it may allow significant savings to be made in some other achiral but very expensive process intermediate.**

vii. **Improved cost efficacy.**

viii. **The physical characteristics of enantiomers versus racemates may confer processing or formulation advantages.**

### 1.1.1 Importance of Chiral α-Hydroxy Ketones

Enantiomerically pure compounds are becoming increasingly more important in the production of pharmaceuticals, agrochemicals (e.g. pesticides, fungicides, herbicides) and flavors.

Optically active α-hydroxy ketones are indispensable building blocks for asymmetric synthesis due to the presence of stereogenic center and their versatile functional groups, which may be easily transformed to other functionalities, e.g. diols, halo or amino derivatives and epoxides.

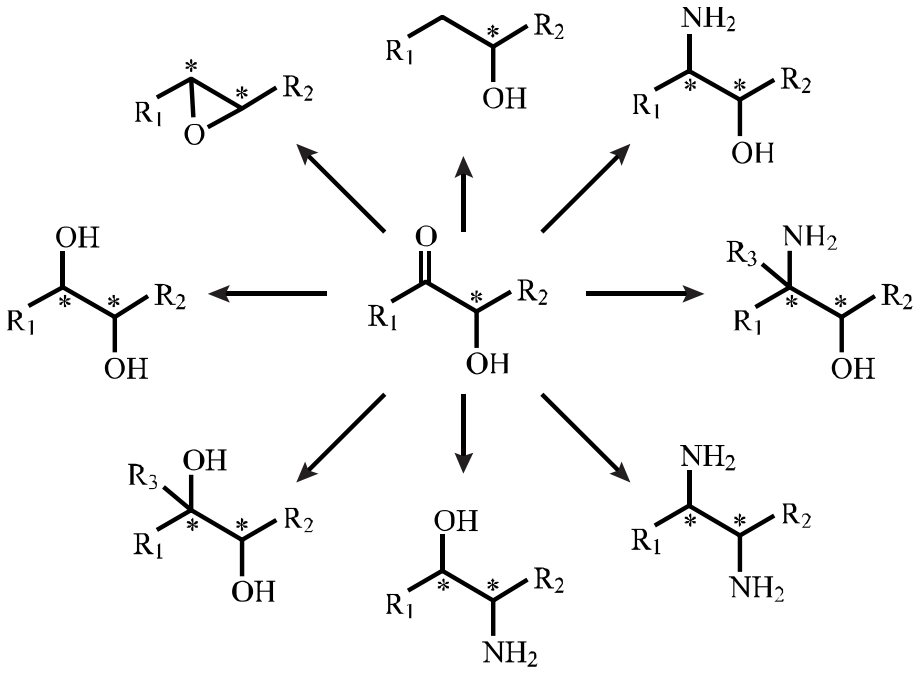



**Figure 4.** α-Hydroxy ketones as building blocks for several active compounds

Indeed, optically active α-hydroxy ketones have been successfully utilized as starting materials for the asymmetric synthesis of a variety of biologically active molecules. Clearly, the convenient and efficient synthesis of optically active α-hydroxy ketones is of timely significance and in urgent demand. [5]

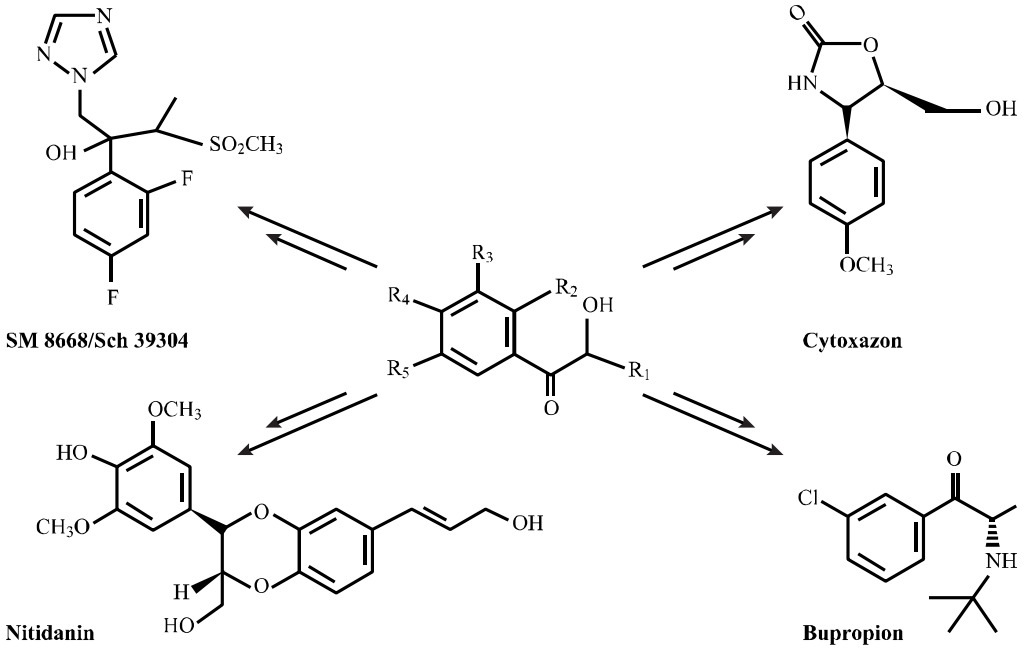



**Figure 5.** Pharmacologicals Based on α-Hydroxy Ketones Derivatives

Several methods have been developed for the preparation of the optically active α-hydroxy ketones. For example, the stereoselective oxidation of optically active enolates [6], oxidation of prochiral enolates using optically active oxaziridines [7], selective oxidation of chiral titanium enolates [8] and asymmetric oxidation of silyl enol ethers. [9]

Enantioenriched *vic*-diols, which could be obtained through the use of asymmetric Sharpless dihyroxylation, are treated with the combination of $RuCl_3$/Oxone/ $NaHCO_3$, provides the enantiomerically pure hydroxy ketones. [10]

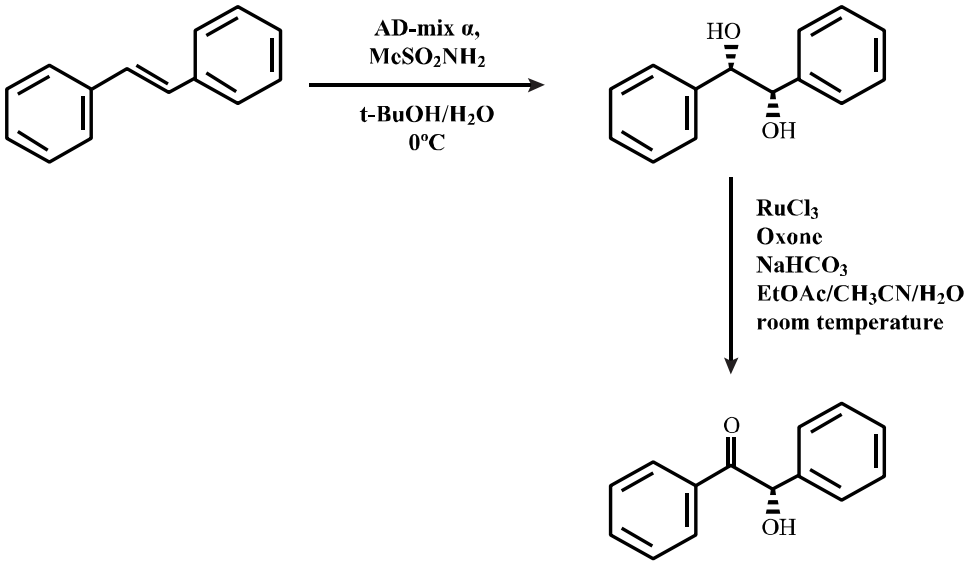



**Figure 6.** The $RuO_4$ catalyzed mono-oxidation

As an alternative to chemical methods, optically active α-hydroxy ketones are prepared enzymatically by reduction of α-diketones [11], by kinetic resolution of racemic α-hydroxy and α-acetoxy ketones [12], enantioselective enzymatic hydrolysis of acetoxy ketones by *Rhizophus Oryzae* [13]

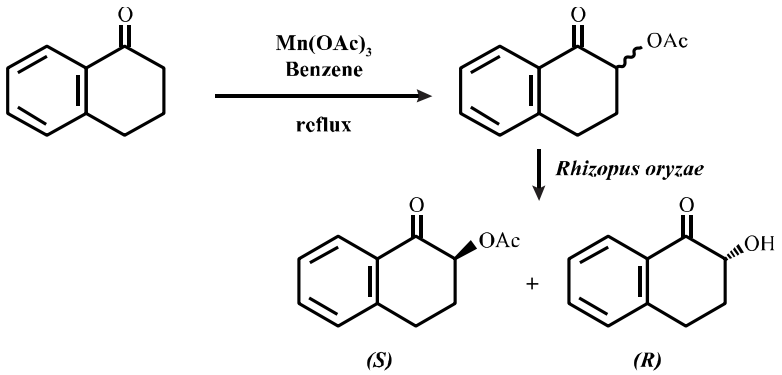



**Figure 7.** Fungi mediated hydrolysis of acetoxy ketones [13]

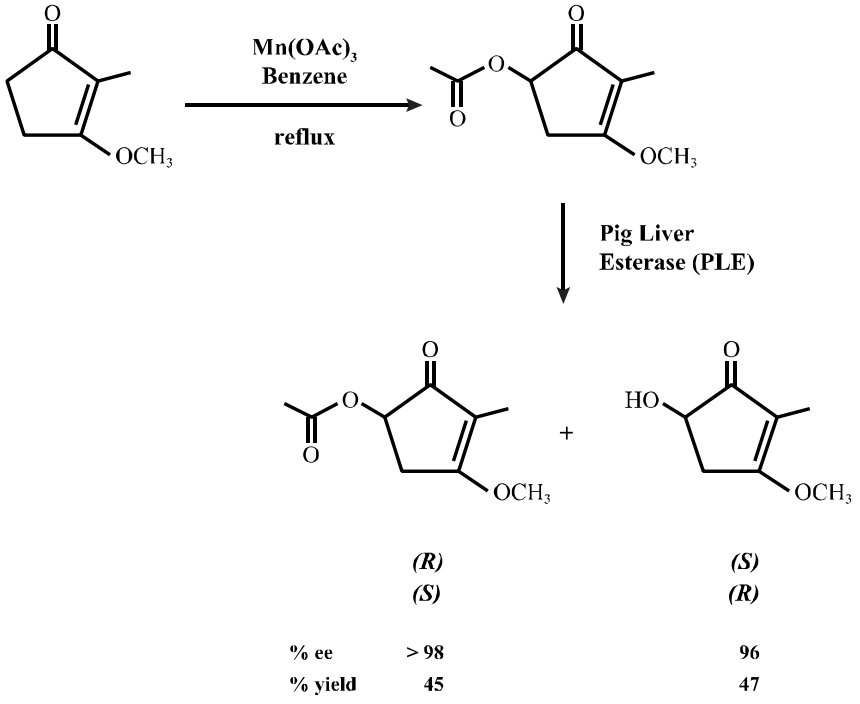



**Figure 8.** Enzyme catalyzed hydrolysis of acetoxy ketones [14]

## 1.2. Introduction to Asymmetric Synthesis

### 1.2.1 Asymmetric C-C Bond Forming Reactions

The importance of being able to synthesize enantiomerically pure compounds has continued to increase ever since Louis Pasteur about 150 years ago realized that molecular asymmetry causes optical activity not the least through the experience that the biological activity, of enantiomers can differ dramatically in its kind and intensity because of the chiral nature of life processes. Current regulatory requests for the potential benefit of all enantio- and/or diastereomers of new xenobiotic agents, especially of pharmaceuticals, call for economical means for their independent, directed synthesis. [15]

### 1.2.2 Asymmetric C-C Bond Forming Reactions Using Chemical Catalysts

Chiral triazolium and tetrazolium salts are frequently used for the purpose of C-C bond formation in modern organic chemistry.

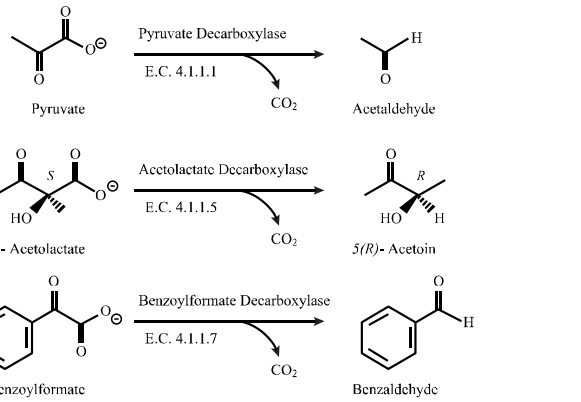
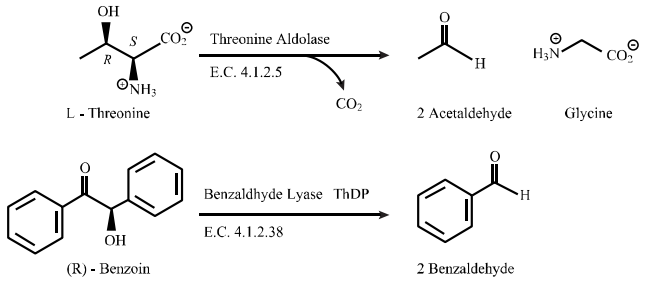
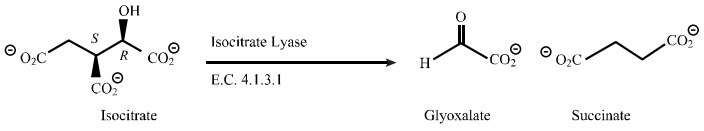
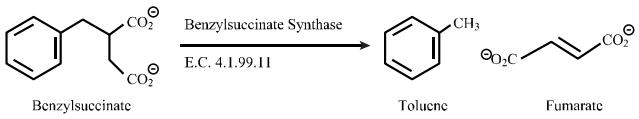
### 1.2.3 Asymmetric C-C Bond Forming Reactions Using Enzymes

As an alternative to classical chemical methodology, enzymes are finding increasing acceptance in modern chemical research and production as catalysts for the *in-vitro* synthesis of asymmetric compounds because they are intrinsically chiral and optimized by the evolution for a high catalytic efficiency. In contrast to most classical chemical techniques, biocatalytic conversions can usually be performed on underivatized substrates, thus making tedious and costly protecting group manipulations superfluous, because of the high levels of selectivity offered by enzymes and because of the usually

very mild reaction conditions that are compatible with most functional groups. In parallel with regulatory demands for pharmaceuticals and with increasing environmental concerns, biocatalysis offers an attractive opinion for the development of new economical and ecologically acceptable processes for the synthesis of the compounds with high optical purity. [15]

An asymmetric C-C coupling, one of the most important and challenging problems in synthetic organic chemistry, seems to be most appropriate for the creation of a complete set of diastereomers because of the applicability of a convergent, combinatorial strategy. In nature, such reactions are facilitated by lyases which catalyze the (usually reversible) addition of carbon nucleophiles to C=O double bonds, in a manner mechanistically categorized as aldol and Claisen additions or acyloin reactions. The most frequent type is the aldol reaction, and some 30 lyases of the aldol type have been identified so far, of which the majority are involved in carbohydrate, amino acid, or hydroxy acid metabolism. [15]

**Table 1.** Representative Examples of Reactions Catalyzed by Carbon - Carbon Lyases

| EC Code<br>Systematic Names | Reaction |
|---|---|
| **4.1 Carbon - Carbon Lyases** | |
| 4.1.1 Carboxy Lyases |  |
| 4.1.2 Aldehyde Lyases |  |
| 4.1.3 Oxo-acid Lyases |  |
| 4.1.99 Other Carbon - Carbon Lyases |  |

It is rather difficult to control the stereochemistry in the synthesis of C-C bonds using a chemical synthetic approach. However, some lyases catalyze the reaction very efficiently and, therefore, are attracting quite a lot of attraction from organic chemists.

### i. Acyloin Condensation

Closely related to the transketolase-reaction are acyloin condensations effected by Baker's yeast, which was first observed by Liebig in 1913. A few years later, Neuberg and Hirsch reported the formation of phenyl acetyl carbinol (3-hydroxy-3-phenylpropan-2-one) from benzaldehyde by fermenting Baker's yeast. This biotransformation assumed industrial importance when it was shown that the acyloin could be converted into (-)-ephedrine by reductive amination.

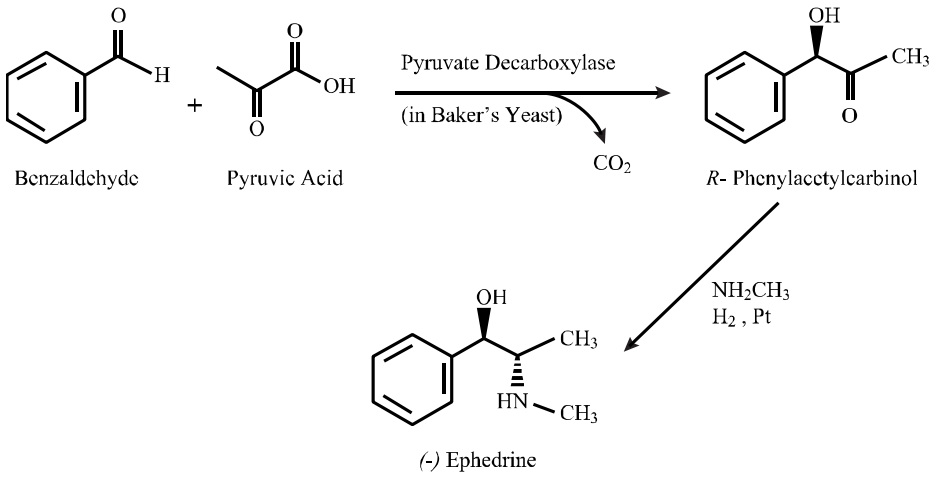



**Figure 9.** Enzymatic synthesis of (-)-Ephedrine Using an Acyloin Reaction

The acyloin reaction involves the condensation of two aldehydes to yield α-hydroxy ketones (acyloins), which are versatile synthons for the fine-chemical industry. Several lyases are dependent on thiamine diphosphate (ThDP) and divalent metal ions; for example, propion synthase and acetohydroxy synthase, catalyze acyloin-condensation reactions. Two other lyases, pyruvate decarboxylase and benzoylformate decarboxylase, which are ThDP-dependent 2-oxo acid decarboxylases, are able to catalyze acyloin condensation reaction as a side reaction.

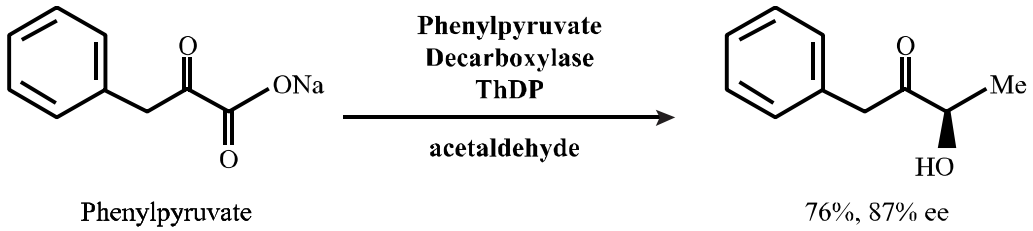



**Figure 10.** Asymmetric acyloin condensation catalyzed by phenylpyruvate decarboxylase

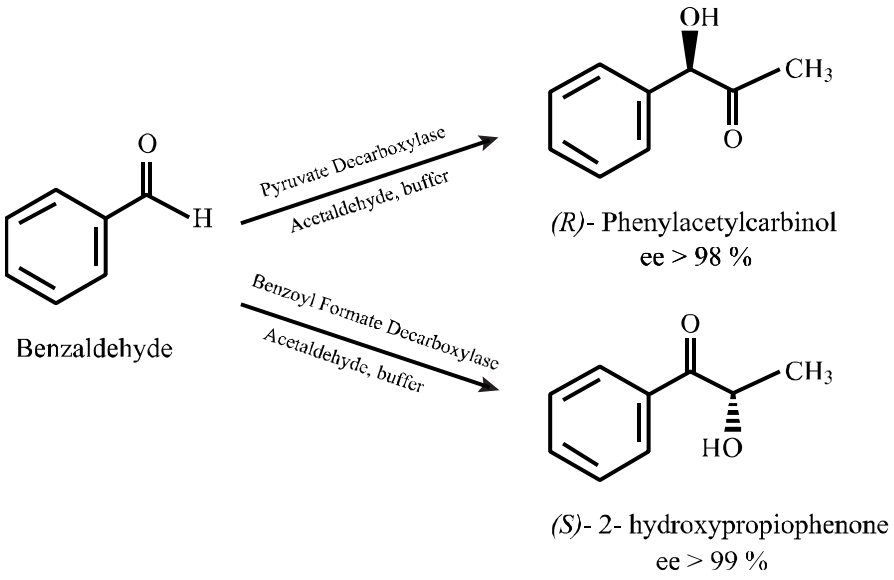



**Figure 11.** Synthesis of (R) - phenylacetyl carbinol and (S)-2-HPP by using the same substrate, but different lyases - Pyruvate Decarboxylase [16] & Benzoylformate Decarboxylase. [17]

### ii. Cyanohydrin Formation

Oxynitrilases (α-hydroxynitrile lyases) catalyze the addition of hydrogen cyanide to an aldehyde or a ketone to form cyanohydrin. Both (R) and (S)-oxynitrilases have been described, and can be used to produce large amount and variety of pure cyanohydrins.

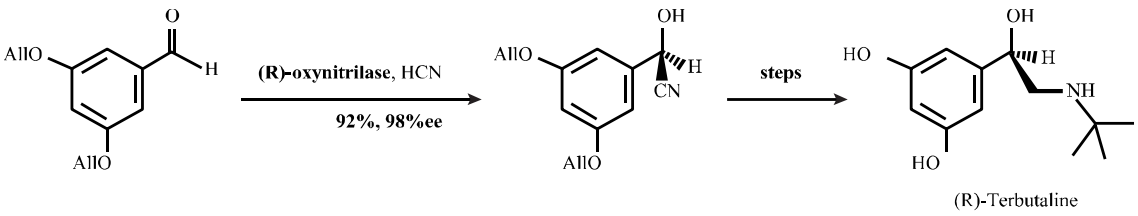



**Figure 12.** Terbutaline synthesis via Oxynitrilase enzyme [18]

Oxynitrilase-catalyzed, highly enantioselective HCN addition to benzaldehydes is the key step to adrenergic pharmaceuticals such as terbutaline.

### iii. Aldol Condensation

The reversible aldol condensation reactions involve the attack by the deprotonated α-carbon atom of an aldehyde or a ketone on the carbonyl carbon of another aldehyde or ketone, resulting in a β-hydroxy aldehyde or a β-hydroxy ketone. This reaction is catalyzed by aldolases, which are involved in the degradation or transformation of sugars, amino acids and aromatic compounds.

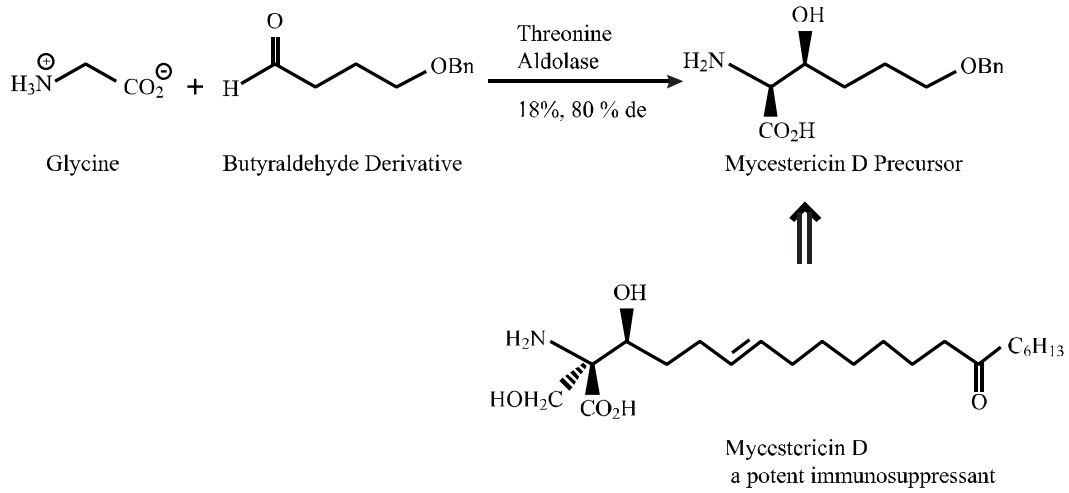



**Figure 13.** Synthesis of Mycestericin D precursor by Threonine Aldolase [18]

### iv. Claisen-type Condensation

Enzymes catalyzing condensation reactions in which the nucleophiles is the α-carbanion of a thioester and the electrophilic component can be a variety of α-keto acids are called 'Claisen Enzymes'. Although the reactions are not formally Claisen Condensations, they are distinct from aldolases in terms of their reaction mechanisms.

### v. Pyridoxal-phosphate-dependent Amino Acid Lyases

Pyridoxal phosphate is a cofactor required by the enzymes catalyzing the synthesis, degradation and interconversion of amino acids. A group of ~25 different PLP-dependent lyases are able to catalyze α,β-elimination, β-replacement, α,γ-elimination and/or γ-replacement reactions. Many of these enzymes are multifunctional, i.e. one enzyme is able to catalyze more than one of the reactions.

### vi. Decarboxylation

Decarboxylases constitute a major class of lyases. These enzymes are particularly suitable for resolving mixture of enantiomers as they exhibit absolute stereospecificity and the reactions that they catalyze are not reversible. Another potentially increasing application of decarboxylases is the decarboxylation of prochiral compounds.

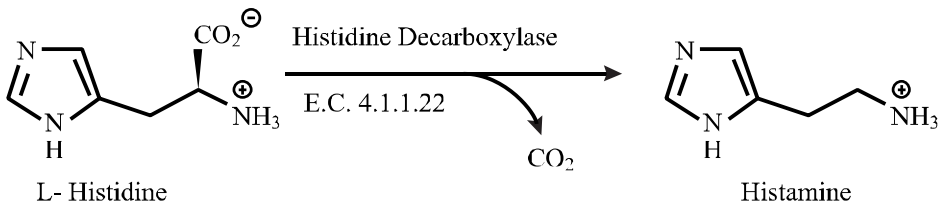


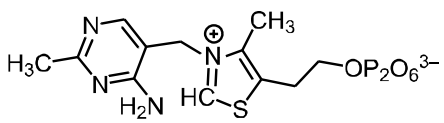

**Figure 14.** Histidine Decarboxylase mediated synthesis of Histamine from L-Histidine

### 1.2.4 Enzyme catalyzed C-C Bond Cleavage Reactions

Lyases catalyze the reversible cleavage of C-O, C-N, C-C or other bonds by elimination to produce double bonds or, conversely catalyze the addition of groups to double bonds. C-C bond cleavage is specifically important for obtaining chiral compounds as this process may be beneficial for racemic resolution of enantiomers.

## 1.3. ThDP-dependent Enzymes in Organic Synthesis

Thiamin diphosphate (ThDP), the biologically active form of vitamin B1, is an essential cofactor that is involved in numerous metabolic pathways. ThDP consists of a thiazolium nucleus, an aminopyrimidine ring, and a diphosphate moiety that is required to provide tight binding to the target enzyme. The electrophilic nature of the positively charged thiazolium ring is key to the stabilization of carbanion states of the cofactor (i.e. the ThDP ylide) and of covalent substrate-ThDP conjugates in terms of a **'Umpolung - or polarity inversion'** mechanism predestining ThDP as an effective participant in the reversible catalytic cleavage of carbon-carbon bonds of vicinal dicarbonyl or α-hydroxy ketone groups, exhibiting a chemical ability not possessed by protein functional groups. [19]




Thiamine diphosphate (ThDP)

**Figure 15.** Structure of Thiamine Diphosphate

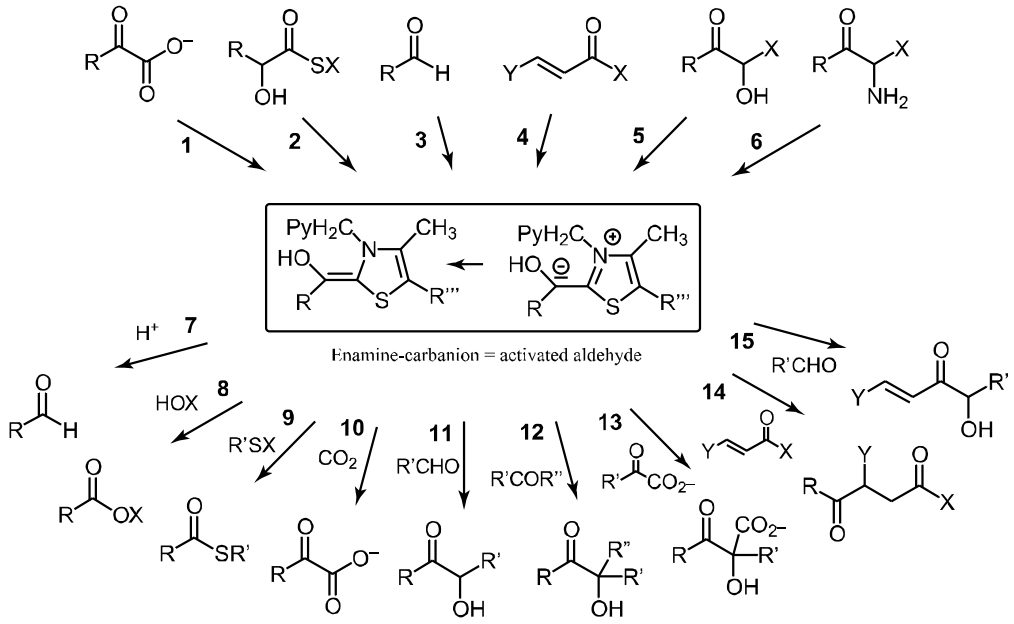



**Figure 16.** The Reactions catalyzed by ThDP [20] (for correlation to specific enzymes see Table 2)

The enzymes that use ThDP as cofactor are listed below: [20]

***Carbon-Carbon Ligation***

- 2-Ketoacid Decarboxylases - Pyruvate Decarboxylase (PDC), Benzoylformate Decarboxylase (BFD)
- Acetohydroxyacid Synthases - Acetolactate Synthase (ALS), Acetohydroxyacid Synthase (AHAS)
- Benzaldehyde Lyase (BAL)
- (1R,6R)-2-Succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate Synthase (SHCHCS)

***Carbon-Nitrogen Ligation***

- $N^2$-(2-Carboxyethyl)arginine Synthase
- Transketolase and 1-deoxy-D-xylulose-5-phosphate synthase

**Table 2.** Selected information on ThDP-dependent enzymes with respect to their origin and application in biotransformations. [20]

| Enzyme | Organism/organelle | Pathway | EC | Donor (see above Figure) | Acceptor |
|---|---|---|---|---|---|
| PDC | Bacteria ( *Zymomonas mobilis* and *Zymobacter palmae* ) | Glycolysis | 4.1.1.1 | 1,3 | 7,11 |
| | Diverse yeast strains, especially *S. cerevisiae* | | | 1 | 7,11 |
| | Various plants | | | 1 | 7 |
| BFD | *Pseudomonas putida*, diverse bacteria | Mandelate catabolism | 4.1.1.7 | 1,3 | 7,11,15 |
| PhePDC | *S. cerevisiae*, *Achromobacter eurydice*, *Acinetobacter calcoaceticus*, *Thauera aromatica* | Ehrlich pathway | 4.1.1.43 | 1 | 7,11 |
| InPDC | *Enterobacter cloacae*, diverse bacteria | Tryptophan catabolism | 4.1.1.74 | 1 | 7 |
| PPDC | *Bacteroides fragilis*, diverse *Streptomyces* species | Biosynthesis of bialaphos, fosfomycin, phosphinothricin and tripeptide antibiotic | 4.1.1.– | 1 | 7 |
| SPDC | *Archaea* | Coenzyme M biosynthesis | 4.1.1.79 | 1 | 7 |
| BAL | *Pseudomonas fluorescens* | nd | 4.1.2.38 | 3,4,5 | 7,11,15 |
| CEAS | *Streptomyces clavuligerus* | Clavulanic acid biosynthesis | – | 3 | |
| AHAS | Bacteria, fungi, diverse plants, mammals, chloroplasts, mitochondria | Branched-chain amino acid biosynthesis | 4.1.3.18 | 1 | 11,13 |
| ALS | *Klebsiella pneumoniae* | 2,3-Butanediol pathway | 2.2.1.6 | 1 | 13 |
| GXC | *E. coli* | Glyoxylate catabolism | 4.1.1.47 | 1 | 13 |
| DXS | Various bacteria, plants | Isoprenoid biosynthesis | 4.1.3.37 | 1,3,5 | 7 |
| TK | Bacteria, yeasts, plants, mammals | Pentose-phosphate pathway | 2.2.1.1 | 1,3,5 | 1 |
| 2-HPCL | *Arabidopsis thaliana*, mammals (peroxisomes) | a-Oxidation pathway of 3-methylbranched fatty acids | 4.1.– | 2 | 7 |
| YerE | *Yersinia pseudotuberculosis* | Yersiniose biosynthesis | – | 1 | 12 |
| SAAT | *Alcaligenes defragrans*, *Alcaligenes xylosoxydans*, *Desulfonispora thiosulfatigenes*, *Rhizobium meliloti* | Anaerobic taurine degradation | 2.3.3.15 | 3 | |
| PK | Diverse bacteria | Pentose phosphate pathway | 4.1.2.– | xylul-5-P, | |
| SHCHCS | Bacteria, *E. coli*, *Arabidopsis thaliana* | Menaquinone biosynthesis protein MenD | 2.5.1.64 | 1 | |

### 1.3.1 Mechanism of ThDP-dependent Catalysis

All biochemical reactions in participation of thiamine start with C-C bond cleavage of 2-oxo carbonyl-compounds and proceed with formation of an activated aldehyde. Thiamine diphosphate dependent enzymes catalyze cleavage and C-C bond formation, the formation of chiral 2-hydroxyketones and transferase reactions. New activities such as the formation of carbon-nitrogen, carbon-sulfur and carbon-oxygen bonds are reported recently. [20]

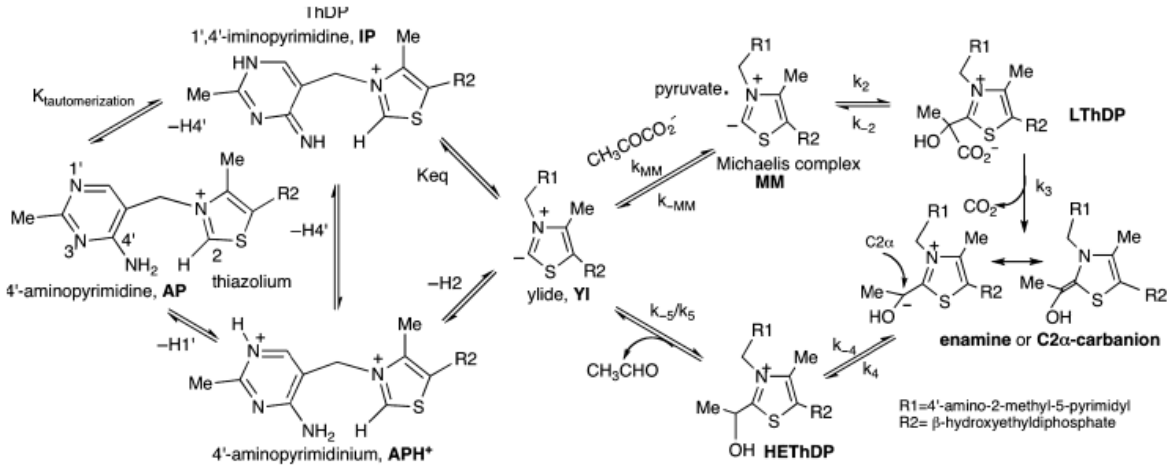



**Figure 17.** Mechanism of yeast pyruvate decarboxylase YPDC [21]

The reactions catalyzed by ThDP can be classified in the sequence: the addition of donors via nucleophilic addition of the carbonyl group to the C2 atom of ThDP; the formation of a common activated intermediate; and subsequent reaction with different acceptors. [20]

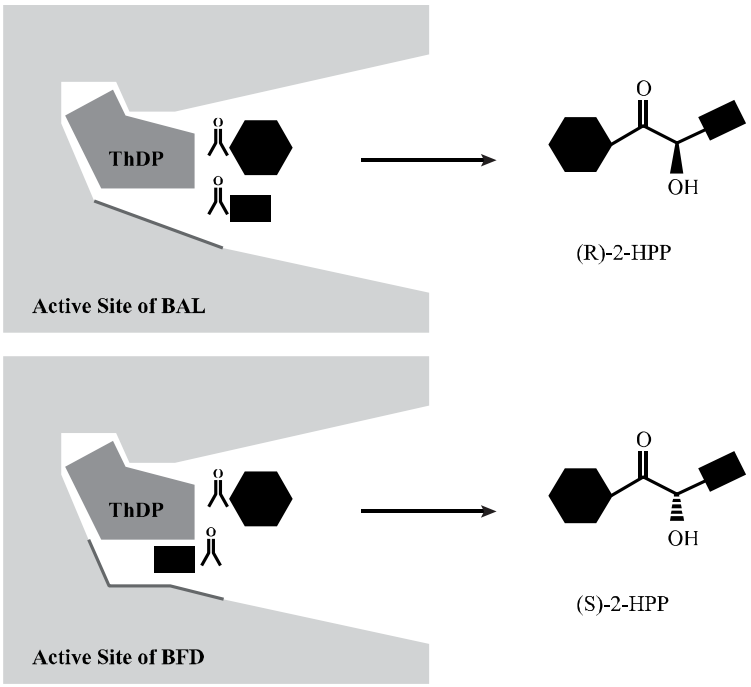



**Figure 18.** Illustrations of active sites of two different ThDP-dependent enzymes [22]

Above figure describes the schematic presentation of the detailed illustrations showing the shape of the binding site for BAL and BFD, two ThDP-dependent enzymes, therefore they are displaying different stereospecificity. [22]

The structure of the active site of the enzyme is another criterion for ThDP-dependent enzymes. The above scheme displays the different stereochemical outcome of products. Due to steric restraints and other interactions, acetaldehyde interacts with ThDP from different directions, thus yielding different enantiomers.

## 1.4. Benzaldehyde Lyase

### 1.4.1 Properties of Benzaldehyde Lyase

Benzaldehyde lyase (BAL, EC 4.1.2.38) from *Ps. Fluorescens* Biovar I was first reported by Gonzales and Vicuna. [28]

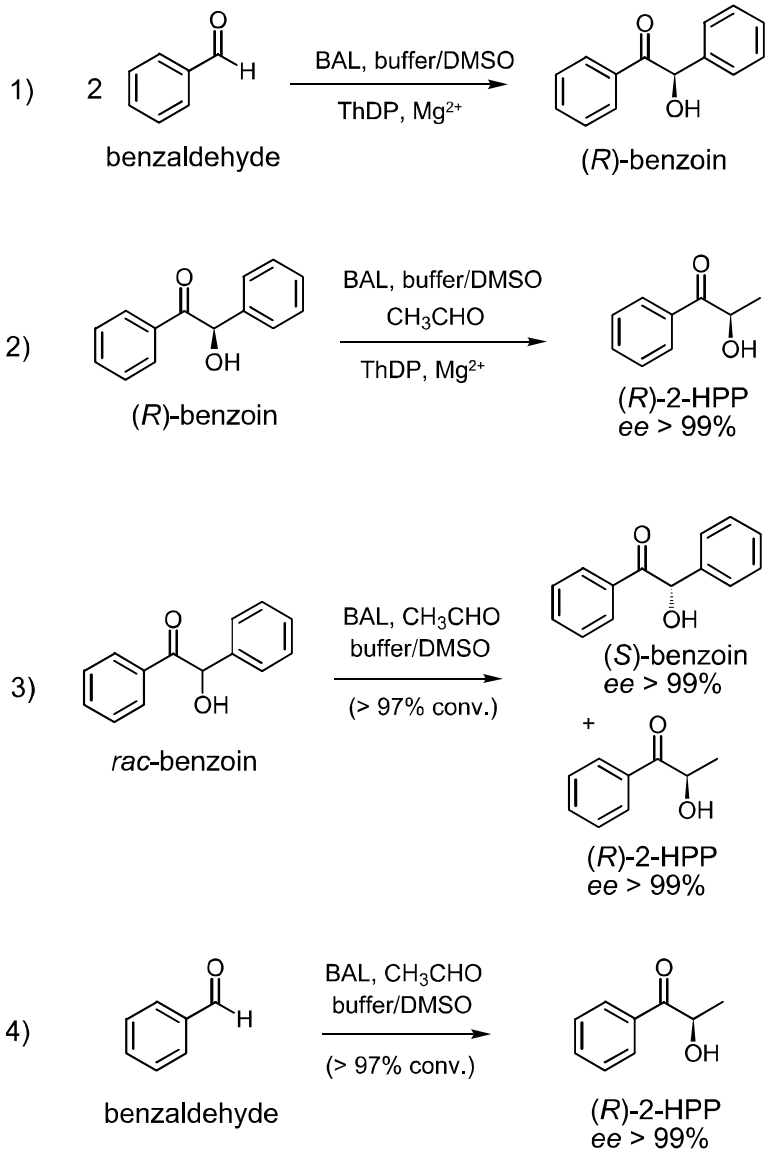



**Figure 19.** Different Type of Reactions Catalyzed by Benzaldehyde Lyase [23]

They showed that the strain can grow on (R)-Benzoin as the sole carbon and energy source due to the ability of BAL to catalyze the cleavage of the acyloin linkage using thiamine diphosphate (ThDP) and $Mg^{2+}$ ion as cofactors. In the reverse reaction, this lyase catalyzes the carboligation of two aldehydes with high substrate selectivity and stereospecificity. [23]

Benzaldehyde lyase utilizes benzoins and acetaldehyde as well as aromatic aldehydes as substrates. When (R)-benzoin is treated with BAL in the presence of acetaldehyde quantitative formation of enantiopure (R)-2-HPP occurs. The same reaction starting from (S)-benzoin fails. Repeating this reaction with racemic benzoin affords enantiopure (R)-2-HPP and (S)-benzoin. Substituted and heteroaromatic benzoins are potential substrates for the kinetic racemic resolution through C-C bond cleavage. The reactions work well in organic - aqueous medium, overcoming the solubility problem of lipophilic substrates and opening the way for large-scale preparations. [23]

### 1.4.2 Optimal Reaction Conditions for Benzaldehyde Lyase

The application of BAL in chemo-enzymatic synthesis requires the addition of cofactors to the buffer: 2.5 mM $MgSO_4$ and 0.1 mM ThDP are sufficient to keep the enzyme stable at up to maximal 37˚C. The enzyme is stable between pH 6 and 8, with pH 7 being the pH-optima of the lyase and ligase reaction. Optimal buffers are potassium phosphate and Tris.

As water-miscible organic co-solvents both, PEG-400 as well as DMSO is useful additives to enhance the solubility of aromatic substrates and products, however the enzyme is significantly more stable in DMSO. [24]

### 1.4.3 Structure of Benzaldehyde Lyase

Benzaldehyde Lyase is a homotetramer of 4 x 563 amino acid residues corresponding to a molecular mass of 4 x 58 919 Da. Each subunit binds one ThDP molecule using one $Mg^{2+}$ ion. The BAL homotetramer has an overall size of approximately 95 x 95 x 75 A˚. No significant structural differences were found between the four crystallographically independent subunits of the tetramer. Each subunit consists of the three domains; Dom-α, Dom-β and Dom-γ, named as in previous annotations. All three domains consist of a central six-stranded parallel β-sheet flanked by a varying number of α-helices. Residues involved in binding of the cofactor ThDP are located at the C-terminal ends of the β-strands of Dom-γ (diphosphates and $Mg^{2+}$) and of Dom-α of a neighboring subunit (pyrimidine moiety). The active center is defined by the thiazolium ring of ThDP, which sits in a deep pocket opening to the outer surface of the tetramer. [25]

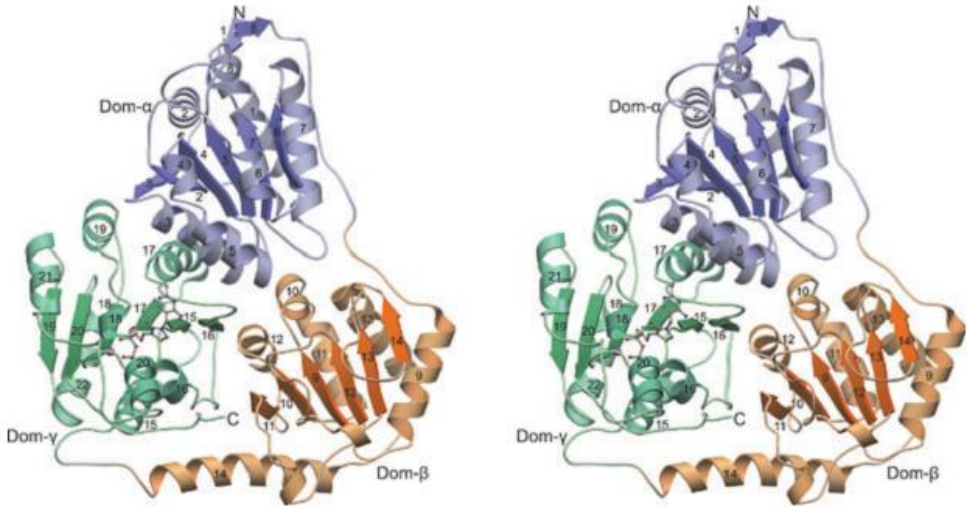



**Figure 20.** The illustration of protein structure of BAL subunit [25]

Stereo ribbon plot of a BAL subunit is composed of the three domains Dom-α (residues 1-183), Dom-β (residues 184-363) and Dom-γ (residues 364-563). The cofactor ThDP is shown in the figure as a ball-and-stick model and $Mg^{2+}$ as a pink sphere. The secondary structures are labeled.

The four subunits A, B, C and D form the two tight dimers A–B and C–D around the molecular axis P, in which each subunit buries a solvent-accessible surface area of 3270 A˚. The two tight dimers are associated much less tightly around the molecular axes Q and R to form a D2-symmetric homotetramer. These secondary interfaces bury 1790 A˚ per subunit. The tight contact is formed by Dom-α and Dom-γ of subunit A with their counterparts in subunit B. It is stabilized by a large number of hydrogen bonds. The weaker contact results from an association of Dom-α and Dom-β of subunit A with the respective domains of subunit D. It contains only few hydrogen bonds. A large cavity lined by the four Dom-α is located at the center of the tetramer. It contains a considerable number of crystallographic water molecules and is not connected to the active center pocket. [25]

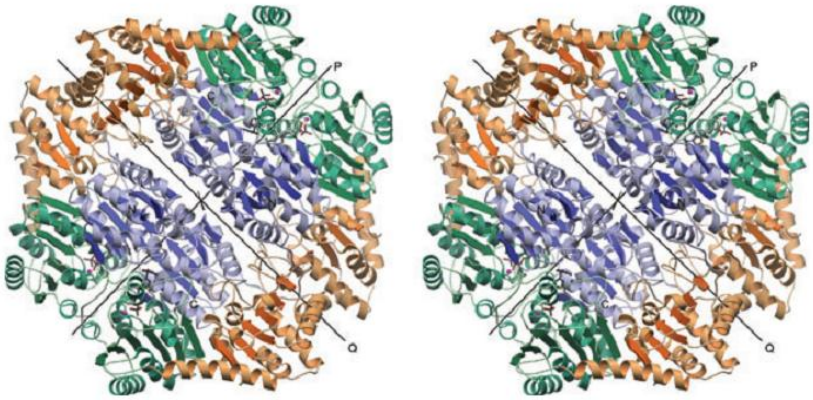



**Figure 21.** Tetrameric protein structure of BAL and interaction of its domains [25]

Above figure illustrates the stereo ribbon plot of the D2-symmetric BAL tetramer with the three molecular twofold axes P, Q and R using the colors of Figure 20. The tetramer should be described as a dimer of dimers. The tightest interfaces are around axis P. Each tight dimer contains two active centers at its interface.

### 1.4.4 Active Center and Reaction Geometry

In crystal structures of BAL a water molecule was identified at a distance of about 3.6 A˚ from the C2 atom of ThDP. This water molecule forms hydrogen bonds with Gln113 and His29, among which Gln113 is known to play an important role in catalysis. The imino group, shown in the below figure, is hydrogen bonded to the carbonyl of Gly419 so that its lone electron pair points to the C2 atom of ThDP. It is therefore most likely that the catalytic cycle starts by transferring a proton from C2 to the imine. The resulting C2 carbanion may then attack the carbonyl carbon of the substrate yielding a covalent ThDP-substrate intermediate. [25]

During acyloin cleavage, the next step is supposedly the deprotonation of the hydroxyl by His29 followed by the dissociation of the first aldehyde. The remaining activated aldehyde is then protonated and also released. The protonation is probably performed by the water attached to His29. During acyloin synthesis, on the other hand, the intermediate is an activated aldehyde that is going to attack an acceptor aldehyde suitably positioned in the active center. Again, His29 is likely to participate in the reaction by forming a hydrogen bond to the oxygen of the acceptor aldehyde, which is eventually converted to a hydroxyl group of the condensation product by deprotonating His29. [25]

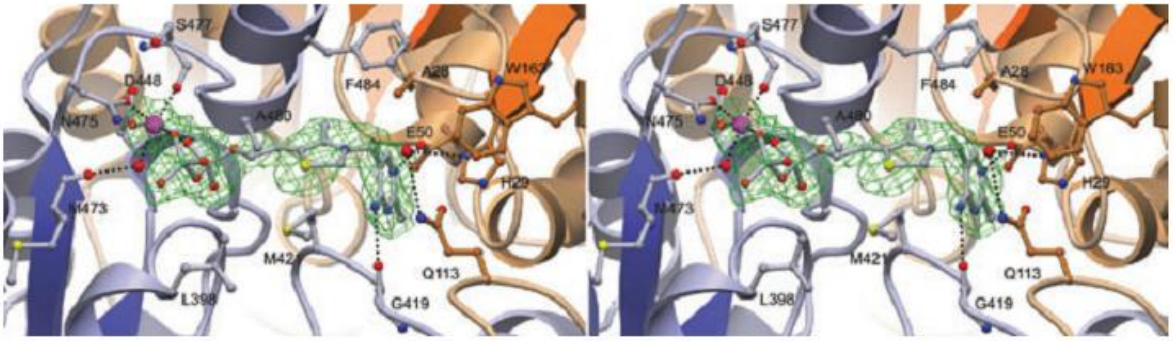



**Figure 22.** The interaction of ThDP and $Mg^{2+}$ at the active site of BAL [25]

Stereoview of ThDP-binding at BAL shows the initial (Fo-Fc)-electron density map of ThDP and $Mg^{2+}$ at the 3 σ contour level. The cofactor binds in the typical V-conformation required for catalysis. BAL residues lining the active center pocket and interacting with the co-factor are shown in blue and orange, corresponding to the two domains they belong to. Hydrogen bonds are given as dotted lines in the figure.

### 1.4.5 Nucleotide Sequence of BAL

The gene encoding benzaldehyde lyase from *Pseudomonas Fluorescens* Biovar I has been cloned and characterized by Hinrichsen and Vicuna in 1994. They characterized the nucleotide sequence containing an open reading frame encoding a protein consists of 563 amino acids. [26]

The Sequence listing below contains the nucleotide sequence encoding a *Pseudomonas Fluorescens* benzaldehyde lyase (Figure 23) and the amino acid sequence which is deduced from it, and also a separate depiction of the amino acid sequence (Figure 24) of the *Pseudomonas Fluorescens* benzaldehyde lyase. [27]

**Number of Sequence ID Nos:2**
**Seq. ID No: 1**
**Length:1689**
**Type: DNA**
**Organism: Pseudomonas Fluorescens**

**Sequence 1**

```
atg gcg atg att aca ggc ggc gaa ctg gtt gtt cgc acc cta ata aag   48
Met Ala Met Ile Thr Gly Gly Glu Leu Val Val Arg Thr Leu Ile Lys

gct ggg gtc gaa cat ctg ttc ggc ctg cac ggc gcg cat atc gat acg   96
Ala Gly Val Glu His Leu Phe Gly Leu His Gly Ala His Ile Asp Thr

att ttt caa gcc tgt ctc gat cat gat gtg ccg atc atc gac acc cgc  144
Ile Phe Gln Ala Cys Leu Asp His Asp Val Pro Ile Ile Asp Thr Arg

cat gag gcc gcc gca ggg cat gcg gcc gag ggc tat gcc cgc gct ggc  192
His Glu Ala Ala Ala Gly His Ala Ala Glu Gly Tyr Ala Arg Ala Gly

gcc aag ctg ggc gtg gcg ctg gtc acg gcg ggc ggg gga ttt acc aat  240
Ala Lys Leu Gly Val Ala Leu Val Thr Ala Gly Gly Gly Phe Thr Asn

gcg gtc acg ccc att gcc aac gct tgg ctg gat cgc acg ccg gtg ctc  288
Ala Val Thr Pro Ile Ala Asn Ala Trp Leu Asp Arg Thr Pro Val Leu

ttc ctc acc gga tcg ggc gcg ctg cgt gat gat gaa acc aac acg ttg  336
Phe Leu Thr Gly Ser Gly Ala Leu Arg Asp Asp Glu Thr Asn Thr Leu

cag gcg ggg att gat cag gtc gcc atg gcg gcg ccc att acc aaa tgg  384
Gln Ala Gly Ile Asp Gln Val Ala Met Ala Ala Pro Ile Thr Lys Trp

gcg cat cgg gtg atg gca acc gag cat atc cca cgg ctg gtg atg cag  432
Ala His Arg Val Met Ala Thr Glu His Ile Pro Arg Leu Val Met Gln

gcg atc cgc gcc gcg ttg agc gcg cca cgc ggg ccg gtg ttg ctg gat  480
Ala Ile Arg Ala Ala Leu Ser Ala Pro Arg Gly Pro Val Leu Leu Asp

ctg ccg tgg gat att ctg atg aac cag att gat gag gat agc gtc att  528
Leu Pro Trp Asp Ile Leu Met Asn Gln Ile Asp Glu Asp Ser Val Ile

atc ccc gat ctg gtc ttg tcc gcg cat ggg gcc aga ccc gac cct gcc  576
Ile Pro Asp Leu Val Leu Ser Ala His Gly Ala Arg Pro Asp Pro Ala

gat ctg gat cag gct ctc gcg ctt ttg cgc aag gcg gag cgg ccg gtc  624
Asp Leu Asp Gln Ala Leu Ala Leu Leu Arg Lys Ala Glu Arg Pro Val

atc gtg ctc ggc tca gaa gcc tcg cgg aca gcg cgc aag acg gcg ctt  672
Ile Val Leu Gly Ser Glu Ala Ser Arg Thr Ala Arg Lys Thr Ala Leu

agc gcc ttc gtg gcg gcg act ggc gtg ccg gtg ttt gcc gat tat gaa  720
Ser Ala Phe Val Ala Ala Thr Gly Val Pro Val Phe Ala Asp Tyr Glu

ggg cta agc atg ctc tcg ggg ctg ccc gat gct atg cgg ggc ggg ctg  768
Gly Leu Ser Met Leu Ser Gly Leu Pro Asp Ala Met Arg Gly Gly Leu

gtg caa aac ctc tat tct ttt gcc aaa gcc gat gcc gcg cca gat ctc  816
Val Gln Asn Leu Tyr Ser Phe Ala Lys Ala Asp Ala Ala Pro Asp Leu

gtg ctg atg ctg ggg gcg cgc ttt ggc ctt aac acc ggg cat gga tct  864
Val Leu Met Leu Gly Ala Arg Phe Gly Leu Asn Thr Gly His Gly Ser

ggg cag ttg atc ccc cat agc gcg cag gtc att cag gtc gac cct gat  912
Gly Gln Leu Ile Pro His Ser Ala Gln Val Ile Gln Val Asp Pro Asp

gcc tgc gag ctg gga cgc ctg cag ggc atc gct ctg ggc att gtg gcc  960
Ala Cys Glu Leu Gly Arg Leu Gln Gly Ile Ala Leu Gly Ile Val Ala

gat gtg ggt ggg acc atc gag gct ttg gcg cag gcc acc gcg caa gat 1008
Asp Val Gly Gly Thr Ile Glu Ala Leu Ala Gln Ala Thr Ala Gln Asp

gcg gct tgg ccg gat cgc ggc gac tgg tgc gcc aaa gtg acg gat ctg 1056
Ala Ala Trp Pro Asp Arg Gly Asp Trp Cys Ala Lys Val Thr Asp Leu

gcg caa gag cgc tat gcc agc atc gct gcg aaa tcg agc agc gag cat 1104
Ala Gln Glu Arg Tyr Ala Ser Ile Ala Ala Lys Ser Ser Ser Glu His

gcg ctc cac ccc ttt cac gcc tcg cag gtc att gcc aaa cac gtc gat 1152
Ala Leu His Pro Phe His Ala Ser Gln Val Ile Ala Lys His Val Asp

gca ggg gtg acg gtg gta gcg gat ggt gcg ctg acc tat ctc tgg ctg 1200
Ala Gly Val Thr Val Val Ala Asp Gly Ala Leu Thr Tyr Leu Trp Leu

tcc gaa gtg atg agc cgc gtg aaa ccc ggc ggt ttt ctc tgc cac ggc 1248
Ser Glu Val Met Ser Arg Val Lys Pro Gly Gly Phe Leu Cys His Gly

tat cta ggc tcg atg ggc gtg ggc ttc ggc acg gcg ctg ggc gcg caa 1296
Tyr Leu Gly Ser Met Gly Val Gly Phe Gly Thr Ala Leu Gly Ala Gln

gtg gcc gat ctt gaa gca ggc cgc cgc acg atc ctt gtg acc ggc gat 1344
Val Ala Asp Leu Glu Ala Gly Arg Arg Thr Ile Leu Val Thr Gly Asp

ggc tcg gtg ggc tat agc atc ggt gaa ttt gat acg ctg gtg cgc aaa 1392
Gly Ser Val Gly Tyr Ser Ile Gly Glu Phe Asp Thr Leu Val Arg Lys

caa ttg ccg ctg atc gtc atc atc atg aac aac caa agc tgg ggg gcg 1440
Gln Leu Pro Leu Ile Val Ile Ile Met Asn Asn Gln Ser Trp Gly Ala

aca ttg cat ttc cag caa ttg gcc gtc ggc ccc aat cgc gtg acg ggc 1488
Thr Leu His Phe Gln Gln Leu Ala Val Gly Pro Asn Arg Val Thr Gly

acc cgt ttg gaa aat ggc tcc tat cac ggg gtg gcc gcc gcc ttt ggc 1536
Thr Arg Leu Glu Asn Gly Ser Tyr His Gly Val Ala Ala Ala Phe Gly

gcg gat ggc tat cat gtc gac agt gtg gag agc ttt tct gcg gct ctg 1584
Ala Asp Gly Tyr His Val Asp Ser Val Glu Ser Phe Ser Ala Ala Leu

gcc caa gcg ctc gcc cat aat cgc ccc gcc tgc atc aat gtc gcg gtc 1632
Ala Gln Ala Leu Ala His Asn Arg Pro Ala Cys Ile Asn Val Ala Val

gcg ctc gat ccg atc ccg ccc gaa gaa ctc att ctg atc ggc atg gac 1680
Ala Leu Asp Pro Ile Pro Pro Glu Glu Leu Ile Leu Ile Gly Met Asp

ccc ttc taa                                                     1689
Pro Phe
```

**Figure 23.** Nucleotide sequence listing of BAL from *Pseudomonas Fluorescens* [27]

**Seq. ID No: 2**
**Length:562**
**Type: Protein**
**Organism: Pseudomonas Fluorescens**

**Sequence 1**

```
Met Ala Met Ile Thr Gly Gly Glu Leu Val Val Arg Thr Leu Ile Lys  16
Ala Gly Val Glu His Leu Phe Gly Leu His Gly Ala His Ile Asp Thr  32
Ile Phe Gln Ala Cys Leu Asp His Asp Val Pro Ile Ile Asp Thr Arg  48
His Glu Ala Ala Ala Gly His Ala Ala Glu Gly Tyr Ala Arg Ala Gly  64
Ala Lys Leu Gly Val Ala Leu Val Thr Ala Gly Gly Gly Phe Thr Asn  80
Ala Val Thr Pro Ile Ala Asn Ala Trp Leu Asp Arg Thr Pro Val Leu  96
Phe Leu Thr Gly Ser Gly Ala Leu Arg Asp Asp Glu Thr Asn Thr Leu 112
Gln Ala Gly Ile Asp Gln Val Ala Met Ala Ala Pro Ile Thr Lys Trp 128
Ala His Arg Val Met Ala Thr Glu His Ile Pro Arg Leu Val Met Gln 144
Ala Ile Arg Ala Ala Leu Ser Ala Pro Arg Gly Pro Val Leu Leu Asp 160
Leu Pro Trp Asp Ile Leu Met Asn Gln Ile Asp Glu Asp Ser Val Ile 176
Ile Pro Asp Leu Val Leu Ser Ala His Gly Ala Arg Pro Asp Pro Ala 192
Asp Leu Asp Gln Ala Leu Ala Leu Leu Arg Lys Ala Glu Arg Pro Val 208
Ile Val Leu Gly Ser Glu Ala Ser Arg Thr Ala Arg Lys Thr Ala Leu 224
Ser Ala Phe Val Ala Ala Thr Gly Val Pro Val Phe Ala Asp Tyr Glu 240
Gly Leu Ser Met Leu Ser Gly Leu Pro Asp Ala Met Arg Gly Gly Leu 256
Val Gln Asn Leu Tyr Ser Phe Ala Lys Ala Asp Ala Ala Pro Asp Leu 272
Val Leu Met Leu Gly Ala Arg Phe Gly Leu Asn Thr Gly His Gly Ser 288

Gly Gln Leu Ile Pro His Ser Ala Gln Val Ile Gln Val Asp Pro Asp 304
Ala Cys Glu Leu Gly Arg Leu Gln Gly Ile Ala Leu Gly Ile Val Ala 320
Asp Val Gly Gly Thr Ile Glu Ala Leu Ala Gln Ala Thr Ala Gln Asp 336
Ala Ala Trp Pro Asp Arg Gly Asp Trp Cys Ala Lys Val Thr Asp Leu 352
Ala Gln Glu Arg Tyr Ala Ser Ile Ala Ala Lys Ser Ser Ser Glu His 368
Ala Leu His Pro Phe His Ala Ser Gln Val Ile Ala Lys His Val Asp 384
Ala Gly Val Thr Val Val Ala Asp Gly Ala Leu Thr Tyr Leu Trp Leu 400
Ser Glu Val Met Ser Arg Val Lys Pro Gly Gly Phe Leu Cys His Gly 416
Tyr Leu Gly Ser Met Gly Val Gly Phe Gly Thr Ala Leu Gly Ala Gln 432
Val Ala Asp Leu Glu Ala Gly Arg Arg Thr Ile Leu Val Thr Gly Asp 448
Gly Ser Val Gly Tyr Ser Ile Gly Glu Phe Asp Thr Leu Val Arg Lys 464
Gln Leu Pro Leu Ile Val Ile Ile Met Asn Asn Gln Ser Trp Gly Ala 480
Thr Leu His Phe Gln Gln Leu Ala Val Gly Pro Asn Arg Val Thr Gly 496
Thr Arg Leu Glu Asn Gly Ser Tyr His Gly Val Ala Ala Ala Phe Gly 512
Ala Asp Gly Tyr His Val Asp Ser Val Glu Ser Phe Ser Ala Ala Leu 528
Ala Gln Ala Leu Ala His Asn Arg Pro Ala Cys Ile Asn Val Ala Val 544
Ala Leu Asp Pro Ile Pro Pro Glu Glu Leu Ile Leu Ile Gly Met Asp 560
Pro Phe                                                         562
```

**Figure 24.** Corresponding amino acid sequence listing of BAL [27]

### 1.4.6 Substrate Specificity

BAL shows a general preference for non-polar substrates and is highly stereospecific with respect to benzoin, cleaving only R-benzoin out of a racemic mixture. Moreover, BAL reacts with benzaldehyde and acetaldehyde to yield (R)-2-hydroxypropiophenone, in contrast to Benzoylformate Decarboxylase, which uses the same educts to produce the (S)-enantiomer. In order to explore the geometry of the reaction catalyzed by BAL, R-benzoin was modeled into its active center. The resulting model accounts for a nucleophilic attack from the deprotonated C2 atom of the thiazolium ring onto the carbonyl carbon of (R)-Benzoin. Fulfilling this restraint, the substrate is uniquely defined with respect to general location and conformation because all alternatives met severe steric obstacles. In contrast to (R)-Benzoin, any model of the (S)-enantiomer gave rise to major sterical clashes, which explains the stereospecificity of BAL. [25]

### 1.4.7 Reaction Mechanism of Benzaldehyde Lyase Catalyzed Acyloin Formation and Cleavage

The first step of the catalytic cycle is the deprotonation of the C2 atom to yield a ThDP ylide that subsequently attacks a carbonyl compound (e.g., benzaldehyde or benzoin). The intermediate products are stabilized by deprotonation (in the case of benzaldehyde), or acyloin cleavage (in the case of benzoin) to yield a carbanion-enamine, that can be regarded as a ThDP-bound, activated donor aldehyde. Subsequently, the carbanion-enamine can attack an acceptor aldehyde to form a 2-hydroxyketone. Alternatively, protonation of the carbanion-enamine results in the release of benzaldehyde. In the end, ThDP is regenerated during the catalytic cycle. [22]

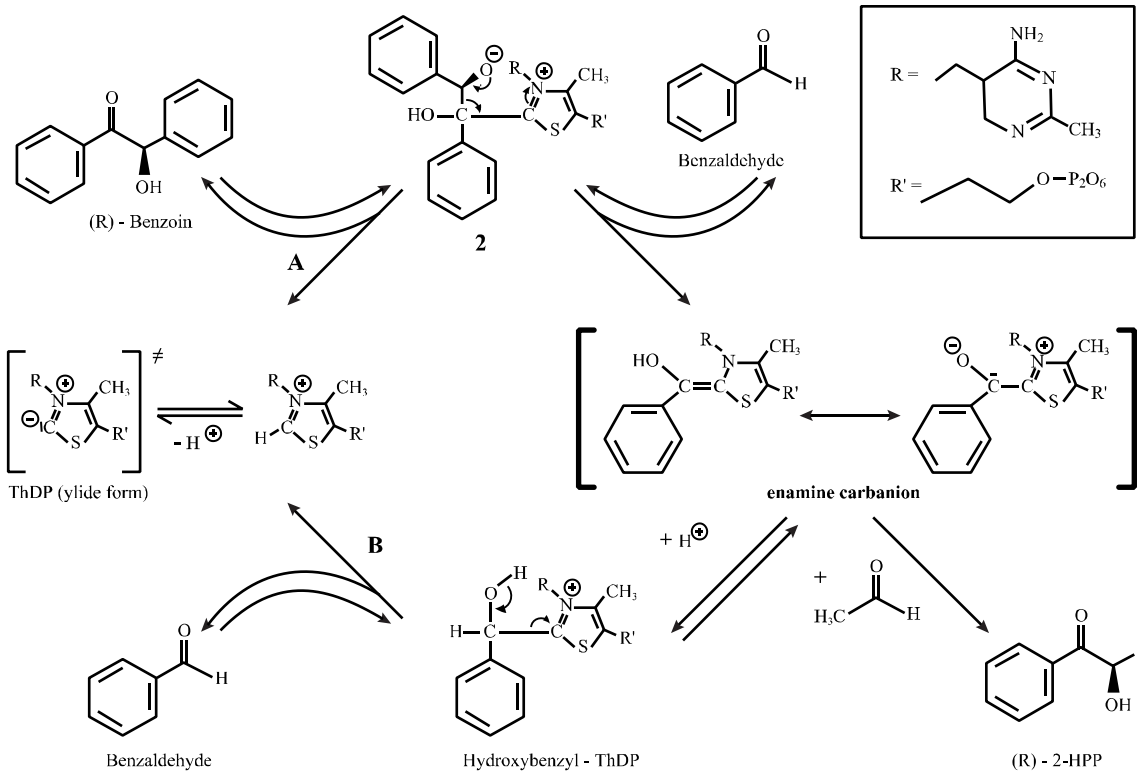



**Figure 25.** Proposed mechanism for BAL catalyzed acyloin formation and cleavage based on observations with other ThDP-dependent enzymes. [23]

## 1.5 Aim of the Study

BAL is a valuable tool for chemoenzymatic syntheses because it generates various enantiomerically pure 2-hydroxy ketones through aldehyde ligation or by partial decomposition of racemic mixtures. [23] In this study, the above-mentioned potentials of Benzaldehyde Lyase will be investigated. This thiamine diphosphate dependent enzyme's substrate profile and its capacity for producing enantiopure benzoin and 2-hydroxypropiophenone derivatives and racemic resolution of benzoins yielding (S)-benzoins will be questioned.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1. Materials

Benzaldehyde Lyase and the cofactor Thiamine diphosphate is supplied from the Institut fur Biotechnologie, Julich, Germany.

Dimethyl Sulfoxide (DMSO) and acetaldehyde were purchased from Merck, Darmstadt, Germany.

Benzaldehyde and the derivatives were commercially available by, Aldrich, Germany and did not require further purification.

Benzoin and the derivatives were synthesized in the laboratory using the Benzoin Condensation procedure.

All other chemicals were of the analytical grade and were obtained from commercial sources at the highest grade of purity available.

Solvents are either in technical or higher grade, when necessary; they were purified and dried with drying agents and by distillation.

## 2.2. Methods

### 2.2.1 Analytical Procedures

#### 2.2.1.1 Chiral Column HPLC

The enantiomeric excess values of benzoin and 2-hydroxypropiophenones and their retention times (RT) are determined by chiral phase HPLC analysis (Chiralpak AD column, UV detection at 254 nm, 20°C). The eluent system is n-hexane/2-propanol (90:10) and the relative proportion of solvents are in volume:volume ratio. The optimum flow rate is determined as 0.80 mL/min. However, for some special analysis were carried using different eluent ratio systems and different flow rates.

#### 2.2.1.2 NMR

Nuclear Magnetic Resonance ($^1$H-NMR) spectra were recorded with a Bruker Instrument DPX-400, 400 MHz High Performance Digital FT-NMR Spectrometer by using deuterochloroform ($CDCl_3$) as a solvent and tetramethylsilane (TMS) as internal standard, respectively. Chemical shifts are as in parts per million (δ) downfield from an internal standard TMS. Spin multiplicities are mentioned as: s (singlet), d (doublet), dd (doublet of doublet), t (triplet), q (quartet), m (multiplet).

#### 2.2.1.3 Measurement of Optical Rotations

Optical rotations of the purified enantiomers were measured with a Bellingham & Stanley P20 polarimeter or a Perkin-Elmer 241 polarimeter. Mps were measured on a capillary tube apparatus and are uncorrected.

### 2.2.2 Procedure of Chemical Synthesis

#### 2.2.2.1 Benzoin Condensation and Purification of Benzoin Derivatives

In a 250 ml flask, 1.5 g of potassium cyanide was dissolved in 15 ml of water. Upon this solution, 30 ml of ethanol and 15 ml of benzaldehyde were added and altogether, they were allowed to reflux gently on a steam bath with the precaution set-up of sodium hydroxide trap. After forty minutes, the flask was cooled on an ice bath for the purpose of crystallization. The precipitation of benzoin was collected on a Buchner funnel and washed with 100 ml of water. Remaining water was removed by suction and the filtered benzoin crystals were washed with 10 ml of cold ethanol and 10 ml of ether, respectively. The benzoin crystals were dried with pump for about half an hour.

### 2.2.3 Procedure of Enzymatic Reaction

#### 2.2.3.1 Preparation of BAL

Hexahistidine-tagged BAL was obtained from recombinant E.Coli SG13009 cells. One unit (U) of activity is defined as the amount of enzyme which catalyzes the cleavage of 1μmol benzoin (1.5 mM) into benzaldehyde in potassium phosphate buffer (50 mmol/L, pH 7.0, containing $MgSO_4$ (2.5 mmol/L), ThDP (0.15 mmol/L) and 15 % PEG 400 (v/v) in 1 min at 30°C.

#### 2.2.3.2 Synthesis of (R)-Benzoin Derivatives

Benzaldehyde (318 mg, 3 mmol) was dissolved in a mixture of dimethyl sulfoxide (20 mL) and potassium phosphate buffer [80 mL, 50 mM, pH 7.0, containing $MgSO_4$ (2.5

mM) and ThDP (0.15 mM)]. After addition of BAL (20 U) the reaction mixture was allowed to stand at 25°C for 48 hours before a further 20 U of BAL were added. After 62 hours, no more benzaldehyde was detected by GC-MS. The reaction mixture was extracted with dichloromethane (250 mL) and the organic layer washed with water (25 mL) and brine (25 mL) and dried over $Na_2SO_4$. The solvent is evaporated and 305 mg of (R)-2-hydroxy-1,2-diphenylethan-1-one as a colorless solid is obtained with a yield percentage of 96% and enantiomeric excess of 99% . [23]

#### 2.2.3.3 Synthesis of (R)-2-Hydroxy-1-phenylpropan-1-one Derivatives

Benzaldehyde (212 mg, 2 mmol) was dissolved in a mixture of dimethyl sulfoxide (20 ml) and potassium buffer (80 ml, 50mM, pH 7.0, containing $MgSO_4$ (2.5 mmol/L), ThDP (0.15 mmol/L). To this solution 88 mg (2 mmol) acetaldehyde was added. After addition of 20 U of BAL, the reaction mixture was allowed to stand at 25°C. After 24 hours, 20 U of BAL and 176 mg (4 mmol) of acetaldehyde were added. This was repeated every 24 hours. After 96 hours, the conversion was determined as 97 % via GC-MS. [23]

#### 2.2.3.4 Enzymatic Racemic Resolution of Benzoins to yield (S)-Benzoin derivatives and the corresponding enzymatic synthesis of (R)-2-Hydroxy-1-phenylpropan-1-one Derivatives from (R)-Benzoin

Approximately 2 mmol substrate of racemic benzoin (424 mg) was dissolved in a mixture of dimethyl sulfoxide (20 ml) and potassium buffer (80 ml, 50mM, pH 7.0, containing $MgSO_4$ (2.5 mmol/L), ThDP (0.15 mmol/L). To this solution 88 mg (2 mmol) acetaldehyde was added. After addition of BAL (20 U), the reaction mixture was

allowed to stand at 25°C. After 24 hours, 20 U of BAL and 176 mg (4 mmol) of acetaldehyde were added. This was repeated every 24 hours until no more (R)-Benzoin was observed. The conversion of (R)-Benzoin to (R)-2-Hydroxy-1-phenylpropan-1-one was determined as 97 % by GC-MS. The racemate of benzoin was resolved to obtain (S)-Benzoin with an ee>99%.

### 2.2.4 Purification of the Compounds

Column chromatography was employed using thick-walled glass columns with flash grade silica-gel (Merck Silica Gel 60, particle size: 0.004-0.063 mm, 230-400 mesh ASTM). Thin layer chromatography was carried out on aluminum sheets precoated with silica gel 60 $F_{254}$ (Merck), and the spots were visualized with UV light ($\lambda$= 254 nm).

The relative proportion of solvents is in volume: volume ratio used in column chromatography as eluent.

# CHAPTER 3

## RESULTS AND DISCUSSION

Starting with different kind of substrates, there are four kinds of reactions that Benzaldehyde Lyase can catalyze:

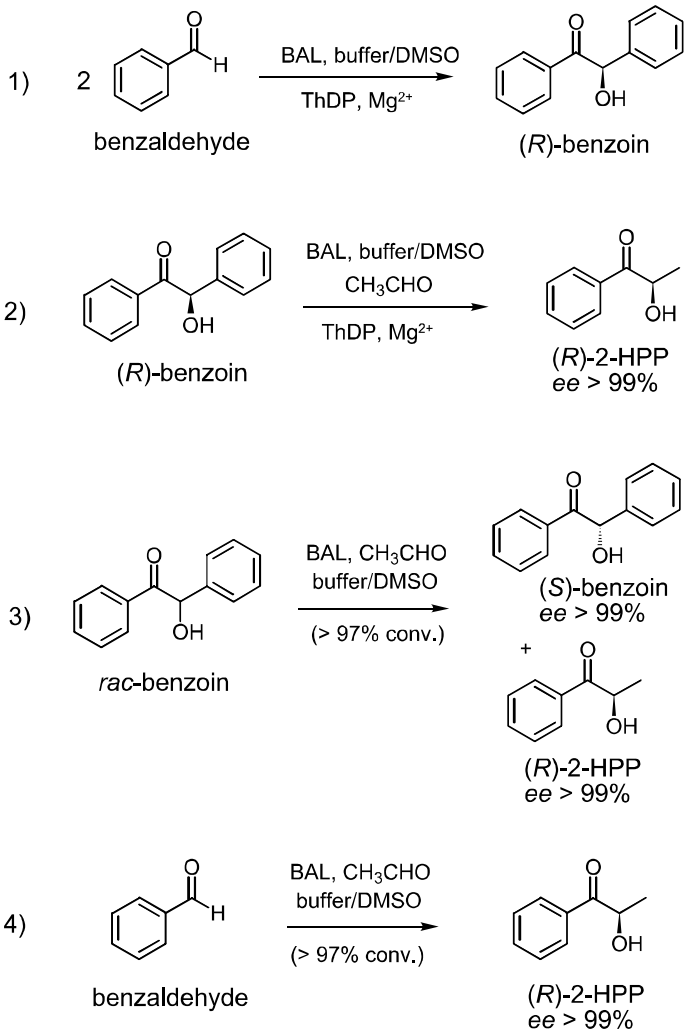



**Figure 26.** Benzaldehyde Catalyzed Reactions

1.Enantioselective C-C bond formation starting with benzaldehyde derivatives

2.Enantioselective C-C bond cleavage and formation reaction starting with (R)-Benzoin and acetaldehyde

3.Enantioselective C-C bond cleavage and formation reaction given racemic benzoin together with acetaldehyde (kinetic racemic resolution reaction)

4.Enantioselective C-C bond formation starting with benzaldehyde derivatives and acetaldehyde

## 3.1. C-C Bond Formation Reactions Using Benzaldehyde Lyase

Benzaldehyde lyase (BAL, EC 4.1.2.38) from *Pseudomonas Fluorescens* Biovar I was first reported by Gonzales and Vicuna. [28] They showed that this strain can grow on benzoin as a sole carbon and energy source due to the ability of BAL to catalyze the cleavage of the acyloin linkage of benzoin yielding benzaldehyde.

Inspired by the cleavage reaction performed by the enzyme, the reverse reaction is questioned in terms of substrate profile and stereospecificity. The potential of BAL for catalyzing C-C bond formation is investigated with different substrates given to the enzyme.

### 3.1.1 The Reaction of BAL with Benzaldehyde Derivatives

As shown in Figure 26, the carboligation reaction with BAL by using benzaldehyde as a sole substrate in potassium phosphate buffer (50 mmol $L^{-1}$, pH 7.0) containing $MgSO_4$ (2.5 mmol $L^{-1}$) and ThDP (0.15 mmol $L^{-1}$) at 21°C is performed and the reaction is

monitored by HPLC using a chiral stationary phase column with authentic samples as reference. The results showed the formation of (R)-benzoin with an ee >99% [23]

Due to the low solubility of the benzaldehyde derivatives in water, DMSO is thought to be employed in the reaction medium. Accordingly, addition of DMSO (20%, v/v) to the aqueous medium containing BAL resulted in the formation of (R)-benzoin starting from benzaldehyde. This conversion worked almost quantitatively and (R)-benzoin was obtained in optically pure form (ee >99%). During the reaction most of the benzoin precipitates from the reaction mixture. A small amount of benzaldehyde was present at the end of the reaction (1 - 2%). [23]

### 3.2. BAL-Catalyzed Enantioselective Synthesis of (R)- Benzoins

Benzaldehyde Lyase has proven itself to have the potential to couple the substrate benzaldehyde and its derivatives to stereospecifically produce (R)-Benzoins.

#### 3.2.1 Synthesis of (R)-2-hydroxy-1,2-diphenylethan-1-one from Benzaldehyde

Benzaldehyde Lyase catalyzes the C-C bond formation reaction between two molecules of benzaldehyde to yield (R)-Benzoin.

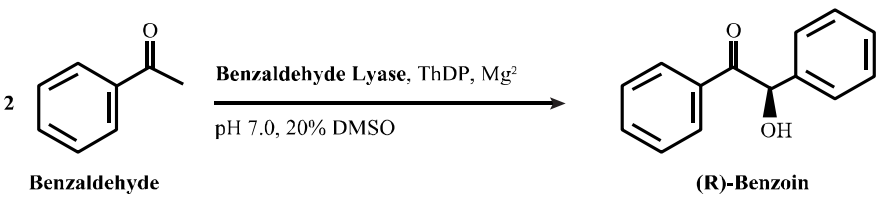



**Figure 27.** (R)-Benzoin formation reaction

Benzaldehyde (318 mg, 3 mmol) was dissolved in a mixture of dimethyl sulfoxide (20 mL) and potassium phosphate buffer [80 mL, 50 mM, pH 7.0, containing $MgSO_4$ (2.5 mM) and ThDP (0.15 mM)]. After addition of BAL (20 U) the reaction mixture was allowed to stand at 25°C for 48 hours before a further 20 U of BAL were added. After 62 hours, no more benzaldehyde was detected by GC-MS. The reaction mixture was extracted with dichloromethane (250 mL) and the organic layer washed with water (25 mL) and brine (25 mL) and dried over $Na_2SO_4$. Evaporation of the solvent and purification of the crude product by crystallization afforded 305 mg (96%) of (R)-Benzoin with an enantiomeric excess value greater than 99%. [23]

(R)-2-hydroxy-1,2-diphenylethan-1-one is a colorless solid with $[a]^{22}_{D}$ : -113.8 (c 1.5, $CH_3COCH_3$) and it melts between 133°-134°C. The HPLC (AD Chiralpak) retention time for (R)-Benzoin is 27.1 minutes when the eluent is isohexane/2-propanol (90: 10, flow 0.80 mL min-1) at 20°C.

The $^{1}H$ NMR (400 MHz, $CDCl_3/CCl_4$) results for (R) Benzoin is: δ=7.92 (d, *J*=7.9 Hz, 2H), 7.29 - 7.52 (m, 8H), 5.97 (d, *J*=6.1 Hz, 1H), 4.58 (d, *J*=6.1 Hz, 1H). $^{13}C$ NMR (100 MHz, $CDCl_3/CCl_4$) results are: δ=198.9, 139.6, 134.1, 134.0, 129.5, 129.4, 128.9, 128.8, 128.2, 76.5.

### 3.2.2 Synthesis of (R)-1,2-Bis(2-fluorophenyl)-2-hydroxyethan-1-one from 2-Fluoro Benzaldehyde

Benzaldehyde Lyase catalyzes:

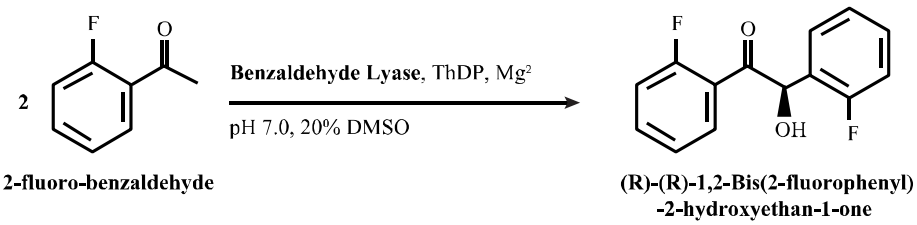



**Figure 28.** (R)-1,2-Bis(2-fluorophenyl)-2-hydroxyethan-1-one formation reaction

2-Fluoro-benzaldehyde (372 mg, 3 mmol) was dissolved in a mixture of dimethyl sulfoxide (20 mL) and potassium phosphate buffer [80 mL, 50 mM, pH 7.0, containing $MgSO_4$ (2.5 mM) and ThDP (0.15 mM)]. After addition of BAL (20 U) the reaction mixture was allowed to stand at 25°C for 48 hours before a further 20 U of BAL were added. After 62 hours, no more 2-fluoro-benzaldehyde was detected by GC-MS. The reaction mixture was extracted with dichloromethane (250 mL) and the organic layer washed with water (25 mL) and brine (25 mL) and dried over $Na_2SO_4$. Evaporation of the solvent and purification of the crude product by crystallization afforded 252 mg (68%) of (R)-1,2-Bis(2-fluorophenyl)-2-hydroxyethan-1-one with an enantiomeric excess value greater than 96%. [23]

(R)-1,2-Bis(2-fluorophenyl)-2-hydroxyethan-1-one is a colorless solid with $[a]^{20}_{D}$: -261.4 (c 0.5, $CH_3OH$). Its melting point is between 60°- 62°C. The HPLC (AD Chiralpak) retention time for (R) enantiomer is 20.2 minutes when the eluent is isohexane/2-propanol (90: 10, flow 0.80 mL $min^{-1}$) at 20°C.

The $^{1}H$ NMR (400 MHz, $CDCl_3/CCl_4$) results for (R)-1,2-Bis(2-fluorophenyl)-2-hydroxyethan-1-one is: δ= 7.80 - 7.91 (m, 1H), 7.55 - 7.67 (m, 1H), 6.98 - 7.30 (m, 6H), 5.91 (d, *J*= 5.6 Hz, 1H), 4.33 (d, J= 5.6 Hz, 1H); $^{13}C$ NMR (100 MHz, $CDCl_3/CCl_4$): δ= 198.6, 164.2 (d, J= 253 Hz), 163.5 (d, *J*= 251 Hz), 134.7 (d, *J*= 13 Hz), 134.1 (d, *J*= 12 Hz), 130.6, 131.2, 129.2, 128.9, 124.4, 123.6, 116.2 (d, *J*= 24 Hz), 115.4 (d, *J*= 23 Hz), 75.9.

### 3.2.3 Synthesis of (R)-1,2-Bis(2-methoxyphenyl)-2-hydroxyethan-1-one from 2-Methoxy-Benzaldehyde

Benzaldehyde Lyase catalyzes:

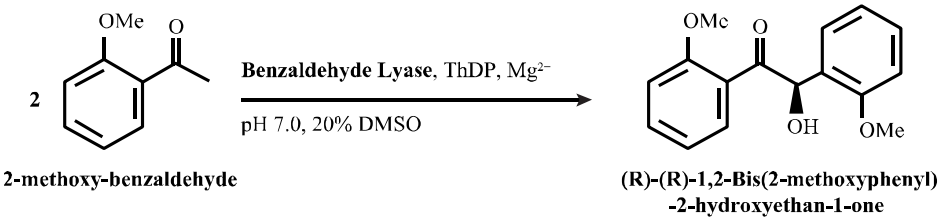



**Figure 29.** (R)-1,2-Bis(2-methoxyphenyl)-2-hydroxyethan-1-one formation reaction

2-Methoxy-benzaldehyde (408 mg, 3 mmol) was dissolved in a mixture of dimethyl sulfoxide (20 mL) and potassium phosphate buffer [80 mL, 50 mM, pH 7.0, containing $MgSO_4$ (2.5 mM) and ThDP (0.15 mM)]. After addition of BAL (20 U) the reaction mixture was allowed to stand at 25°C for 48 hours before a further 20 U of BAL were added. After 96 hours, no more 2-methoxy-benzaldehyde was detected by GC-MS. The reaction mixture was extracted with dichloromethane (250 mL) and the organic layer washed with water (25 mL) and brine (25 mL) and dried over $Na_2SO_4$. Evaporation of the solvent and purification of the crude product by crystallization revealed 354 mg (87%) of (R)-1,2-Bis(2-methoxyphenyl)-2-hydroxyethan-1-one with an enantiomeric excess value greater than 99%. [23]

(R)-1,2-Bis(2-methoxyphenyl)-2-hydroxyethan-1-one is a colorless solid with $[a]^{20}_D$: -125 (c 0.9, $CHCl_3$). The racemic compound has a melting point is between 98°- 99°C. The HPLC (AD Chiralpak) retention time for (R) enantiomer is 31.2 minutes when the eluent is n-hexane/2-propanol (98: 2, flow 0.90 mL $min^{-1}$) at 20°C.

The $^1H$ NMR (400 MHz, $CDCl_3/CCl_4$) results for (R)-1,2-Bis(2-methoxyphenyl)-2-hydroxyethan-1-one is: δ= 6.63 - 7.69 (m, 8H), 5.92 (d, *J*= 5.1 Hz, 1H), 4.29 (d, *J*= 5.1 Hz, 1H), 3.71 (s, 3H), 3.69 (s, 3H); $^{13}C$ NMR (100 MHz, $CDCl_3/CCl_4$): δ= 201.6, 158.4, 157.6, 134.0, 131.1, 130.3, 129.8, 128.2, 125.8, 120.9, 120.8, 111.4, 111.2, 76.1, 55.5, 55.4.

### 3.3. C-C Bond Cleavage Reactions Using Benzaldehyde Lyase

Knowing that Benzaldehyde Lyase uses benzoin derivatives as secondary substrates (primary substrates are benzaldehyde derivatives), different kinds of benzoin derivatives are introduced to the enzyme. The main purpose is to define the substrate profile of the enzyme and understand the synthesis efficiency of BAL.

#### 3.3.1 The Reaction of BAL with Benzoin Derivatives

Benzaldehyde Lyase is shown to accept only the (R)-Benzoin derivatives as substrates and to stereospecifically cleave the acyloin linkage of (R)-Benzoins to yield benzaldehyde derivatives. If an aldehyde (aliphatic or aromatic) is introduced to the reaction medium, the enzyme's ability is questioned if it produces new enantiopure alpha hydroxy ketones. In this study, acetaldehyde is the secondary aldehyde to be introduced into the reaction medium in the presence of benzoin derivatives.

The above mentioned ability of the enzyme will be used for two aims in this study:

1. Synthesis of (R)-2-HPP derivatives
2. Racemic Resolution of Benzoin Derivatives

### 3.4. BAL-Catalyzed Enantioselective Synthesis of (R)-2-HPP and (S)-Benzoins Starting with Racemic Benzoins

The substrate profile of the enzyme for the (R)-2-HPP synthesis starting from racemic benzoin was analyzed. Before dealing with the preparative studies, the enzyme is first tested with 5mM of substrate (benzoin derivatives) with addition of 5 µl of acetaldehyde

in 1500 µl reaction medium. The control reactions were allowed to stand for 96 hours and every 24 hour the reaction solutions were analyzed by TLC.

### 3.4.1 2-Hydroxy-1,2-diphenylethan-1-one

The Benzaldehyde Lyase is expected to catalyze the (R)-2-HPP formation following the racemic resolution of the benzoin which is introduced to enzyme with both enantiomers.

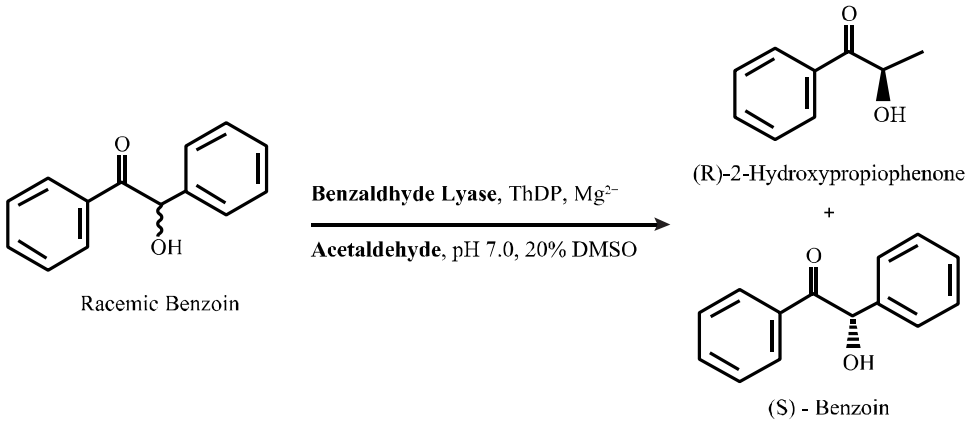



**Figure 30**. BAL catalyzed formation of (R)-2-HPP and resolution of racemic Benzoin to yield (S)-Benzoin

In a 1500 µl Eppendorf tube, the reaction is tested with 5mM of substrate (1.6 mg benzoin) with addition of 5 µl of acetaldehyde in 1500 µl reaction medium. The reaction solution consists of 900 µl potassium phosphate buffer (pH 7.0), 100 µl of thiamine diphosphate solution, 250 µl of enzyme solution, 250 µl of DMSO solution. Due to its highly volatile nature, acetaldehyde is dissolved in 250 µl of DMSO first. The control reactions were allowed to stand for 96 hours at room temperature and every 24 hour, 5 µl of acetaldehyde and 1 U enzyme is added. In order to observe the (R)-2-HPP formation

with TLC, a blank solution excluding the enzyme is prepared. The solvent ratio for TLC is 3 Hexane: 1 Ethyl acetate. The formation of (R)-2-HPP is observed by a separate dot on the TLC sheet under UV light.

For the preparative studies, approximately 424 mg substrate of racemic benzoin (2 mmol) was dissolved in a mixture of dimethyl sulfoxide (20 ml) and potassium buffer (80 ml, 50mM, pH 7.0, containing $MgSO_4$ (2.5 mmol/L), ThDP (0.15 mmol/L). To this solution 88 mg (2 mmol) acetaldehyde was added. After addition of BAL (20 U), the reaction mixture was allowed to stand at room temperature. After 24 hours, 20 U of BAL and 176 mg (4 mmol) of acetaldehyde were added. This was repeated every 24 hours until no more (R)-Benzoin was observed. The conversion was determined as 49 % (GC-MS). The racemate of benzoin was resolved to obtain (S)-Benzoin with an enantiomeric excess of more than 99 %.

**(S)-Benzoin** *((S)-2-hydroxy- 1,2-diphenylethan-1-one)* is a colorless solid and it melts between 132 - 133ºC. According to the results from polarimeter recordings, the (S) enantiomer has $[a]^{22}_{D}$ : +113.8 (c 2.5, $CH_3COCH_3$). The HPLC (Chiralpak AD, isohexane/2-propanol 90:10, flow 0.80 mL.min$^{-1}$, 20ºC) results displayed the separation of (R) and (S) enantiomers whose retention times are 27.1 and 34.5 minute, respectively.

The $^{1}$H NMR (400 MHz, $CDCl_3/CCl_4$) results for (S) Benzoin is: δ=7.92 (d, *J*=7.9 Hz, 2H), 7.29 - 7.52 (m, 8H), 5.97 (d, *J*=6.1 Hz, 1H), 4.58 (d, *J*=6.1 Hz, 1H). $^{13}$C NMR (100 MHz, $CDCl_3/CCl_4$) results are: δ=198.9, 139.6, 134.1, 134.0, 129.5, 129.4, 128.9, 128.8, 128.2, and 76.5.

**(R)-2-HPP** is viscous oil. The yield for the reaction is determined as 47% with an enantiomeric excess of more than 99%. According to the results from polarimeter recordings, the (R)-2-HPP has $[a]^{20}_{D}$ : +85.1 (c 2.0, $CHCl_3$). The HPLC (Chiralpak AD, isohexane/2-propanol 90:10, flow 0.80 mL $min^{-1}$, 20 ºC) results showed the formation of (R)-2-HPP which has a Rt(R) value at 14.3 minute.

The $^{1}H$ NMR (400 MHz, $CDCl_3/CCl_4$) results for (R)-2-HPP is: δ=7.90 (dd, *J*=1.4, 8.2 Hz, 2H), 7.40 - 7.60 (m, 3H), 5.13 (q, *J*=6.0 Hz, 1H), 3.80 (br.s, 1 H), 1.41 (d, *J*=6.0 Hz, 3H). $^{13}C$ NMR (100 MHz, $CDCl_3/CCl_4$) results are: δ=202.7, 134.4, 134.0, 128.9, 128.7, 69.2, 22.0.

### 3.4.2 1,2-Bis(2-fluorophenyl)-2-hydroxyethan-1-one

The Benzaldehyde Lyase is expected to catalyze the (R)-1-(2-Fluorophenyl)-2-hydroxypropan-1-one formation following the racemic resolution of the 1,2-Bis(2-fluorophenyl)-2-hydroxyethan-1-one which is introduced to enzyme in the presence of acetaldehyde.

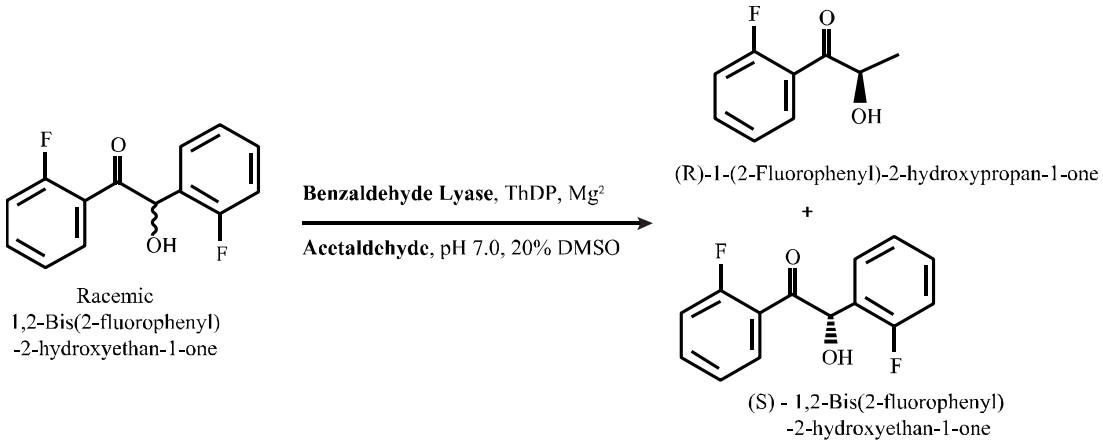



**Figure 31.** BAL catalyzed formation of (R)-1-(2-Fluorophenyl)-2-hydroxypropan-1-one

In a 1500 µl Eppendorf tube, the reaction is tested with 5mM of substrate (1.87 mg 1,2-Bis(2-fluorophenyl)-2-hydroxyethan-1-one) with addition of 5 µl of acetaldehyde in 1500 µl reaction medium. The reaction medium was allowed to stand for 96 hours at room temperature and every 24 hour, 5 µl of acetaldehyde and 1 U enzyme is added. In order to observe the (R)-1-(2-Fluorophenyl)-2-hydroxypropan-1-one formation with TLC, a blank solution excluding the enzyme is prepared. The formation of (R)-1-(2-Fluorophenyl)-2-hydroxypropan-1-one is observed by a separate dot on the TLC (solvent system 3H: 1 EA) sheet under UV light.

For the preparative studies, approximately 476 mg substrate of racemic 1,2-Bis(2-fluorophenyl)-2-hydroxyethan-1-one (2 mmol) was dissolved in a mixture of dimethyl sulfoxide (20 ml) and potassium buffer (80 ml, 50mM, pH 7.0, containing $MgSO_4$ (2.5 mmol/L), ThDP (0.15 mmol/L). To this solution 88 mg (2 mmol) acetaldehyde was added. After addition of BAL (20 U), the reaction mixture was allowed to stand at room temperature. After 24 hours, 20 U of BAL and 176 mg (4 mmol) of acetaldehyde were added. The conversion of (R)- 1,2-Bis(2-fluorophenyl)-2-hydroxyethan-1-one to ((R)-1-(2-Fluorophenyl)-2-hydroxypropan-1-one was determined as 34 % (GC-MS). The racemate of benzoin was resolved to obtain (S)-Benzoin with an enantiomeric excess of 96%.

**(S)-1,2-Bis(2-fluorophenyl)-2-hydroxyethan-1-one** is a colorless solid and it melts between 61 - 62 ºC. According to the results from polarimeter recordings, the (S) enantiomer has $[a]^{20}_D$ : +264.1 (c 0.5, $CH_3OH$). The HPLC (Chiralpak AD, isohexane/2-

propanol 90:10, flow 0.80 mL $min^{-1}$, 20 ºC) results displayed the separation of (R) (Rt 20.2 minute) and (S) (Rt 17.8 minutes) enantiomers.

The $^{1}H$ NMR (400 MHz, $CDCl_3/CCl_4$) results for (S)-1,2-Bis(2-fluorophenyl)-2-hydroxyethan-1-one is: δ = 7.80 - 7.91 (m, 1H), 7.55 - 7.67 (m, 1H), 6.98 - 7.30 (m, 6H), 5.91 (d, *J* =5.6 Hz, 1H), 4.33 (d, *J* =5.6 Hz, 1H). $^{13}C$ NMR (100 MHz, $CDCl_3/CCl_4$) results are: δ = 198.6, 164.2 (d, *J*= 253 Hz), 163.5 (d, *J* = 251 Hz), 134.7 (d, *J* = 13 Hz), 134.1, 131.2, 129.2, 128.9, 124.4, 123.6, 116.2 (d, *J* = 24 Hz), 115.4 (d, *J* = 23 Hz), 75.9.

**(R)-1-(2-Fluorophenyl)-2-hydroxypropan-1-one** is a viscous oil. The yield for the reaction is determined as 32% with an enantiomeric excess of 97 %. According to the results from polarimeter readings, the (R)-1-(2-Fluorophenyl)-2-hydroxypropan-1-one has $[a]^{20}_{D}$ : +105.3 (c 0.5, $CHCl_3$). The HPLC (Chiralpak AD, isohexane / 2-propanol 90:10, flow 0.80 mL.$min^{-1}$, 20 ºC) results showed the formation of (R)-1-(2-Fluorophenyl)-2-hydroxypropan-1-one which has a Rt (R) value at 11.1 minute.

The $^{1}H$ NMR (400 MHz, $CDCl_3/CCl_4$) results for (R)-1-(2-Fluorophenyl)-2-hydroxypropan-1-one is: δ=7.81 - 7.90 (m, 1H), 7.56 - 7.67 (m, 1H), 7.12 - 7.31 (m, 2H), 4.96 (q, *J* =6.8 Hz, 1H), 3.72 (br.s, 1H), 1.29 (d, *J* =6.8 Hz, 3H). $^{13}C$ NMR (100 MHz, $CDCl_3/CCl_4$) results are: δ=198.2, 163.8 (d, *J* =251 Hz), 134.5 (d, *J* =12 Hz), 131.3, 129.9, 124.5, 115.9 (d, *J* =23 Hz), 72.2, 21.1.

### 3.4.3 1,2-Bis(2-methoxyphenyl)-2-hydroxyethan-1-one

The Benzaldehyde Lyase is expected to catalyze the (R)-1-(2-methoxyphenyl)-2-hydroxypropan-1-one formation following the racemic resolution of the 1,2-Bis(2-methoxyphenyl)-2-hydroxyethan-1-one which is introduced to enzyme in the presence of acetaldehyde.

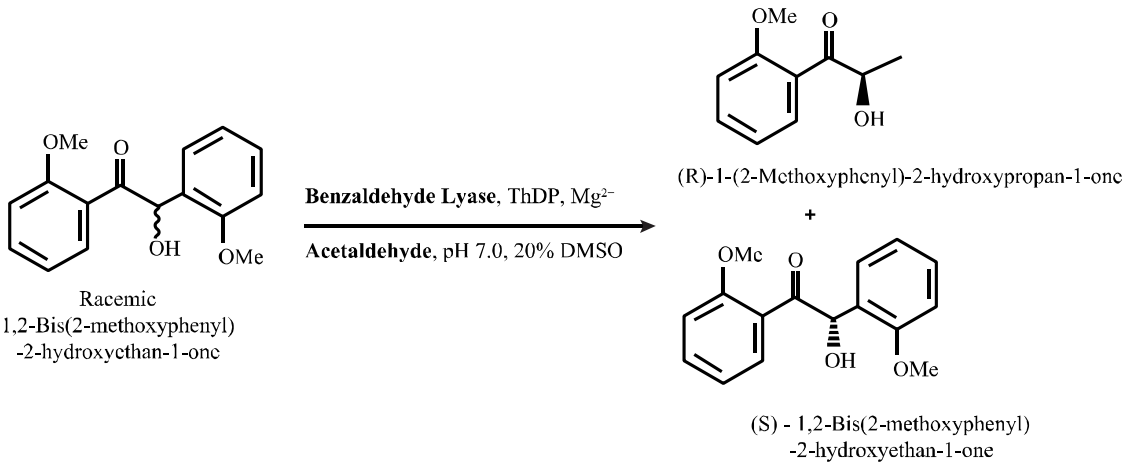



**Figure 32**. BAL catalyzed formation of (R)-1-(2-methoxyphenyl)-2-hydroxypropan-1-one

In a 1500 µl Eppendorf tube, the reaction is tested with 5mM of substrate (2 mg 1,2-Bis(2-methoxyphenyl)-2-hydroxyethan-1-one) with addition of 5 µl of acetaldehyde in 1500 µl reaction medium. The reaction medium was allowed to stand for 96 hours at room temperature and every 24 hour, 5 µl of acetaldehyde and 1 U enzyme is added. In order to observe the (R)-1-(2-Methoxyphenyl)-2-hydroxypropan-1-one formation with TLC, a blank solution excluding the enzyme is prepared. The formation of (R)-1-(2-Methoxyphenyl)-2-hydroxypropan-1-one is observed by a separate dot on the TLC (solvent system 3H: 1 EA) sheet under UV light.

For the preparative studies, approximately 544 mg substrate of racemic 1,2-Bis(2-methoxyphenyl)-2-hydroxyethan-1-one (2 mmol) was dissolved in a mixture of dimethyl sulfoxide (20 ml) and potassium buffer (80 ml, 50 mM, pH 7.0, containing $MgSO_4$ (2.5 mmol/L), ThDP (0.15 mmol/L). To this solution 88 mg (2 mmol) acetaldehyde was added. After addition of BAL (20 U), the reaction mixture was allowed to stand at room temperature. After 24 hours, 20 U of BAL and 176 mg (4 mmol) of acetaldehyde were added. The conversion was determined as 25% (GC-MS). The racemate of benzoin was resolved to obtain (S)-Benzoin with an enantiomeric excess of 99 %.

**(S)-1,2-Bis(2-methoxyphenyl)-2-hydroxyethan-1-one** is a colorless solid which has a melting point value between 98 - 99 ºC .According to the results from polarimeter recordings, the (S) enantiomer has $[a]^{20}_{D}$ : +125 (c 0.9, $CHCl_3$). The HPLC (Chiralpak AD, n-hexane / 2-propanol 98:2, flow 0.90 $mL.min^{-1}$, 20 ºC) results displayed the separation of (R) (Rt: 31.2 minute) and (S) (Rt: 42.2 minutes) enantiomers.

The $^{1}H$ NMR (400 MHz, $CDCl_3/CCl_4$) results for (S)-1,2-Bis(2-methoxyphenyl)-2 hydroxyethan-1-one is: δ = 6.63 - 7.69 (m, 8H), 5.92 (d, *J* =5.1 Hz, 1H), 4.29 (d, *J* =5.1 Hz, 1H), 3.71 (s, 3H), 3.69 (s, 3H). $^{13}C$ NMR (100 MHz, $CDCl_3/CCl_4$) results are: δ = 201.6, 158.4, 157.6, 134.0, 131.1, 130.3, 129.8, 128.2, 125.8, 120.9, 120.8, 111.4, 111.2, 76.1, 55.5, 55.4.

**(R)-1-(2-methoxyphenyl)-2-hydroxypropan-1-one** is a viscous oil. The yield for the reaction is determined as 32% with an enantiomeric excess of more than 99%. According to the results from polarimeter readings, the (R)-1-(2-methoxyphenyl)-2-hydroxypropan-1-one has $[a]^{20}_{D}$ : +143 (c 0.8, $CHCl_3$). The HPLC (Chiralpak AD,

isohexane / 2-propanol 98:2, flow 0.75 mL.min$^{-1}$, 20 ºC) results showed the formation of (R)-1-(2-Methoxyphenyl)-2-hydroxypropan-1-one which has a Rt (R) value at 42.7 minute.

The $^{1}$H NMR (400 MHz, $CDCl_3/CCl_4$) results for (R)-1-(2-methoxyphenyl)-2-hydroxypropan-1-one is: : δ = 7.70 - 7.78 (m, 1H), 7.39 - 7.51 (m, 1H), 6.91 - 7.01 (m, 2H), 5.05 (q, *J* = 6.8 Hz, 1H), 3.89 (s, 3H), 3.68 (br.s, 1H),1.42 (d, J_6.8 Hz, 3H). $^{13}$C NMR (100 MHz, $CDCl_3/CCl_4$) results are: δ = 203.7, 158.2, 134.5, 131.3, 125.1, 121.1, 111.3, 72.9, 55.2, 20.7

# CHAPTER 4

## CONCLUSION

In conclusion, the method described here presents a convenient one-enzyme-catalyzed, highly selective synthesis of (R)-Benzoin and (R)-2-HPP analogues. The reactions work in organic-aqueous medium, overcome the solubility problem with organic substrates, and open the way for large-scale preparation. The products are obtained in high yield starting from simple, easily available aromatic aldehydes, benzoins and acetaldehyde via carboligation reactions. In this way, BAL represents a valuable alternative to BFD concerning the formation of (R)-HPP derivatives.

**Table 3.** Results for the Reactions Catalyzed By BAL In Terms of Yield and Enantiomeric Excess

| Substrate | Product | Yield(%) | Enantiomeric Excess(%) |
|---|---|---|---|
| Benzaldehyde | (R)-Benzoin | 96 % | > 99 % |
| 2-Fluoro-benzaldehyde | (R)-1,2-Bis(2-Fluorophenyl)-2-hydroxyethan-1-one | 68 % | 96 % |
| 2-Methoxy-benzaldehyde | (R)-1,2-Bis(2-Methoxyphenyl)-2-hydroxyethan-1-one | 87% | > 99 % |
| *rac*-Benzoin | (R)-2-hydroxy-propiophenone | 47 % | > 99 % |
| | (S)-Benzoin | 49 % | > 99 % |
| *rac*-1,2-Bis(2-Fluorophenyl)-2-hydroxyethan-1-one | (R)-1-(2-Fluorophenyl)-2-hydroxypropan-1-one | 32 % | 97 % |
| | (S)-1,2-Bis(2-Fluorophenyl)-2-hydroxyethan-1-one | 34 % | 96 % |
| *rac*-1,2-Bis(2-Methoxyphenyl)-2-hydroxyethan-1-one | (R)-1-(2-Methoxyphenyl)-2-hydroxypropan-1-one | 32 % | > 99 % |
| | (S)-1,2-Bis(2-methoxyphenyl)-2-hydroxyethan-1-one | 25 % | > 99 % |

Benzaldehyde lyase from *Pseudomonas Fluorescens* Biovar I is the first ThDP-dependent enzyme, which catalyzes the synthesis of optically pure α-hydroxy ketones via resolution of racemic compounds by C-C bond cleavage and concomitant C-C bond formation.

Benzaldehyde Lyase is a promising enzyme that can be used to synthesize optically pure benzoin and 2-HPP derivatives.

BAL can be easily used for kinetic resolution of racemic benzoins through the (R) – 2-HPP formation reaction.

It is an advantageous enzyme as:

1. It utilizes a cheap type coenzyme-ThDP.
2. It is easy to handle and work with.
3. It has broad substrate specificity.
4. It is highly enantioselective.

The chiral products by Benzaldehyde Lyase can be used for many purposes such as synthesis of biologically active compounds and important chirons for pharmaceutical and other industrial compounds.

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