ASYMMETRIC SYNTHESIS OF BETA-HYDROXY PHOSHPONATES VIA
BENZALDEHYDE LYASE CATALYZED CROSS ACYLOIN REACTIONS

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

BY

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

JANUARY 2014

Approval of the thesis

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ABSTRACT

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January 2014, 86 pages

Synthetically useful asymmetric cross acyloin reactions with functionalized aliphatic aldehyde have a broad range of application. The products of acyloin reactions are α-hydroxy ketone which are the subunits in several compounds and can be found in many biologically important compounds. Phosphonates can be used as an intermediate in the synthesis of potentially significant peptide analogues, catalytic antibodies, and phosphonic acid-based antibiotics which are also acknowledged as biologically important compounds. On the other hand, lyases are an important class of enzymes that achieve several important reactions including C-C bond formations. In this study asymmetric synthesis of novel β-hydroxy phosphonates with cross acyloin reactions was achieved via benzaldehyde lyase (BAL) catalyst. By making a contribution to β-hydroxy phosphonate synthesis methods, this application can also be a preference to chemoenzymatic methods for the synthesis of β-hydroxy phosphonates. In this study, first of all diethyl 2,2-diethoxyethylphosphate was synthesized by Michaelis-Arbuzov reaction of triethylphosphite and bromoacetaldehyde diethylacetal. Then by hydrolyzing phoshorylated diethylacetal, phosphonoacetaldehyde which was used as aliphatic substrate in chemoenzymatic reaction was synthesized.

Secondly, benzaldehyde lyase was produced by using recombinant E.coli. Finally, by using ThDP dependent benzaldehyde lyase in DMSO solvent, C-C bond formation

was achieved between benzaldehyde derivatives and aliphatic phosphonoacetaldehyde and asymmetric novel β-hydroxy phosphonates was synthesized. As a result, high enantiomeric excesses up to 98% ee were obtained. These results made a significant contribution to both the hydroxy phosphonates synthesis methods and the aliphatic substrate range of benzaldehyde lyase catalyzed reactions.

Keywords: α-hydroxy ketone, β-hydroxy phosphonates, benzaldehyde lyase, acyloin reactions, chemoenzymatic methods, asymmetric synthesis

ÖZ

BENZALDEHİT LİYAZ KATALİZÖRLÜĞÜNDE GERÇEKLEŞEN ÇAPRAZ ASİLOİN REAKSİYONLARI İLE BETA HİDROKSİ FOSFONATLARIN ASİMETRİK SENTEZİ

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Ocak 2014, 86 pages

Fonksiyonel alifatik aldehitlerle gerçekleştirilen sentetik olarak kullanılabilir asimetrik çapraz asiloin tepkime ürünlerinin geniş bir kullanım alanı vardır. Asiloin tepkimelerinin ürünleri olan alfa hidroksi ketonlar çeşitli bileşiklerin ara ürünleridir ve bunlar biyolojik olarak önemli birçok bileşikte bulunurlar. Potensiyel öneme sahip peptit analoglarının, katalitik antibodilerin ve fosfonik asit esaslı antibiyotiklerin sentezlenmesinde aracı olarak kullanılan fosfanatlar da biyolojik açıdan önemli bileşikler olarak bilinirler. Önemli bir enzim sınıfı olan liyazlar karbon karbon bağının oluşumunu da içeren birçok önemli tepkimeyi gerçekleştirirler. Bu çalışmada asimetrik beta hidroksi fosfanatların çapraz asiloin tepkimeleri ile sentezi benzaldehit liyaz katalizörlüğünde ilk kez gerçekleştirilmiştir. Hidroksi fosfanat sentez yöntemlerine yeni bir katkı sağlayan bu uygulama hidroksi fosfanatların kemoenzimatik sentez yöntemlerinde de öncelikli olarak tercih edilebilir. Bu çalışmada öncelikle trietil fosfit ile bromoasetaldehit dietil asetalin Michaelis-Arbuzov tepkimesi sonucu dietil 2,2-dietoksietilfosfat sentezlendi.

Sonra fosforlanmış dietilasetalimizin hidrolizi gerçekleştirilerek enzim tepkimesinde kullanılacak alifatik ürün olan fosfonaasetaldehit elde edildi. Daha sonra recombinant E.coli yardımıyla benzaldehit liyaz enzimi üretildi. Son olarak ThDP bağımlı benzaldehit liyaz enzimini DMSO solventi içinde kullanarak, benzaldehit

türevleri ve alifatik fosfonoaldehit arasında C-C bağı oluşumu sağlandı ve denenmemiş asimetrik beta hidroksi fosfanat türevlerinin üretimi gerçekleştirildi. Sonuç olarak, %98'e varan yüksek enantiyomerik saflık elde edildi. Bu sonuçlar hidroksi fosfanatların sentez yöntemlerine ve benzaldehit liyaz enziminin katalizlediği tepkimelerin alifatik substrat aralığına önemli bir katkı sağladı.

Anahtar Kelimeler: alfa hidroksi keton, beta hidroksi fosfonat, benzaldehit liyaz, asiloin kondenzasyonu, kemoenzimatik yöntemler, asimetrik sentez

To my beloved family and Prof. Dr. Ayhan Sıtkı Demir

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

AHAS: Acetohydroxy acid synthase

BAL: Benzaldehyde Lyase

BHP: Beta-hydroxy phosphonates

BFD: Benzoylformate decarboxylase

DMSO: Dimethyl sulfoxide

FDA: Food and Drug Administration

FPLC: Fast protein liquid chromatography

HPP: Hydroxypropiophenones

HRMS: High-resolution mass spectrometry

HPLC: High pressure liquid chromatography

IPTG: Isopropyl β-D-1-thiogalactopyranoside

LB: Luria broth

NADH: Nikotinamid adenin dinükleotit

NMR: Nuclear magnetic resonance spectroscopy

PAC: Phenylacetylcarbinol

PDC: Pyruvate dehydrogenase

ThDP: Thiamine diphosphate

THF: Tetrahydrofuran

TLC: Thin layer chromatography

TPP: Thiamine pyrophosphate

CHAPTER 1

INTRODUCTION

1.1 Biotransformation

Biotransformation is basically useful discipline that transforms organic substrates by using enzymes and whole cells as catalysts [1]. Biotransformation technology has been confirmed an alternative tool to synthetic organic chemistry [2]. This technology is raising very rapidly, applications varying from pharmaceuticals and fine chemicals to food additives, cosmetic constituents and more recently biofuels [3]. For thousands of years, microorganisms and their extracts have been manipulated for chemical reactions, mostly for fermentation reactions producing alcoholic beverages, vinegar and foodstuffs such as yoghurt. Therefore, in the nineteenth century, historical study of biotransformation in brewing process has similarities with the study of the chemistry.

After the discovery of penicillin, early notice in biotechnology was encouraged both by the use of microorganisms to produce bulk chemicals, such as sugars and acids, and also in development of microbes for pharmaceutical production for the chemical industry in the twentieth century [4].

Such developments in biotransformation inspired research into the optimization of large-scale fermentations of microorganisms and the enzyme biochemistry, and also inspired the development of microbial culture collections that assist a precious reservoir of biocatalyst today. After this, microbes and enzymes have been accepted as keys for many of the challenges confronted synthetic chemistry with respect to their competences as catalysts of single-step reactions that are comparable to standard organic chemistry [5].

Usage of biocatalysts has been increased due to three major respects. First, in the late twentieth century, the evolving field that the synthesis of single enantiomer forms of chiral drugs was going to be progressively important in industrial chemistry, enzymes was illustrated as chiral catalyst. Second, these developments gave pressure

to provide environmentally friendly products, 'green chemistry', that would be achieved by natural characteristics of enzymes and microbes.

Third, innovations in gene and protein engineering give a rise to the manipulation of enzymes that has become a technique in laboratories worldwide [3].

1.2 Enzymes in synthetic organic chemistry

Enzyme catalysis in organic synthesis is a term that today this technology is accepted as a main break for the formulation of a wide scope of chemical compounds. Particularly, this is proper not only for theoretical syntheses but also for industrial applications [6]. Enzyme catalysis is matched with typical chemical and chemo catalytic synthetic advances, have worked out to be reasonable for several molecules. Furthermore, the traditional organic disciplines, metal catalysis and organocatalysis, enzymatic catalysis are progressively well known by organic chemists in both academia and industry as an appealing synthetic tool [7].

Enzymes can catalyze broad range of transformations, involving, redox reactions, C-C bond forming reactions, and hydrolytic reactions. However, enzyme catalysis was not recognized as an alternative to organic syntheses because of drawbacks such as limited substrate range, partial stability of enzymes under organic reaction conditions, low efficiency due to wild type strains so, these are cause of low efficiency in volumetric productivity. Though, remarkable developments in enzyme engineering and process provide several organic syntheses with enzymes that leading to decrease these disadvantages [8].

Successes in microbiology and molecular biology give an extensive range of enzymes exposing a tremendous performance. Today, these enzymes are obtained very economically and large scale by cell density fermentation. This cost-effective approach to biocatalysts facilitates an increasing demand for the development of organic syntheses processes with enzymes.

Advancing from these improvements, isolated enzymes and recombinant microorganisms have been applied very effectively and results found to be superior

to competitive chemical or chemo catalytic approaches, especially for the production of chiral compounds used as drug intermediates [9].

Incorporation of microbiology, genetics, molecular biology, organic synthesis and reaction engineering provide enzyme catalysis to see an interdisciplinary field and combination of these fields give an opportunity for the development of bio-catalytic processes. Catalytic activities of enzymes lead important selectivity due their complex three-dimensional structures. Enzymes easily detect specific substrates and apart from chemoselectivity, stereoselectivity of enzymes is high to excellent [10].

1.2.1 Selectivity—The strength of biocatalysis

Enzymes display high selectivity that is a prerequisite in synthetic organic chemistry. The basic strength of biocatalysis is regioselectivity, this provide an easy way without any need the number of synthetic steps and reduces occupation time of chemical reactors, which is important for the cost of pharmaceutical and chemical production [11].

1.2.1.1 Regioselectivity and chemoselectivity

Regioselective reactions catalyzed by enzymes, act only one chemically equivalent site in a substance. Hydroxylation of progesterone by the fungus Rhizopus arrhizus is an example of regioselectivity, this occurs only at the 11 position of the hormone [3].
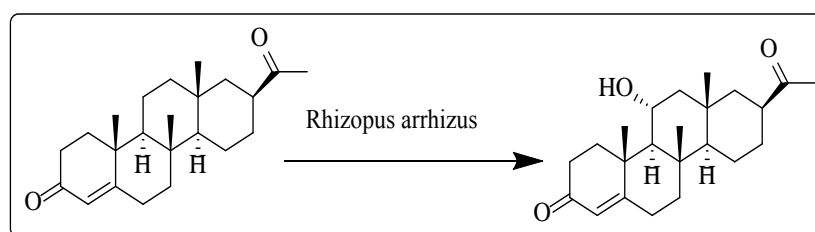
Scheme 1. Hydroxylation of progesterone by Rhizopus arrhizus.

Another example shows the both regio and chemoselectivity of reaction. Acylation of purine, which is an anti-leukaemic agent, using a lipase from Candida Antarctica, type B, and vinyl acetate as acyl donor, leads to the 5-monoacetate.

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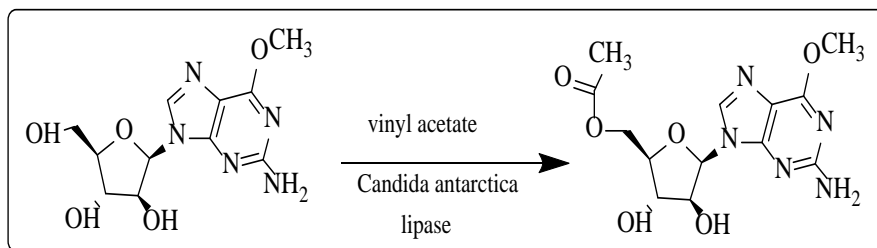
Scheme 2. Acylation of purine, regio and chemo selective reaction.

Biocatalysts are also capable of catalyzing chemoselective reactions, chemoselectivity is the preferential reaction of a chemical reagent with one of two or more different functional groups. A reagent has a high chemoselectivity if reaction occurs with only a limited number of different functional groups such as in the preferential hydrolysis of a nitrile over a carboxy-ester, two acid-labile groups, by a strain of bacterium known as Rhodococcus [3].
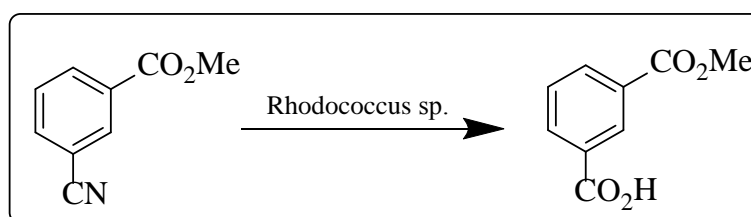
Scheme 3. Hydrolysis of a nitrile over a carboxy-ester by a strain of Rhodococcus sp.

1.2.1.2 Stereoselectivity

The preferential formation in a chemical reaction of one stereoisomer over another is an important specialty of enzymes. Particularly in pharmaceuticals obtaining single stereoisomer drug has improved performance of enzymes in organic synthesis [11]. Although enantiomers have same chemical and physical properties, their natural properties such as crop protection products, flavor compound, pheromones and drugs can vary extremely [12]. For example, role of chirality on odorants such as (4S)-(+)-carvone, which has a different caraway odor, as compared to (4R)-(-)-carvone has a characteristically sweet spearmint odor [13].

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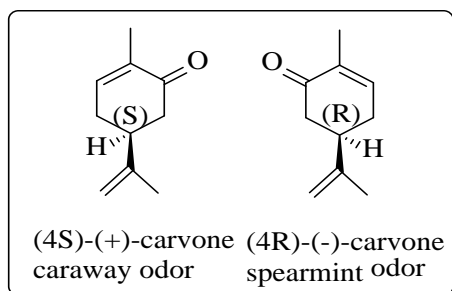
Figure 1. Enantiomers of carvone.

Modern area of interest noted that more than 285 enantiomers are known to show different odors [14].

Biological properties of pharmaceutically active compounds have same principals like herbicides and pesticides containing chiral stereogenic centers. These are can be a source of problems due to differences not only natural properties but also pharmacokinetics of enantiomers. For example, the (R)-(+)-enantiomer of the herbicide dichlorprop is the active enantiomer in killing the weeds, while the (S)-(-)-dichlorprop enantiomer is inactive as an herbicide [15].
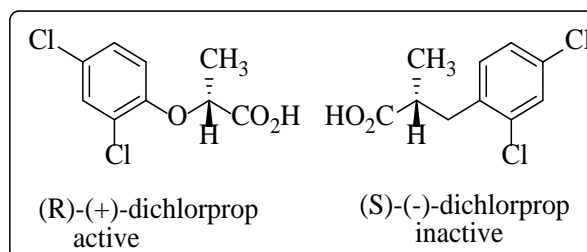
Figure 2. Enantiomers of dichlorprop.

Production of racemic compounds has adverse results that mark optically active drugs crucial in the marketing. Therefore, since 2001 no new racemic drugs have been located on the market [16, 17, 18].

Today, enantiomers are reflected distinctly different compounds, like enantiomers of drug substances have different biological dealings that are the result of extremely diverse pharmacological, toxicological activities [19].

Piutti made first detection on the taste differences between two enantiomers of asparagines in 1886. Colorless crystalline asparagine is found in the cell sap of

5

plants. Enantiomers of asparagine exist in asparagus, wheat and levo- form is tasteless but dextro- form is sweet [20].
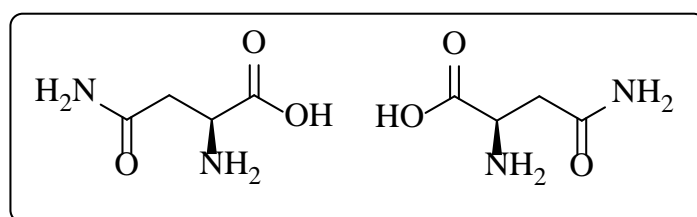
Figure 3. Enantiomers of asparagines.

Thalidomide is another classical example after asparagine discovery. It was first produced as a racemate in 1953 and was widely recommended for morning sickness from 1957 to 1962 in the European countries and Canada [21]. The sedative-hypnotic drug thalidomide exhibited irreversible neurotoxicity and teratological (mutagenic) effects in which babies were born deformed. The drug was suggested to pregnant women to counter morning sickness. However later it was realized that these effects were caused by the S-enantiomer but the R- enantiomer contained the desired therapeutic activity [2].
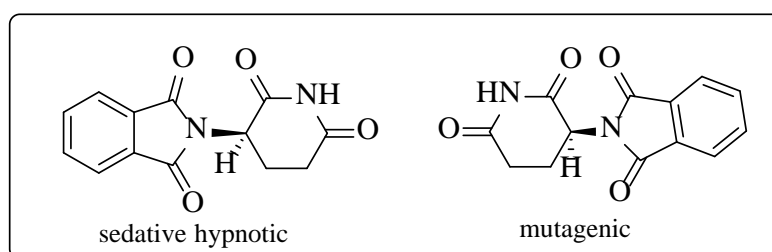
Figure 4. Enantiomers of Thalidomide.

Chiral selectivity of body cooperates with each racemic drug differently and processes each of them in separate pathway to generate different pharmacological activity. One isomer may produce the required therapeutic activity, while the other may be inactive or give unwanted side effects. Studying the properties of enantiomers of chiral drug molecule gives an advantage in terms of therapeutic efficiency and danger.

In the light of this, properties of each enantiomers should be studied before marketed the drug as one of the enantiomers or as a racemate that is taken an obligatory by the

FDA and the European Committee for Proprietary Medicinal Products since 1992. Therefore, since 2001 no new racemic drugs have been marketed [16].

There are basically three methods to achieve chiral substances:
1. Racemic mixture separation (e.g. kinetic resolution, deracemization)
2. Natural source isolation
3. Asymmetric synthesis
Asymmetric synthesis is the most effective methods that achiral precursors processed to synthesize selective chiral molecules very economically.

1.3 Advantages and disadvantages of enzymes

Nature has formed outstanding catalysts by advancement over millions of years. Biocatalysts use only enzymes as natural catalysts. The properties of enzymes, compared with catalysts normally used in chemical processes, are remarkable. Enzymes exist for nearly all reactions known in organic chemistry.

Many organic reactions can take place freely, but some of them need a substantial rate to be catalyzed. Therefore, during the conversion of reactant to product, catalyst reduces the level of the energy barrier. This energy barrier is called by thermodynamically as a free-energy change.
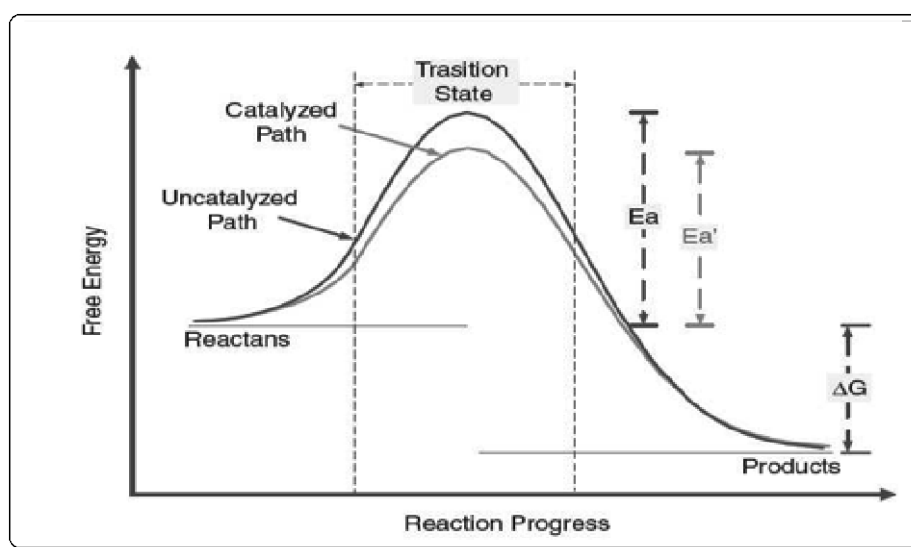
Figure 5. A reaction coordinate diagram for catalyzed and uncatalyzed reactions [24].

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Ea and Ea' are the energies of activation of the uncatalyzed and catalyzed reaction. ΔG is the free energy change of the reaction.

Apart from that, biocatalysts commonly progress high chemo-, regio-, and enantio-selectivity up to >99% ee because of enzymes' chirality. This unique property provides the formation chiral products otherwise chemical synthesis often gives racemic mixtures. Such high selectivity is very desirable in chemical synthesis as it may offer several benefits such as reduced or no use of protecting groups, minimized side reactions, easier separation, and fewer environmental problems [24].

Advantages and disadvantages of enzyme catalyzed reactions that are outlined in Table 1.

Table 1. Characteristics of biocatalysis.

Advantages	Disadvantages
-Enzymes catalyze broad spectrum of reactions and accelerate the rate of reactions	-Availability of enzymes is limited
	-Limited number of enzymes exist
	-Protein catalyst stability is limited
-Enzymes are more selective; chemo-selectivity, regio-selectivity,diastereo-selectivity and enantio-selectivity	-Enzymes require cosubstrates such as cofactors
-Enzymes act under mild conditions such as pH range 5-8 and temperature range 20–40°C	-Inactivation occur at high temperatures, extreme pH and organic solvents
-Byproducts are low	-Inhibition may occur by substrate, product and metal ions
-Environmentally acceptable, they are non-toxic	-Enzyme can cause allergic reactions
-Large scale production is possible through fermentation	
-Recycling is possible	
-Enzymes can be modified to a certain extent	

Enzymes used in industrial processes; however, well-known limitations related with biocatalysts are that enzymes act under mild conditions, so they are unstable and can be damaged by extreme reaction conditions. Nevertheless, in the last 20 years enzyme technology progressed, these barriers have been exceeded [25].

1.4 Enzyme Classes

According to the International Union of Biochemistry and Molecular Biology (IUBMB) nomenclature system, all enzymes are classified into six classes on the basis of the general type of reactions that they catalyze [27].

Table 2. Classification of Enzymes [8].

Enzymes	Type of reactions	Representative subclasses
Oxidoreductases	Catalyze the transfer of hydrogen or oxygen atoms or electrons from one substrate to another	Oxidases, oxygenases, peroxidase, dehydrogenases
Transferases	Catalyze the group transfer reactions	Glycosyltransferases, transketolases, transaldolases, transaminases
Hydrolases	Catalyze the hydrolytic reactions	Esterases, lipases, protases, glycosidases, phosphotases
Lyases	Catalyze the nonhydrolytic removal of groups	Decarboxylases, aldolases, ketolases, hydratases
Isomerases	Catalyze the isomerization reaction	Racemases,epimerases, isomerases
Ligases	Catalyze the synthesis of various type of bonds	Synthetases, carboxylases

1.5 α-Hydroxy ketones

Enantiomerically enhanced α-hydroxy ketones are important building blocks for the production of fine chemicals and pharmaceuticals. These ketones are located in certain antidepressants, in some inhibitor which are used in Alzheimer's disease treatment, in inhibitor of Kurasoin A and B and in several antitumor antibiotics (Olivomycin A, Chromomycin A_3, epothilones). Futhermore, many important structures (amino alcohols, diols, etc) can be produced from α-hydroxy ketones [28].
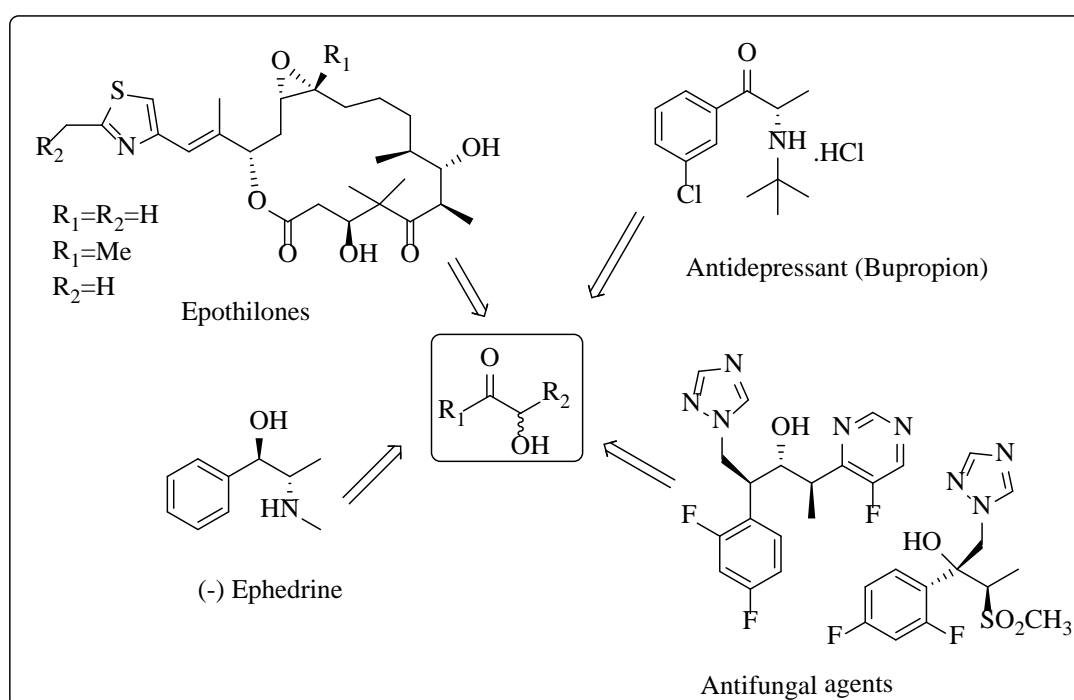
Figure 6. Examples of compounds contains α-hydroxy ketones [28]**.**

Asymmetric functionalities such as diols, halo or amino derivatives, and epoxides can be produced from active crucial building blocks, especially carboxylic acids, aldehydes and ketones.
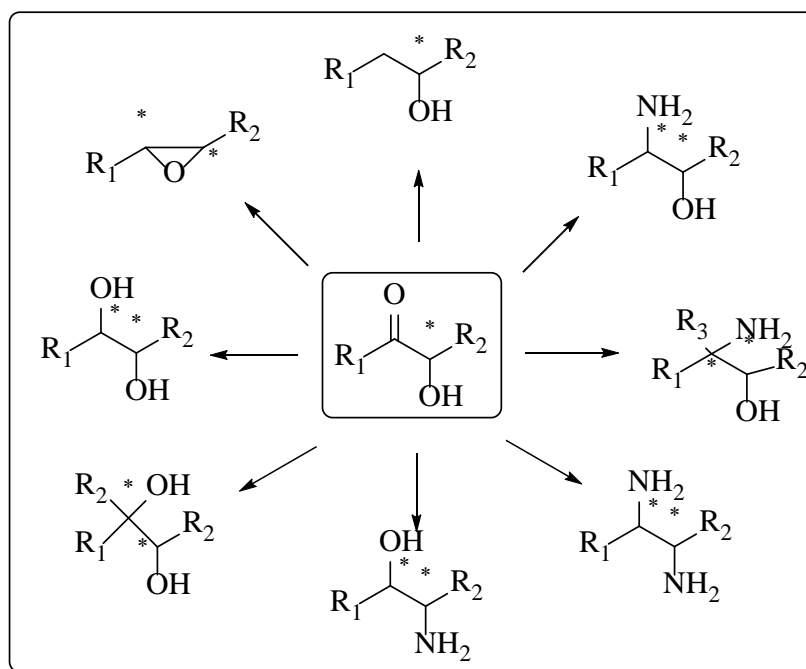
Scheme 4. Hydroxy ketones as building blocks for several active compounds [29].
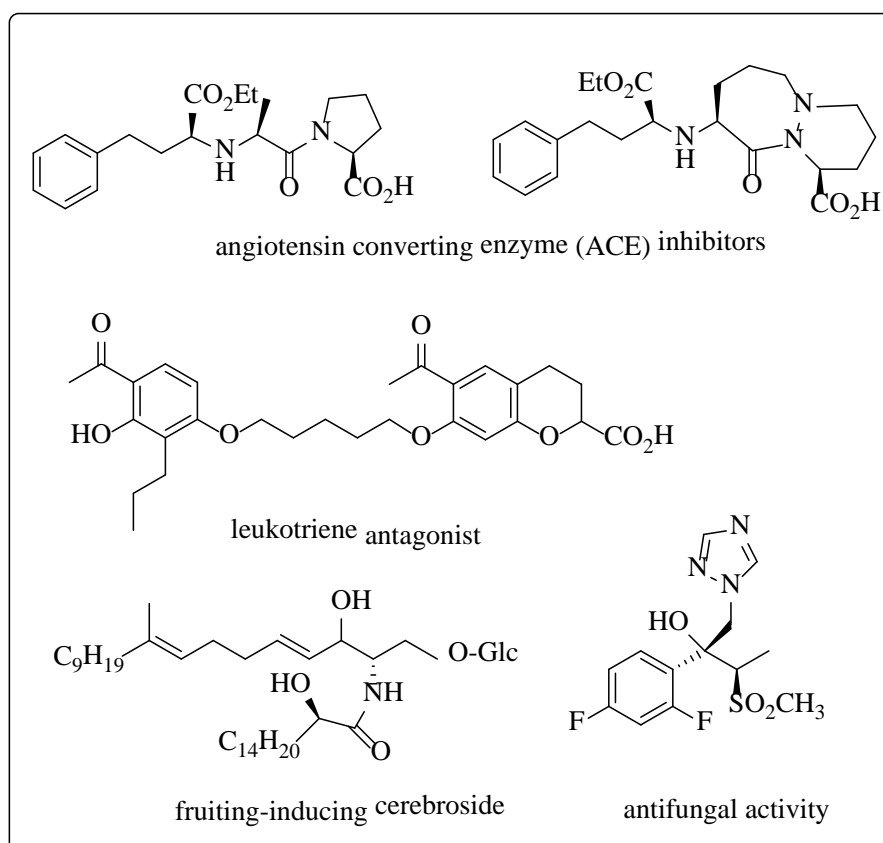
Figure 7. Biologically active molecules prepared from optically active R-oxyfunctionalized carbonyl building blocks [30].

1.6 Chemical asymmetric acyloin synthesis

There are various chemical methods to synthesize asymmetric acyloins have been reported.

1. α- hydroxylation
2. ketohydroxylation
3. asymmetric condensation
4. asymmetric oxidation
5. oxidative kinetic resolution
6. stereoselective reduction
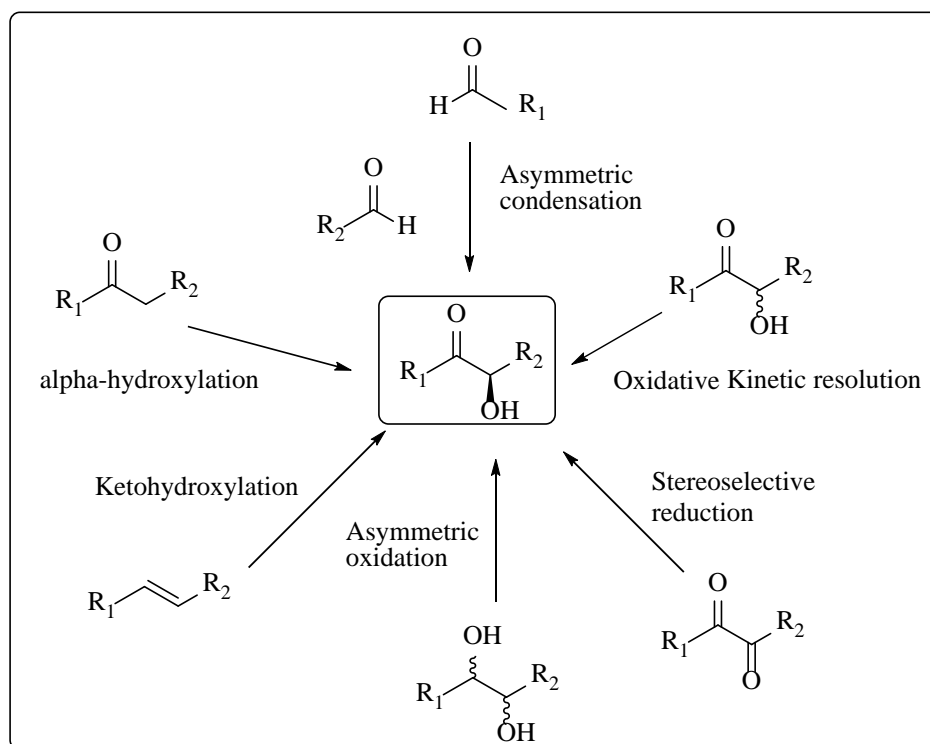
Scheme 5. Chemical strategies for the synthesis of acyloins [31].

Possibly the recorded strategies are the enantioselective enolate oxidation of ketones (formed in situ, catalyzed by chiral oxidants), asymmetric dihydroxylation of ketone [32]. Further chemical methodologies are the ketohydroxylation of olefins, the asymmetric monooxidation of the diols, and oxidative kinetic resolution of racemic

acyloins. Additionally, organocatalytic approaches can be applied to obtain acyloins. Oxygenation of ketones in the presence of proline and alanine and also benzoin type condensation by using chiral thiazolim and triazolium salts [31].

Nevertheless, while there are useful molecules have been produced by chemical strategies, in several cases, requirement of various number of chemical steps are account of uneconomic, lack selectivity, low overall yields, complicated following.

To reduce these difficulties, biocatalysts have capability to process significant chemical structures; they can be achieved with high enatio-, regio-, and chemoselectivities. And also this selectivity can be obtained with more economic and environmental manner [6, 24].

1.7 Enzymatic asymmetric acyloin synthesis

There are three different methods to produce α-hydroxy ketones with biocatalysis.
1. ThDP-lyases
2. Lipases (Dynamic kinetic resolution)
3. Oxido-reductases
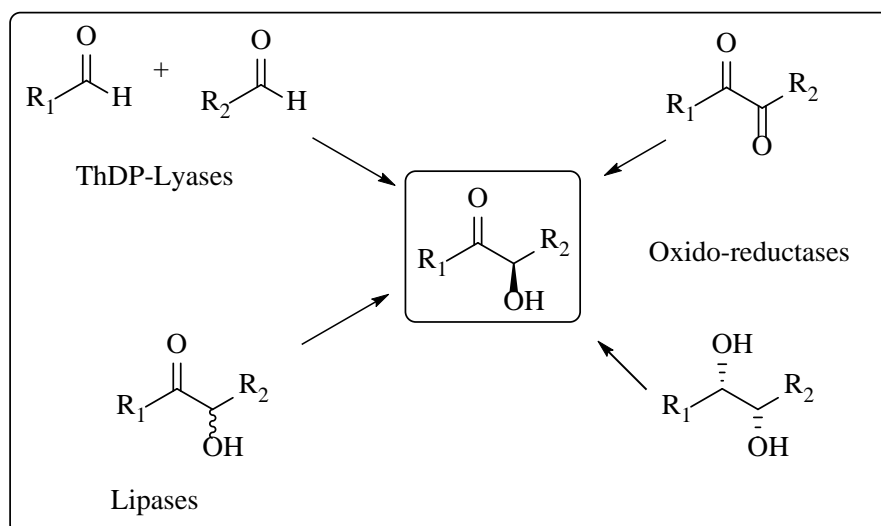
Figure 8. Enzymatic asymmetric acyloin synthesis.

1.6.1 ThDP-lyases

Enantiopure α-hydroxy ketones are formed from umpolung carboligation of aldehydes. Thiamine diphosphate dependent lyases such as pyruvate decarboxylase (PDC), benzoylformate decarboxylase (BFD), and benzaldehyde lyase (BAL) catalyzes these reactions leads to high enantiomeric excesses (up to 99%). Important part of this catalysis is the reaction of cofactor (ThDP) with the donor aldehyde forms an enamine-carbanion intermediate. This intermediate acted as a nucleophile attacks to the acceptor aldehyde to form α-hydroxy ketone. Several donor and acceptor aldehydes can be used and this leads to many useful building blocks which are pharmaceutically important.

Using biocatalyst either as enzyme or whole cell in practical application provides an economical benefit. In this sense, several lyases are practiced and identified with a broad substrate range is remarkably a success.

For instance, benzaldehyde lyase (BAL) from Pseudomonas fluorescens catalyzes the coupling of aromatic-aliphatic aldehydes and forms chiral hydroxy ketones.
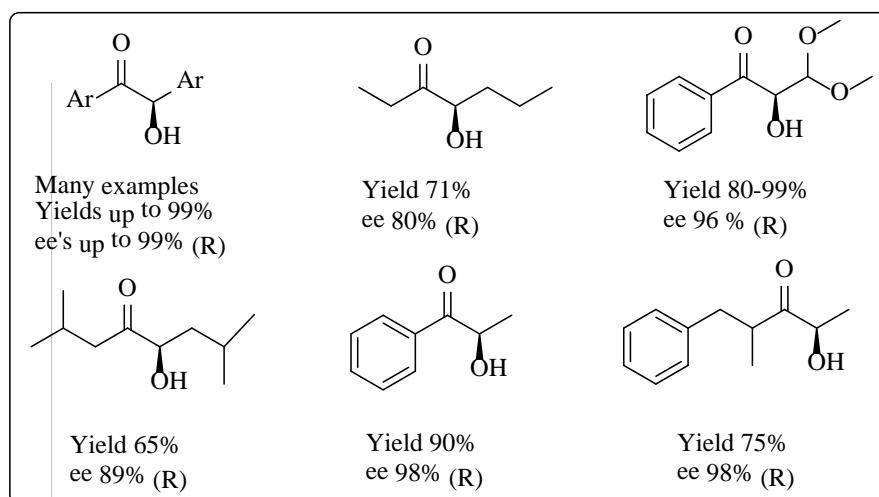
Figure 9. Selected examples of chiral R-hydroxy ketones produced by means of BAL as biocatalyst, starting from aldehydes [29, 34].

1.6.2 Lipases

The uses of lipases and esterases to produce α-hydroxy ketones by resolving of racemates have been addressed. Kinetic resolution of racemates is an efficient method to obtain enantiopure compounds.

Hydrolases has been identified by several structurally different chiral hydroxy ketones. Alpha-hydroxy ketones have been obtained through two methods: Fungus catalyzed kinetic resolution of the acetates and transesterification of α-hydroxy ketones catalyzed by lipases [35,36].
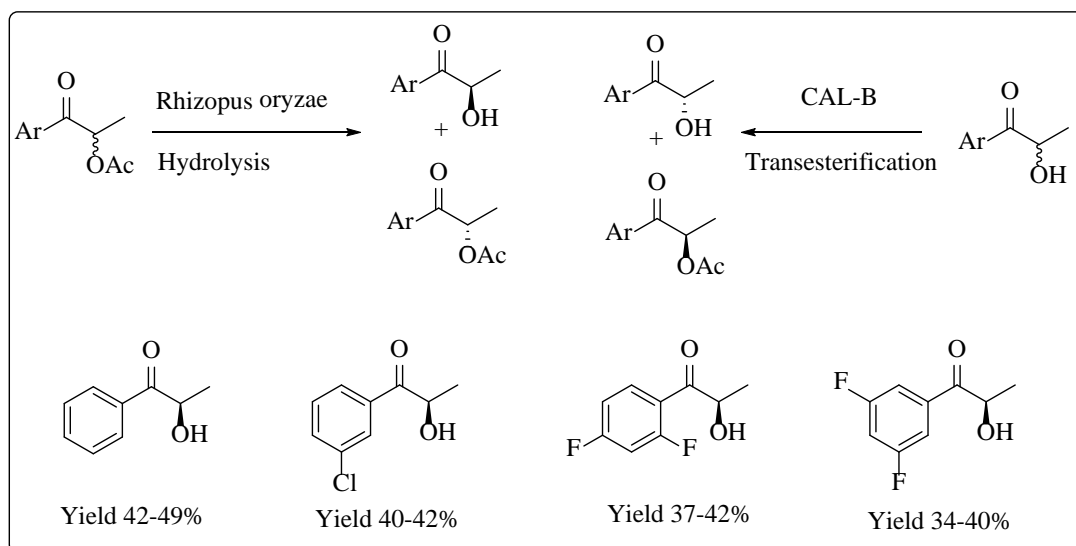
Scheme 6. Hydrolase-Catalyzed Kinetic Resolution of Alkyl-Aryl R-Hydroxy Ketones.

Nevertheless, maximum theoretical yield is 50% can be reached by kinetic resolution.

1.6.3 Oxido-reductases

Oxido-reductases exhibits reduction, oxidation, and deracemization type of processes. To obtain chiral ketones, three types of methods have been applied: reduction of chiral ketones by dehydrogenases, oxidation of diols by oxidases, and deracemization of hydroxy ketones. These reactions can be catalyzed either by enzymes or whole cells.

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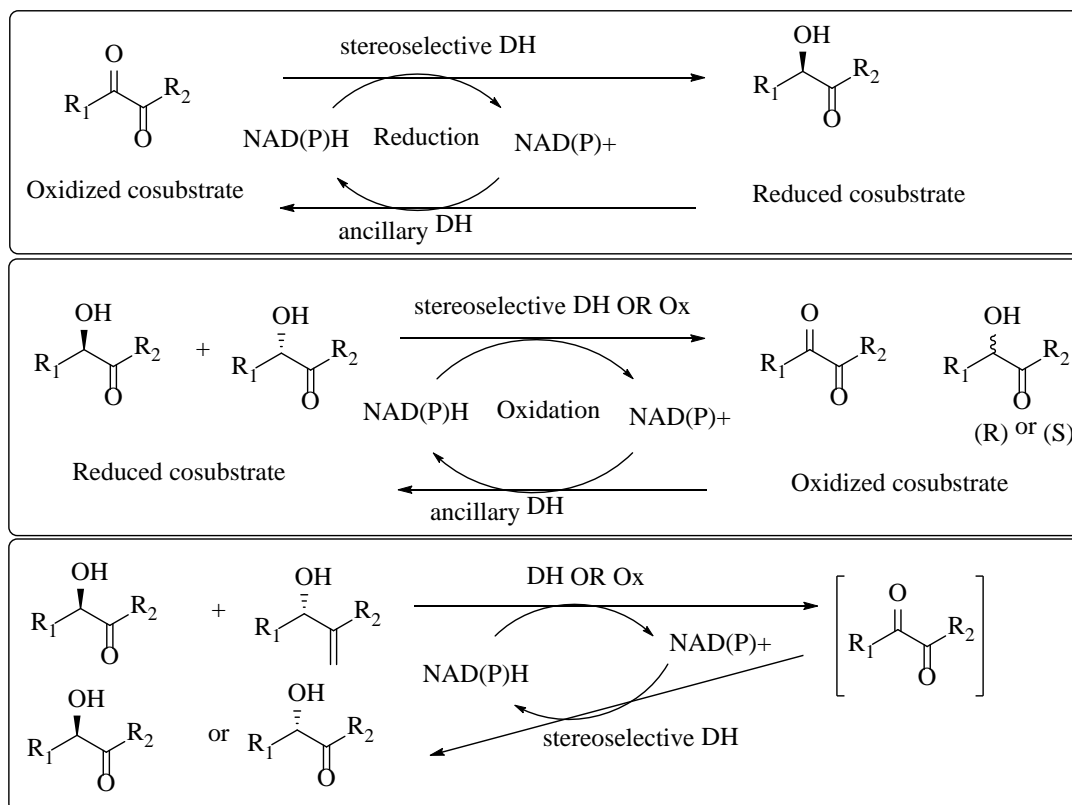
Scheme 7. Synthesis of Enantioenriched α-Hydroxy Ketones by Oxidoreductases [38].

For instance, different type of whole cells are used for the reduction of several benzoins [38].
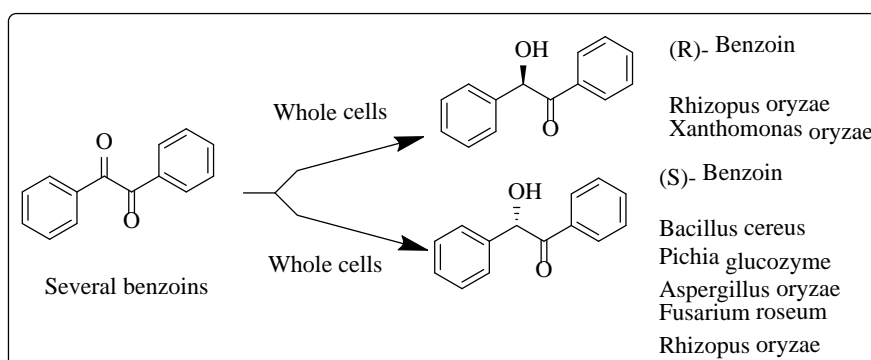
Scheme 8. Stereoselective Reduction of 1,2-Diaryl-Ethanediones to the Corresponding Benzoins Using Whole Microbial Cells.

The last method is deracemization which is tried with whole cell Rhizopus oryzae that is able to catalyze the racemic benzoins to enantiomers depending on the pH of the medium.

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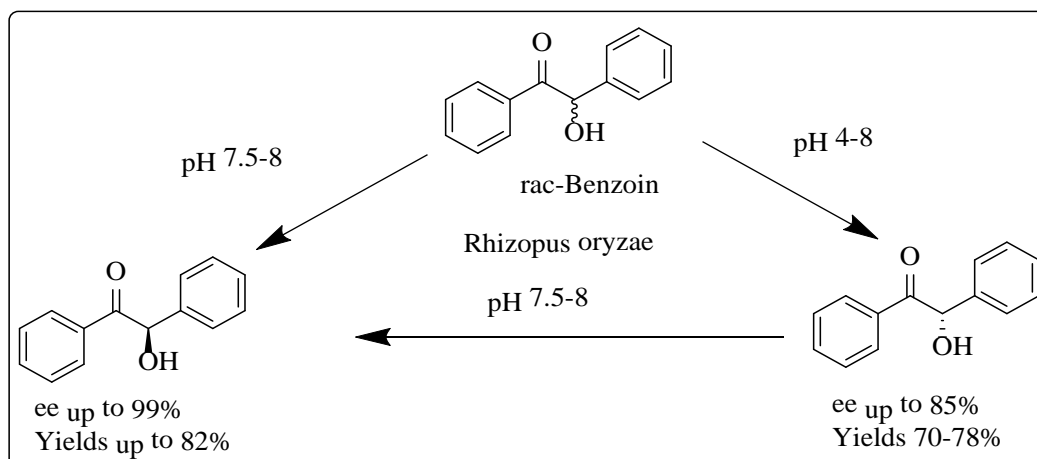
Scheme 9. Benzoin Deracemization, catalyzed by Rhizopus oryzae Whole Cells [39].

1.8 Thiamine diphosphate (ThDP)

The first application of ThDP dependent enzymes for the production of chiral hydroxy ketones had been performed in 1921, when the first whole cell biotransformation method was cogitated. Today this process is still used for production of chiral phenylacetylcarbinol, which is the precursor of (-) ephedrine [40].
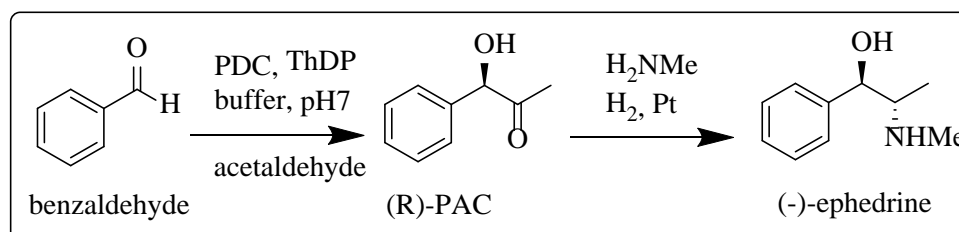
Scheme 10. Enzymatic formation of (R)-PAC used in the production of (-)-ephedrine.

Thiamine diphosphate dependent enzymes mostly perform the cleavage and formation of carbon-carbon bonds apart from that catalyze a broad range of reactions such as formation of C-N, C-O and C-S bond formation. Occurance of polarity change (umpolung) on the carbonyl carbon is used as a donor in both acyloin and benzoin condensation reaction. Carbon carbon bond formation is catalyzed by number of enzymes such as acetohydroxyacid synthase (AHAS), benzaldehyde lyase (BAL), benzoylformate decarboxylase (BFD), phenylpyruvate decarboxylase

(PhPDC), and pyruvate decarboxylase (PDC). All these enzymes are dependent to cofactor, thiamine pyrophosphate (TPP).
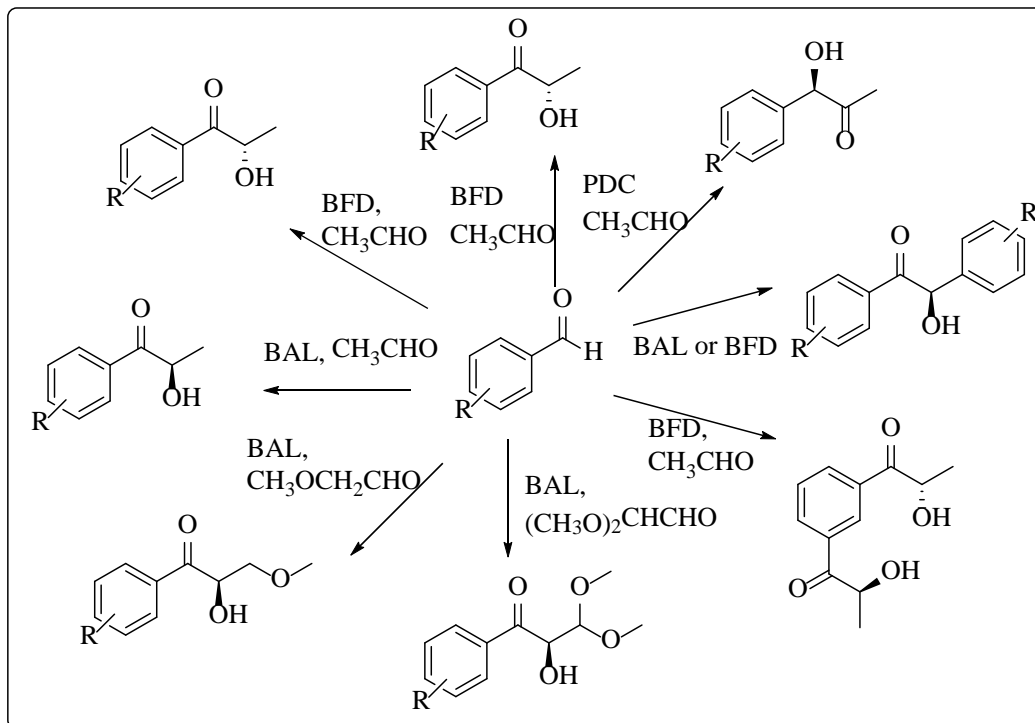
Scheme 11. Examples of ThDP-dependent enzyme-catalysed transformations [41].

Thiamine diphosphate (ThDP) is the biologically active form of vitamin B_1, used as a cofactor in a wide range of reactions. It has three distinctive units, which contain a pyrophosphate part, a thiazolium core, and a pyrimidine unit. Covalent bonding happen interaction of substrate and cofactor. Each subunit of ThDP has a special role in enzymatic catalysis.
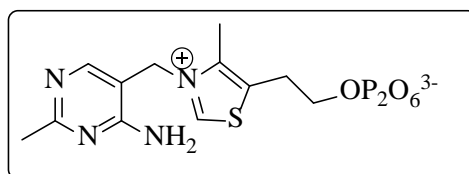
Figure 10. Thiamine diphosphate (ThDP) structure.

Formation of ylide is the first step of process. ThDP of C2-carbanion acts as a nucleophile added to the carbonyl of other substrate. Active site of enzymes contains a glutamic acid residue, which balances the imino tautomer by hydrogen bonding. C2 deprotonation is accomplished by ''V'' confirmation of cofactor with the help of

bulky residues such as leucine, isoleucine or methionine. Thus, the imino group is placed adjacent to the C2 of thiazolium ring that simplify proton abstraction [42].

Chemical structure of ThDP in solution

ThDP in its activated form
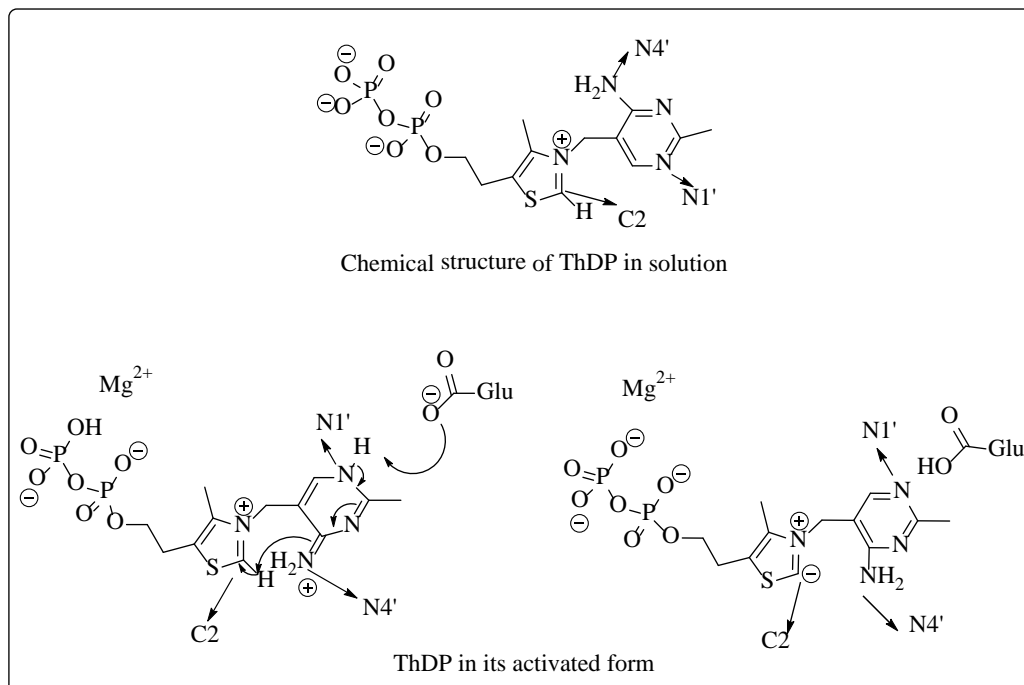
Scheme 12. Formation and stabilization of ThDP ylide.

1.9 Benzaldehyde Lyase

Benzaldehyde lyase was isolated from the strain Pseudomonas fluorescens Biovar I that was found in a cellulose factory and was first reported by Gonzales and Vicuna. They showed that BAL can break the acyloin linkage of chiral benzoin to use these products as a carbon and energy source [43].
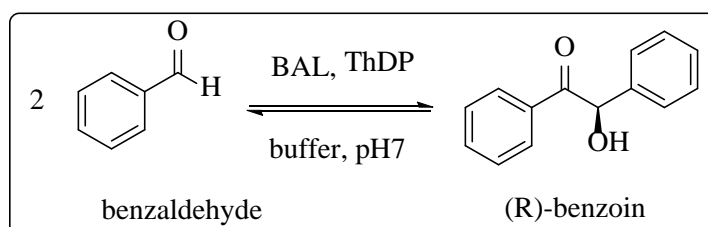
Scheme 13. Benzaldehyde lyase (BAL) catalyzes cleavage and formation of α-benzoin.

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The purified enzyme (BAL) cleaves the chiral hydroxy ketones benzoin and anisoin, in a reaction that requires ThDP and cation. In 2001, first enantioselectivily C-C bond forming reaction synthesized via benzaldehyde. α-benzoin and α-2-hydroxypropiophenone derivatives are the products of reaction were firstly catalyzed. After that, benzaldehyde lyase was used broad substrate spectrum due to the carboligation activity [44].
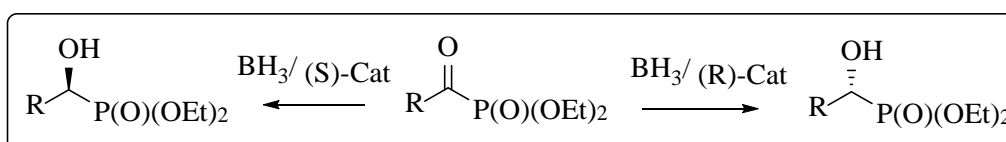
Scheme 14. General carboligation scheme for benzaldehyde lyase.

There is correlation in mechanism of BAL with other ThDP-dependent enzymes. Thus, the first step of the mechanistic approach is the breaking C-C bonds of chiral benzoin due to attacking of the ylide form of ThDP on the carbonyl carbon of benzoin. Nitrogen part of the cofactor is highly electrophilic thus enamine intermediate and free benzaldehyde is formed by electron rearrangement of ThDP. Besides breaking of C-C bonds, enamine intermediate also catalyzes the formation hydroxy ketones. Moreover, in the presence of an acceptor aldehyde, the enamine intermediate processes a bond formation between aldehydes. As a result catalytic cycle is completed with release of formed acyloin, (R)-2-HPP analogous, and renewal of the ylide.
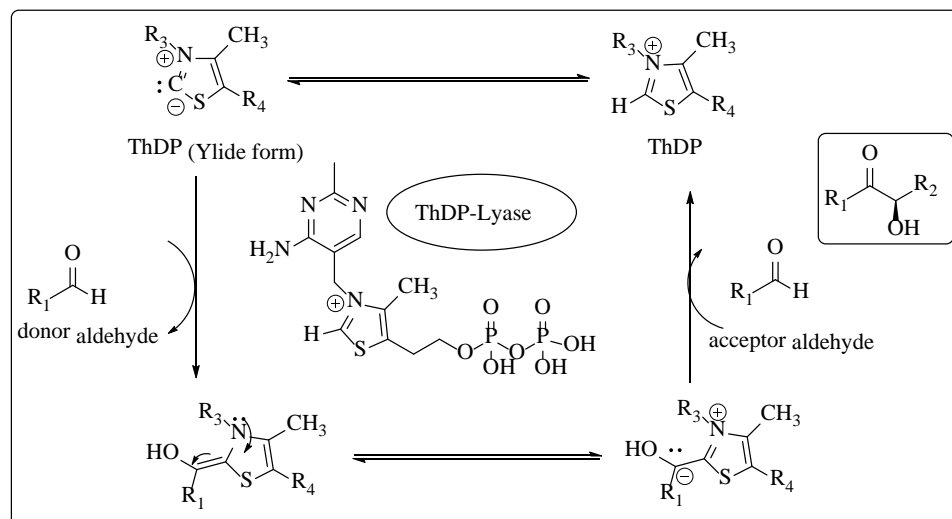
Scheme 15. Proposed Mechanism for the ThDP-Lyase-Catalyzed *Umpolung* Carboligation of Aldehydes [28].

Molecular mass of BAL is 4 x 58,919 Da. It has homotetramer of 4 x 563 amino acid residues. Subunits of BAL separately bind one ThDP molecule exploiting one Mg^{2+} ion. This subunit consists of three domains and these domains consist of a central six-stranded parallel *beta*-sheet connected to a variable number of *alpha* helices.

The active centre is defined by the thiazolium ring of ThDP, which bound in a deep pocket. Residues from Dom-β make interactions with the diphosphate moiety of ThDP and the magnesium with C-terminal, while those from Dom-α of a neighbouring subunit bind to the pyrimidine ring of ThDP.
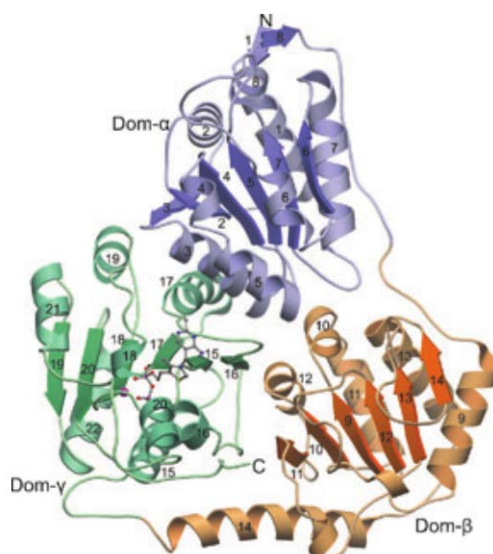
Figure 11. Stereo ribbon plot of a BAL subunit composed of the three domains [46].

ThDP is located at the bottom of a narrow channel. Therefore, thiazolium ring of the ThDP and the C2 atom of the thiazolium ring channels divide bal into two part.
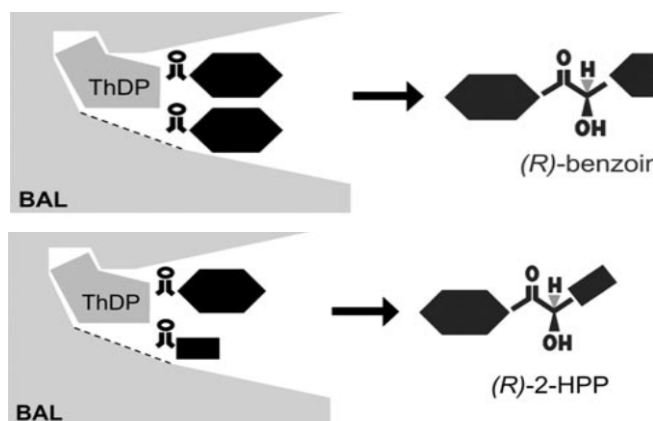
Figure 12. Illustrations showing the shape of the binding site for BAL [47].

1.9.1 Benzaldehyde lyase catalyzed reactions

Benzaldehyde lyase was first identified by its lyase activity. Lyases are an important class of enzymes that achieve several important reactions, including C–C bond formations. The thiamine diphosphate dependent enzyme, benzaldehyde lyase has been indicated to catalyze a broad range of C– C bond formation reactions. BAL has been used broadly to achieve aromatic-aromatic, aromatic-aliphatic, and non-functionalized aliphatic-aliphatic acyloin derivatives. Nowadays, BAL is accepted as one of the most useful and versatile ThDP-dependent enzymes in asymmetric synthesis. The above figure shows the some examples that benzaldehyde lyase catalyze [29].
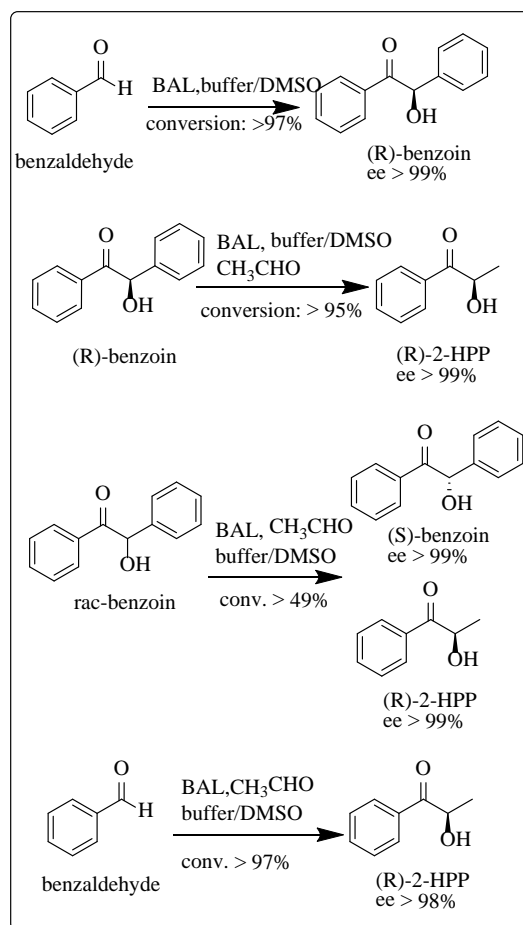
Scheme 16. Several types of reactions catalysed by BAL [29].

Stability of BAL in chemo-enzymatic synthesis needs the addition of cofactors to the buffer. 37°C is the optimum temperature that should not be exceeded. The enzyme is stable between pH 6 and 8. Potassium phosphate was recognized as optimum reaction buffers. DMSO as a solvent is proper to increase the solubility of aromatic substrates and products and provide stability of BAL.
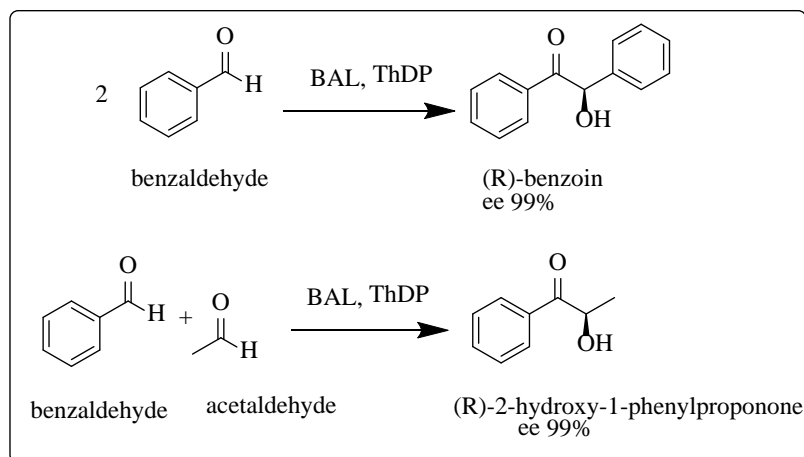
Scheme 17. Self and cross condensation reactions of BAL.

The figure above shows the aromatic-aromatic and aromatic-aliphatic coupling reactions of BAL. Aromatic and aliphatic aldehydes are recognized by the enzyme yielding either benzoins as a product of self-condensation of the aromatic aldehyde or 2-hydroxy ketones resultant by carboligation of the aromatic and the aliphatic aldehyde. Benzaldehyde coupling with acetaldehyde is the result of the HPP formation and this reaction has reversible subreactions as shown in scheme 18. Reaction **1** is the cross coupling of aldehydes. The self-condensation of benzaldehyde in terms of the benzoin reaction is observed in reaction **3**. In the existence of acetaldehyde BAL catalyzes the breaking of (*R*)-benzoin C-C bond yielding HPP and benzaldehyde in reaction **2** [48].
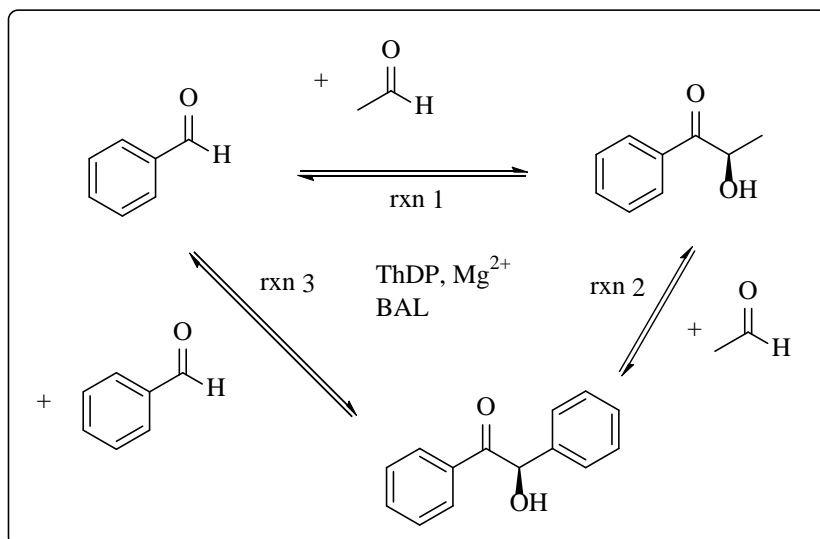
Scheme 18. Benzaldehyde coupling with acetaldehyde is the result of the HPP formation and this reaction has reversible subreactions [48].

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Table 3 shows the reactions with BAL that catalyze a wide range of aromatic aldehydes and hetero aromatic aldehydes, and the resulted acyloin derivatives which were obtained in high enantiomeric excess, reviewed as 2a-j and 3a-c [34].
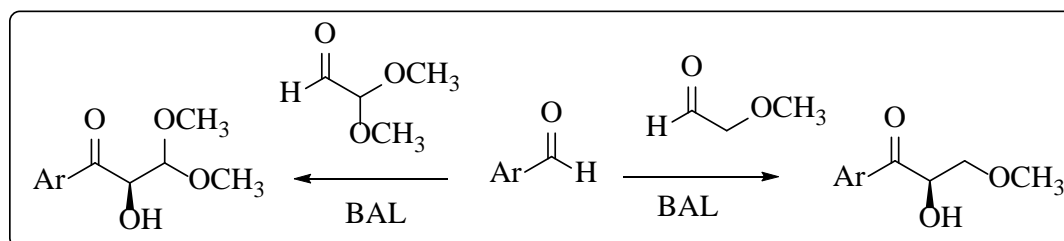
	ARCHO	(R)-2a−j		(R)-3a−c	
	1	yield (%)[a]	ee (%)[b]	yield (%)[a]	ee (%)[b]
a	ph	94	>98[c]	91	>98[i]
b	2,4-F$_2$C$_6$H$_3$	81	94[c]	87	89[j]
c	4-MeOC$_6$H$_4$	87	92[c]	79	>98[k]
d	2-MeOC$_6$H$_4$	91	94[c]		
e	2-furanyl	90	95[d]		
f	4-HOC$_6$H$_4$	94	95[e]		
g	3-OH-4-MeOC$_6$H$_3$	72	nd[f]		
h	3,5-F$_2$C$_6$H$_3$	70	91[g]		
i	3- pyridinyl	<5[h]		<5[h]	
j	4-pyridinyl	<5[h]		<5[h]	

Conversion rates of yielding were different due to the steric and electronic demand of the substituent. For instance, fluorine substitution on the 3,5- and 2,4-positions of the phenyl ring decreased the yield of the reaction. Pyridine carbaldehyde also provided a low yield, but furfural and *o*-methoxy benzaldehyde gave the products in high yields [34].

Besides benzaldehyde, a wide range of aromatic aldehydes substituted at the ortho-, meta-, and para-position with electron-releasing as well as electron-withdrawing properties are accepted as substrates.

Meta- and para-substituted benzaldehydes selectively acted as donors, whereas ortho-substituted analogs served as acceptors in cross-benzoin condensation reactions catalyzed by BAL, cross-benzoin products were obtained with >99% ee and the reactions work well in organic–aqueous medium, overcoming the solubility

problem of lipophilic substrates and opening the way for large-scale preparation.Apart from that, the latest substrate scanning study with BAL was the cross condensation reactions of benzyloxyacetaldehyde with 2-furan-2-carbaldehyde and dimethoxyacetaldehyde. When two different aldehydes are coupled under the catalysis of BAL in a cross condensation manner, four products can be expected which is shown in scheme 19; two self-condensation (**1** & **2**) and two cross condensation products (**3** & **4**). When 1:1 ratio is used between benzyloxyacetaldehyde (**7**) and furan-2-carbaldehyde (**8**) under BAL catalysis, only one cross condensation product 3-(benzyloxy)-1-(furan-2-yl)-2-hydroxypropan-1-one (**9**), was obtained with 90% ee in scheme 20. Furan-2- carbaldehyde acted as donor. Self-condensation products were only detectable when one of the substrates was used in excess amounts. In all cases, (*R*)-configured products are obtained [49].
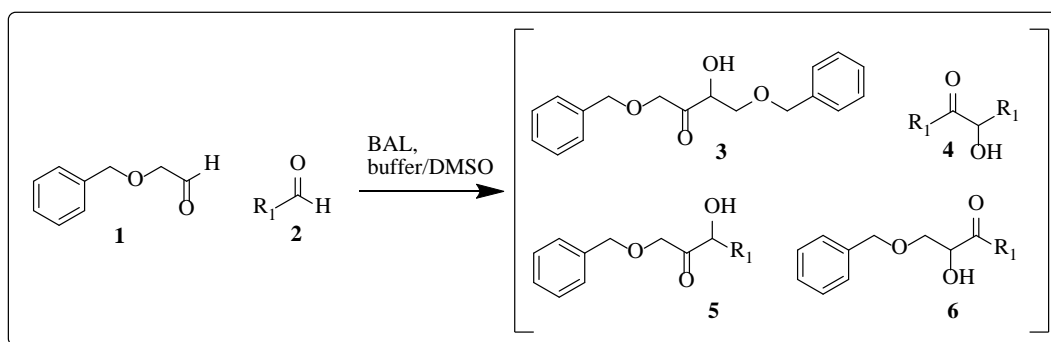
Scheme 19. Two different aldehydes reaction with BAL, four products can be expected.
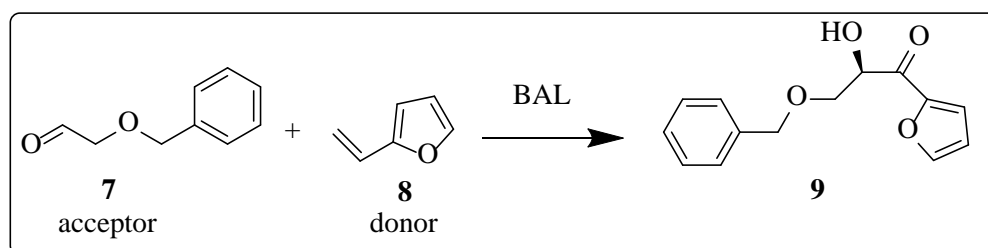
Scheme 20. Reactions of benzyloxyacetaldehyde and furan-2-carbaldehyde under BAL catalysis.

1.10 Importance of hydroxy phosphonates

Phosphonates have been used widely in phosphonate chemistry and biology.Carbon-phosphorus bond in phosphonates reveals stability and longer duration of action under physiological conditions. Biologically important phosphonates can be found in carbohydrates, amino acids, phospholipids, nucleotides, etc.

Hydroxy phosphonic acids are an important class of compounds, some of which are obtained from nature. The best known naturally occuring hydroxy phosphonic acids are 1-hydroxy-2-aminoethylphosphonic acid (HO-AEP), 3-(N-acetyl-N-hydroxyamino)-2-hydroxypropyl- phosphonic acid (FR-33289), 1,5-dihydroxy-2-oxopyrrolidin-3-ylphosphonic acid (SF-2312), phosphonothrixin. Phosphonothrixin, a new herbicidal antibiotic, is a chiral b-hydroxyphosphonic acid also known as fosfomycin.
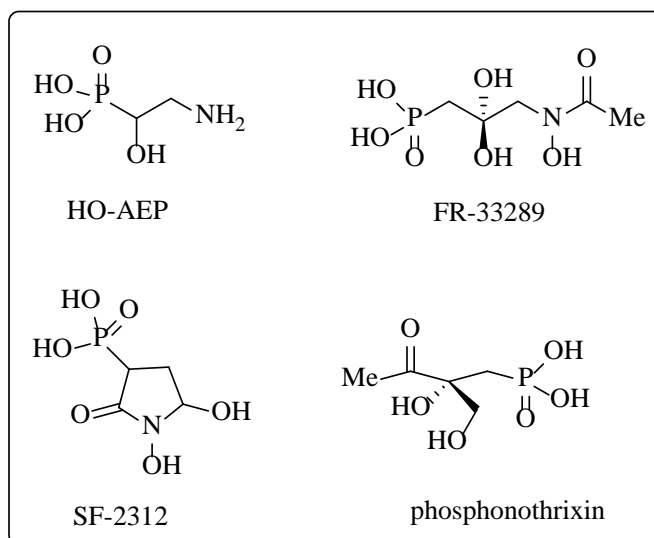
Figure 13. Naturally occurring hydroxyphosphonic acids [50].

Figure 14 shows the biologically active hydroxy phosphonates. Many of these compounds have taken interest because of their antibacterial, antiviral, antibiotic, pesticidal, anticancer, and enzyme inhibitor properties.
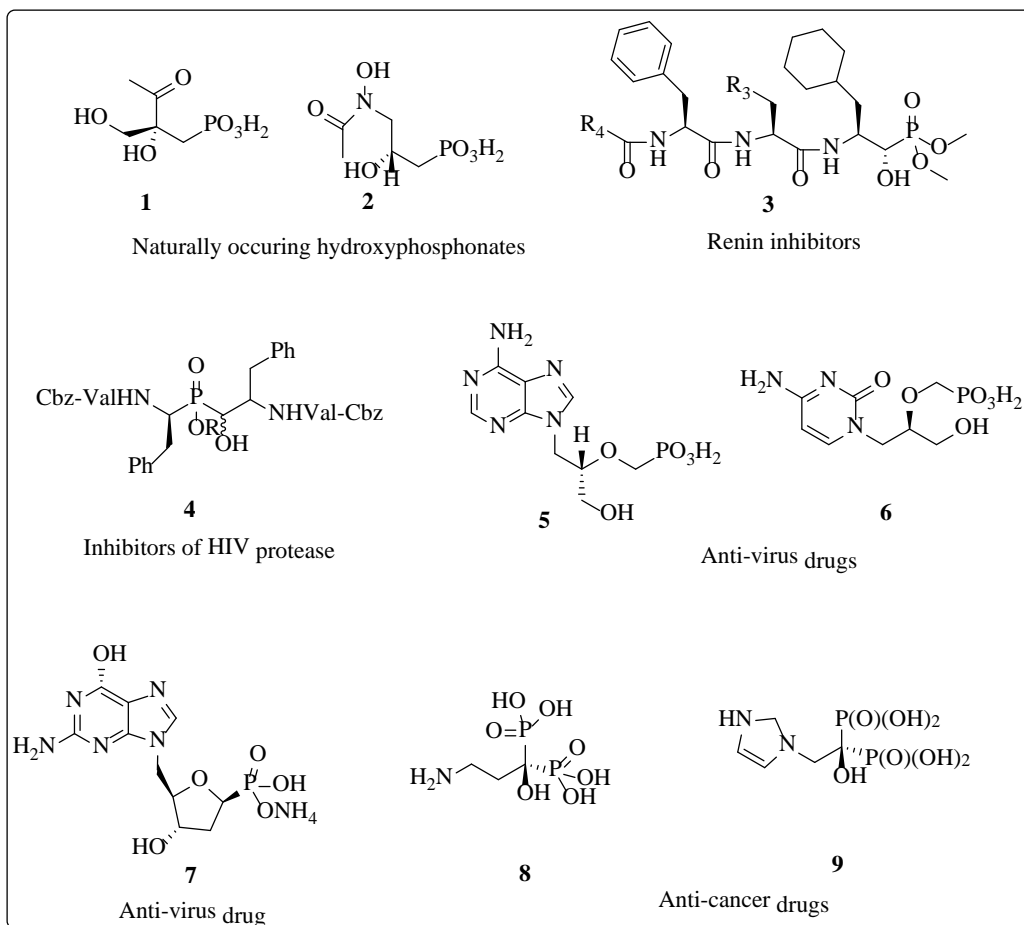
Figure 14. Naturally and biologically active hydroxy phosphonates [51].

For example phosphonopeptide **(3)** efficiently inhibits human renin, the enzyme which specifically catalyses the conversion of angiotensinogen to angiotensin.

Importantly, hydroxyphosphonates exhibit high antibacterial, antiviral, anticancer activity, for instance, compound **(4)** constrains HIV protease, and they can be drugs for the treatment of AIDS. Hydroxy phosphonates **(5–6)** are efficient medications against smallpox. The example **(7-9)** shows antiproliferative activity so holding potent antitumour activity and used for the treatment of cancer [51].

Reported theoretical and practical application of chiral hydroxyphosphonates exhibits the interest asymmetric synthesis of hydroxyphosphonates.

Especially, chiral beta hydroxy phosphonic acids (Figure 15) have acknowledged significant attention lately because of their unique physiological activities as well as their ability to mimic the corresponding hydroxy carboxylic acids or amino acids because they can be used as an intermediate in the syntheses of potentially

significant peptide analogues, catalytic antibodies, and phosphonic acid-based antibiotics [50].
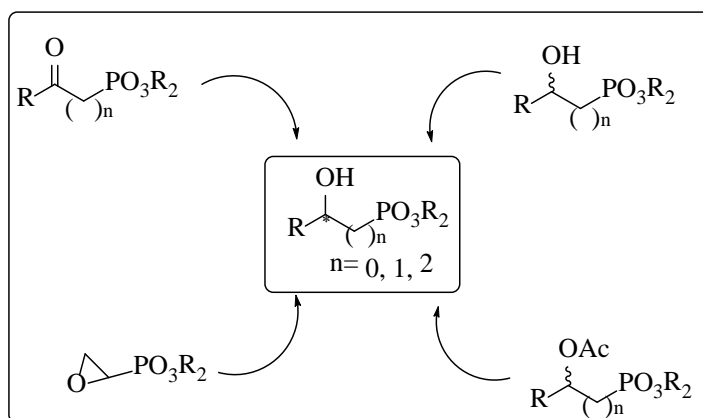
Figure 15. Chiral β-hydroxy phosphonic acid.

1.11 Asymmetric synthesis of hydroxy phosphonates

Chiral hydroxy phosphonic acids can be prepared by a number of methods including kinetic resolution of racemic mixtures or enzymatic and asymmetric synthesis. In recent years, the chemo-enzymatic method for the synthesis of optically active hydroxy phosphonates has been used more and more often.
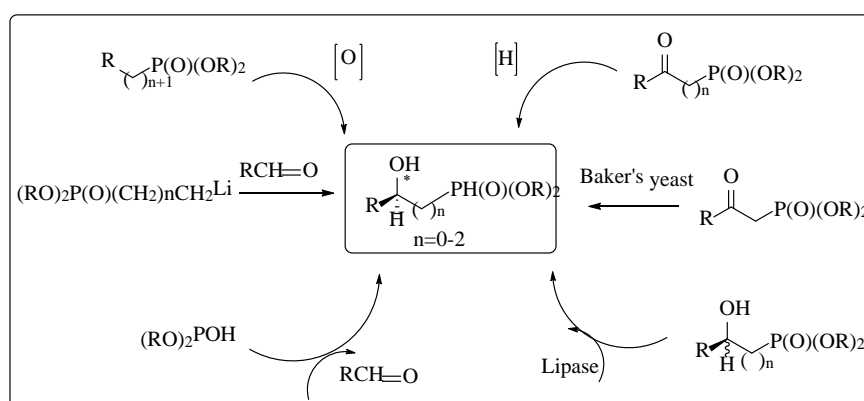
Scheme 21. Synthetic routes to chiral hydroxy phosphonates [51].

Methods to synthesize chiral hydroxy phosphonates

1. Asymmetric addition reactions
2. Asymmetric reduction

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3. Asymmetric oxidation

4. Chemoenzymatic synthesis

1.11.1 Asymmetric addition reactions

Phosphonylation of carbonyl compounds is the main method for the synthesis of hydroxyphosphonates. There are two methods that are used for the asymmetric phosphonylations of carbonyl compounds.

a. phospho-aldol addition (the Abramov reaction)
the reaction of dialkylphosphites with carbonyl reagents, in the presence of base catalyst
b. reaction of phosphonate carbanions with aldehydes or ketones
the addition reaction of phosphoric acid triesters to carbonyl compounds, progressing in the presence of proton donating reagents (phenol, carboxylic acid, etc.), or Lewis acids.

1.11.2 Asymmetric reduction

The asymmetric reduction of ketophosphonates is one of the most convenient methods for the synthesis of chiral hydroxyphosphonates. Chiral auxiliaries, asymmetric catalysts or chiral reagents can be used for the asymmetric reduction of ketophosphonates.

For instance, chiral β-butyloxazoborolidines as a catalyst reduced the diethyl alpha ketophosphonates, produced the diethyl 1-hydroxy- alkylphosphonates enantiomers in good yields and reasonable enantiomeric excesses (53–83% ee) [51].
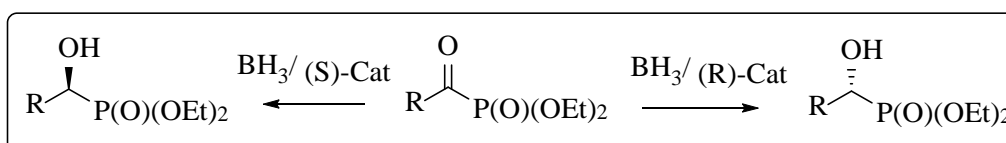
Scheme 22. Borane reduction of ketophosphonates.

1.11.3 Asymmetric oxidation

The asymmetric oxidation of carbon–carbon bonds is a useful route to synthesize a variety of hydroxyphosphonates. For example, oxaziridine mediated hydroxylation of dialkyl benzylphosphonates resulted chiral alpha-hydroxyphosphonates with high enantiomeric excess (96–98% ee) [51].
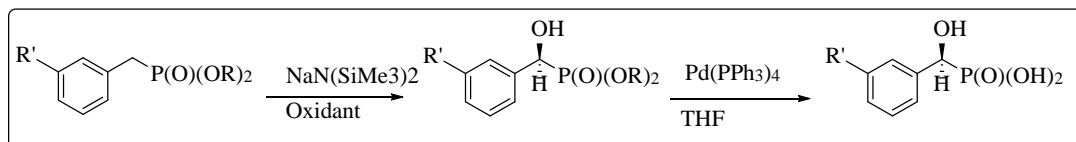
R	R'	Oxidant	X	ee (%)	Refs
Me	H		H	69	46
Me	H		Cl	93	46
Me	NO$_2$		Cl	80	46
Me	Cl		Cl	87	46
Me	MeO		Cl	81	46
Et	CF$_3$		Cl	98	62
CH$_2$=CHCH$_2$	H		Cl	96	62
CH$_2$=CHCH$_2$	PhO		Cl	98	62

Scheme 23. Enantioselective oxidation of benzylphosphonates with chiral oxaziridines. [51].

1.11.4 Chemoenzymatic synthesis

Chemoenzymatic synthesis exhibits an efficient method sometimes and can be an alternative to the standard synthesis of fine chemicals. To obtain optically active hydroxy phosphonates, bacteria, fungi, and various lipases can be used as biocatalysts.

Four processes have been used to synthesize hydroxy phosphonates.
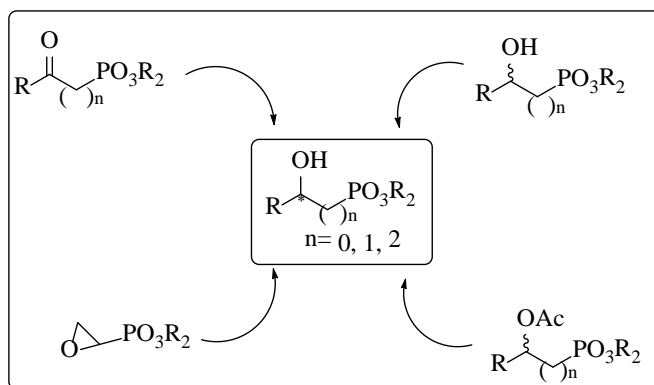
Scheme 24. Enzymatic synthesis of hydroxy phosphonates.

- the use of baker's yeast
- the use of microorganisms and lipases for the enantioselective separation of hydroxy phosphonates via acylation
- the use of lipases either for enantioselective hydrolysis of acyloxyalkanephosphonates
- the use of bacteria and fungi for hydrolytic oxirane ring opening in substituted 1,2-epoxyethanephosphonates [51].

1.12 Aim of the study

Over the last 20 years, a significant number of techniques for the asymmetric synthesis and practical application of chiral hydroxy phosphonates has been stated, which clearly display the theoretical interest and practical importance of hydroxy phosphonates.

Many of these hydroxy phosphonates interest attention due to their remarkable biological properties as antibacterial, antiviral and antitumour agents, antibiotics, enzyme inhibitors, amino acid mimetics and pesticides. Therefore, study of the biological activities of hydroxy phosphonates appears favorable for the development of new bioregulators and drugs [51].

Although the development of efficient catalysts for asymmetric phospho-aldol Abramov reaction and for the reduction of keto phosphonates appears especially important for the synthesis of β-hydroxy phosphonates, asymmetric chemoenzymatic methods for synthesis of β-hydroxy phosphonates are more attractive (non-toxic,

recycable, high enantioselectivity, acts under mild conditions, less byproduct) than the others. Therefore, the search for alternative chemoenzymatic methods opening an easy way to optically active β-hydroxy phosphonates still remains topical. Ligase activity of BAL for the coupling of aldehydes has not been tried for the synthesis of β-hydroxy phosphonates yet.

In the light of this, aim of my study is to synthesize novel chiral β-hydroxy phosphonates by benzaldehyde lyase with high enantiomeric excess. Synthesis of novel β-hydroxy phosphonates with this methodology can be an alternative to chemoenzymatic and other routes. Therefore, this study can be prior route and widen the scope of the substrate range of the benzaldehyde lyase catalyzed reactions.

CHAPTER 2

EXPERIMENTAL

2.1 Materials

Benzaldehyde Lyase is delivered from the Institut für Biotechnologie, Jülich, Germany.

Benzaldehyde and the derivatives are commercially available by, Aldrich, Germany.

Solvents are either in technical or higher grade. They were purchased from Sigma Aldrich when necessary; they were purified and dried with drying agents and by distillation.

2.2 Methods

Nuclear Magnetic Resonance (^{1}H NMR and ^{13}C NMR) spectra were recorded with a Bruker Instrument DPX-400 spectrometer by using deuterochloroform ($CDCl_3$) as a solvent and tetramethylsilane (TMS) as internal reference.

All reactions were analyzed by TLC on silica gel plates (Merk Silica Gel 60 F_{254}) and the spots were visualized with UV light (l=254 nm). Column chromatography was performed on silica gel 60. Chromatographic separation was performed on glass precoated silica gel purchased from Macherey-Nagel. Enantiomeric excesses of all the products were determined by Agilent 1100 series HPLC device using appropriate chiral columns.

HRMS data were recorded with Agilent 6224 TOF LC/ MS at UNAM.

Protein purification was performed with Aktaprime FPLC system.

For disrupting a cell wall sonics, vibra cell instrument was used.

Shaking of cells was provided by Shaker Infors-HT instrument.

Freeze dryer, Telstar Cryodos instrument was used for lyophilization.

2.3 Production and purification of Benzaldehyde lyase

Institute of Biotechnology, Research Centre Jülich arranged the cells of E. coli SG13009/BAL$_{His}$ containing the overexpressed enzyme. Hexahistidine-tagged BAL was obtained from recombinant E.Coli SG13009 cells.

The recombinant E.coli strains were grown on LB agar containing 100 μg/mL ampicilline and 35 μg/mL chloramphenicol incubated in oven for overnight at 37 °C. To produce our enzyme, first cells were received from LB agars by sterile loop and transported to sterile 10 mL LB medium contains 20 μL amphicillin and 20 μL chloramphenicol. This medium was inoculated for 12 hours at 37 °C. In precultivation part growing duration is important since cells begin to die at some point. This part 500 mL Erlenmeyer flask was used for 100 mL LB (90 mL distilled water + 10 mL growth cell) medium that contains 100 μL amphicillin and chloramphenicol inoculation ratio 1/1000. It was grown for 6 hours at 37 °C, then 100 mL transferred to production medium contains 1500 mL LB medium is shaking with 180 rpm. Four hours after the inoculation of the microorganism, production of enzyme was induced with addition of isopropyl-β-D-thiogalacto pyranosid (IPTG). After the induction, enzyme production was continued for 12 hours, cell pellets were collected with centrifugation. In order to break the cell walls to release our enzyme, the cell pellets which are taken from -20 °C has been melted to room temperature and sonicated. Finally, removals of water from cells by lyophilization made with freeze dryer.

2.3.1 Purification of Benzaldehyde lyase

FPLC system (AKTA prime) performed for purification of benzaldehyde lyase. BAL was purified via immobilized metal ion chromatography (Co^{2+}- NTA column) for affinity chromatography and sephadex G-25 resin for desalting of eluted sample from affinity column as described in the literature [52,53].
 Lyophilized E.coli cells were dissolved in phosphate buffer and slurry sonicated with micro prob. After sonication, the mixture is centrifuged at 10000 rpm at 4 °C for 30 min and filtered through 0.45 μm filters.

The mixture is ready for Co^{2+}- NTA column purification. Both columns should be equilibrated pH 7 beforehand. First, sample entered to column, 50 mM potassium phosphate pH7 buffer was used to wash nontagged background proteins then, the same buffer, which had now been supplemented with 50 mM imidazole, low affinity binding proteins is prevented. The benzaldehyde lyase, with its histidine end (histagged-BAL), is obtained by elution of 200 mM imidazole. Proteins bound to the resin are eluted with low pH buffer or by competition with imidazole or histidine. After affinity step, proteins were separated according to their size. Big sizes eluted first and small molecules eluted after proteins. Samples were collected and used for activity assay. Desalting technique is also known as size exclusion or gel permeation chromatography.

2.3.2 Activity assay

According to literature [54], one unit (U) of activity is described as the amount of enzyme which catalyzes the cleavage of 1 μmol benzoin (1.5 mM) into benzaldehyde in potassium phosphate buffer (50 mM, pH7), containing $MgSO_4$ (2.5 mM), ThDP (0.15 mM) and DMSO (20%, v/v) in 1 min at 30 °C.

To perform this assay, different benzaldehyde concentration was prepared with commercially available benzaldehyde and standard curve was drawn with GC-MS analysis to conclude the activity of BAL.

For activity experiment, 2.5μL benzaldehyde was dissolved in 0.5 mL DMSO and 1.5 mL phosphate buffer which contains ThDP and $MgSO_4$ was added to the mixture. Reaction was started with the addition of 5 mg crude enzyme. After 10 min, chloroform was added and centrifuged. Finally, organic layer was obtained and analyzed with GC-MS to conclude activity.

2.4 General procedure of crossed acyloin condensation of aliphatic aldehydes with enzymes (BAL)

Benzaldehyde derivatives or heterocyclic aldehydes (0.5 mmol) and diethyl (2-oxoethyl) phosphonate (1.5 mmol) were dissolved in 5 mL dimethylsulfioxide (25 vol%) then 15 mL (75 vol%) potassium phosphate buffer (50 mM, pH 7) containing 0.15 mM ThDP and 2.5 mM MgSO4 was added to this solution. The reaction was started with the addition of BAL (50 U) at 37 °C (120 rpm). Every 48 hours 50U of BAL was added. The reaction was monitored with TLC and concluded after 72 h. The reaction mixture was extracted with diethylether (8 x 40 mL) and the combined organic layers washed with brine and dried over MgSO4, and the solvent was removed under reduced pressure. The product was purified with column chromatography.

2.4.1 Synthesis of diethyl (2-hydroxy-3-oxo-3-phenylpropyl) phosphonate (BHP-1)
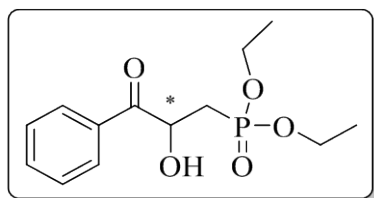
General procedure described above starting from benzaldehyde provided pure product obtained after column chromatography on silica gel, gradients are diethylether : hexane (1:1), (2:1), (18% yield, yellow oil). TLC diethylether: hexane = 2:1 R_f; 0.25

^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.45-1.04 (m, 6H), 2.06-1.89 (m, 1H), 2.32-2.20 (m, 1H), 4.27-3.92 (m, 4H), 5.39-5.26 (m, 1H), 7.44 (t, J = 7.69 Hz, 2H), 7.55 (t, J = 7.42 Hz, 1H), 7.92 (d, J = 7.14 Hz, 2H) **^{13}C NMR** (100 MHz, CDCl$_3$) δ: 16.6, 33.5, 61.9, 68.5, 128.9, 129.1, 133.3, 134.2, 200.3 **HRMS** C$_{13}$H$_{18}$O$_5$P (MH$^+$): Calculated 287.1004, found 287.1008.

Optical rotation of the **BHP-1** was determined as $[\alpha]_D^{16}$= +4.58° (c = 2.5 x 10^{-2} g/mL, CHCl$_3$)

2.4.2 Synthesis of diethyl (2-hydroxy-3-oxo-3-(*m*-tolyl)propyl)phosphonate (BHP-2)

General procedure described above starting from 3-methylbenzaldehyde provided pure product after column chromatography on silica gel. (20% yield, yellow oil). TLC diethylether:hexane= 2:1 R_f; 0.31

^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.39-1.14 (m, 6H), 2.08-1.86 (m, 1H), 2.33-2.16 (m, 1H), 2.35-2.31 (s, 3H), 4.25-3.96 (m, 4H), 5.39-5.22 (m, 1H), 7.33-7.27 (dd, 1H), 7.37-7.33 (d, 1H), 7.73-7.69 (d, 1H), 7.69-7.66 (s, 1H) **^{13}C NMR** (100 MHz, CDCl$_3$) δ: 16.5,21.3, 32.9, 61.9,68.4, 126.1, 128.8, 129.3, 133.8, 135.0, 138.8, 199.6 **HRMS** C$_{14}$H$_{20}$O$_5$P (MH$^+$): Calculated 301.1160, found 301.1164.

Optical rotation of the **BHP-2** was determined as $[\alpha]_D^{16}$= +1.63° (c = 4.8 x 10^{-2} g/mL, CHCl$_3$)

2.4.3 Synthesis of diethyl (2-hydroxy-3-oxo-3-(*p*-tolyl)propyl) phosphonate (BHP-3)

General procedure described above starting from 4-methylbenzaldehyde provided pure product after column chromatography on silica gel, gradients are diethylether : hexane (1:1), (2:1) (18% yield) TLC diethylether: hexane = 2:1 R_f: 0.24

^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.43-1.10 (m, 6H), 2.03-1.86 (m, 1H), 2.32-2.07 (m, 1H), 2.37-2.35 (s, 3H), 4.25-3.90 (m, 4H), 5.50-5.11 (m, 1H), 7.23 (d, J = 8.00 Hz, 2H), : 7.81 (d, J = 8.24 Hz, 2H) **^{13}C NMR** (100 MHz, CDCl$_3$) δ: 16.6, 21.9, 33.3, 62.6, 68.3, 128.5, 129.1, 130.6, 145.8, 199.3 **HRMS** C$_{14}$H$_{20}$O$_5$P (MH$^+$): Calculated 301.1160, found 301.1167.

Optical rotation of the **BHP-3** was determined as $[\alpha]_D^{16}$= +11.4° (c = 5.6 x 10^{-3} g/mL, CHCl$_3$)

2.4.4 Synthesis of diethyl (2-hydroxy-3-(3-methoxyphenyl)-3-oxopropyl) phosphonate (BHP-4)

General procedure described above starting from 3-methoxybenzaldehyde provided pure product after column chromatography on silica gel. (18% yield) TLC diethylether: hexane = 2:1 R_f; 0.18

^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.32-1.22 (m, 6H), 2.01-1.90 (m, 1H), 2.32-2.21 (m, 1H), 3.79-3.74 (s, 3H), 4.25-3.95 (m, 4H), 5.35-5.20 (m, 1H), 6.69-6.58 (s, 1H), 7.04 (s, 2H) **^{13}C NMR** (100 MHz, CDCl$_3$) δ: 16.9, 33.1, 55.7, 62.7, 68.7, 105.4, 106.7, 134.8, 161.2, 199.7 **HRMS** C$_{14}$H$_{20}$O$_5$P (MH$^+$): Calculated 317.1109, found 317.1117.

Optical rotation of the **BHP-4** was determined as $[\alpha]_D^{16}$= +0.71° (c = 11.8 x 10^{-2} g/mL, CHCl$_3$)

2.4.5 Synthesis of diethyl (2-hydroxy-3-(4-methoxyphenyl)-3-oxopropyl) phosphonate (BHP-5)
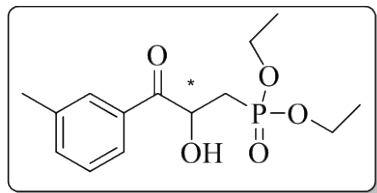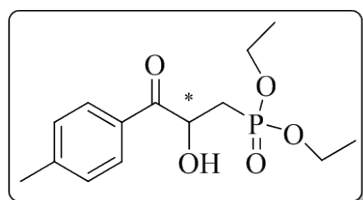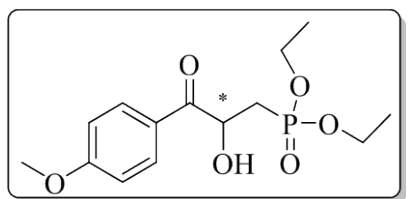

General procedure described above starting from 4-methoxybenzaldehyde provided pure product after column chromatography on silica gel, gradients are diethylether : hexane (2:1)

TLC diethylether: hexane = 2:1 R_f; 0.15 16% yield.

^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.37-1.21 (m, 6H), 2.08-1.80 (m, 1H), 2.35-2.14 (m, 1H), 3.83-3.81 (s, 3H), 4.28-3.91 (m, 4H), 5.38-5.18 (m, 1H), 6.91 (d, J = 8.91 Hz, 2H), 7.91 (d, J = 8.89 Hz, 2H) **^{13}C NMR** (100 MHz, CDCl$_3$) δ: 16.7, 32.5, 55.9, 62.4, 68.1, 114.5, 120.1, 131.3, 164.7, 195.4 **HRMS** C$_{14}$H$_{20}$O$_6$P (MH$^+$): Calculated 317.1109, found 317.1119.

Optical rotation of the **BHP-5** was determined as $[\alpha]_D^{16}$= +17.4° (c = 0.6 x 10^{-2} g/mL, CHCl$_3$)

2.4.6 Synthesis of diethyl (3-(3,5-dimethoxyphenyl)-2-hydroxy-3-oxopropyl) phosphonate (BHP-6)
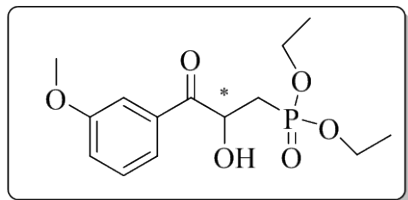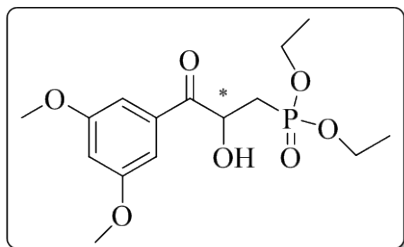

General procedure described above starting from 3,5-dimethoxybenzaldehyde provided pure product after column chromatography on silica gel gradients are diethylether : hexane (2:1). TLC diethylether: hexane = 2:1 R_f; 0.18

^{1}**H NMR** (400 MHz, CDCl$_3$) δ: 1.32-1.22 (m, 6H), 2.01-1.90 (m, 1H), 2.32-2.21 (m, 1H), 3.79-3.74 (s, 3H), 4.25-3.95 (m, 4H), 5.35-5.20 (m, 1H), 6.69-6.58 (s, 1H), 7.04 (s, 2H) ^{13}**C NMR** (100 MHz, CDCl$_3$) δ: 16.9, 33.1, 55.7, 62.7, 68.7, 105.4, 106.7, 134.8, 161.2, 199.7 **HRMS** C$_{15}$H$_{22}$O$_7$P (MH$^+$): Calculated 347.1215, found 347.1218.

Optical rotation of the **BHP-6** was determined as $[\alpha]_D^{16}$= +2.82° (c = 2.9 x 10^{-2} g/mL, CHCl$_3$)

## 2.4.7 Synthesis of diethyl (2-hydroxy-3-(4-hydroxyphenyl)-3-oxopropyl) phosphonate (BHP-7)
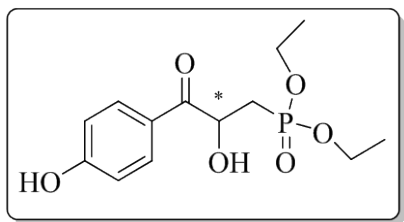
General procedure described above starting from 4-hydroxybenzaldehyde provided pure product after column chromatography on silica gel, gradients are diethylether:hexane (2:1), (25% yield). TLC diethylether:hexane=2:1

R_f; 0.12 ^{1}**H NMR** (400 MHz, CDCl$_3$) δ: 1.40-1.21 (m, 6H), 2.07-1.94 (m, 1H), 2.36-2.23 (m, 1H), 4.23-3.97 (m, 4H), 5.40-5.24 (m, 1H), 6.88 (d, J = 8.79 Hz, 2H), 7.80 (d, J = 8.79 Hz, 2H), 10.21-9.69 (s, 1H) ^{13}**C NMR** (100 MHz, CDCl$_3$) δ: 15.4, 32.7, 62.4, 66.3, 115.1, 123.1, 130.5, 162.6, 196.7

HRMS C$_{13}$H$_{18}$O$_6$P (MNa$^+$): Calculated 325.0771, found 325.0779.

Optical rotation of the **BHP-7** was determined as $[\alpha]_D^{16}$= +0.27° (c = 3.6 x 10^{-2} g/mL, CHCl$_3$)

**2.4.8 Synthesis of diethyl (3-(4-fluorophenyl)-2-hydroxy-3-oxopropyl) phosphonate (BHP-8)**
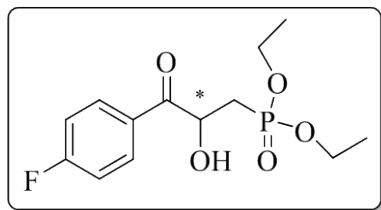
General procedure described above starting from 4-fluorobenzaldehyde provided pure product after column chromatography on silica gel. TLC diethylether : hexane = 2:1 R_f; 0.24, 13% yield.

^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.37-1.20 (m, 6H), 2.05-1.93 (m, 1H), 2.31-2.08 (m, 1H), 4.21-3.95 (m, 4H), 5.37-5.17 (m, 1H), 7.12 (t, J = 8.56 Hz, 2H), 7.98 (t, J = 8.73, 5.37 Hz, 2H) **^{13}C NMR** (100 MHz, CDCl$_3$) δ: 15.2, 32.6, 61.5, 68.7, 115.8, 124.8, 130.6, 161.6, 198.1 **HRMS** C$_{13}$H$_{17}$O$_5$P (MH$^+$): Calculated 305.0909, found 305.0921.

Optical rotation of the **BHP-8** was determined as $[\alpha]_D^{16}$= +0.29° (c = 4.8 x 10^{-2} g/mL, CHCl$_3$)

**2.4.9 Synthesis of diethyl (3-(furan-2-yl)-2-hydroxy-3-oxopropyl) phosphonates (BHP-9)**
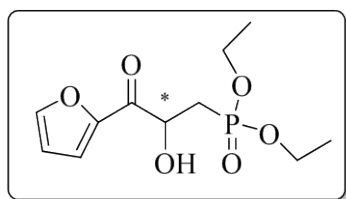
General procedure described above starting from furan-2-carbaldehyde provided pure product after column chromatography on silica gel, gradients are diethylether: hexane (1:3), 12% yield. TLC diethylether: hexane = 2:1 R_f; 0.14

^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.35-1.20 (m, 6H), 2.20-2.03 (m, 1H), 2.44-2.30 (m, 1H), 4.20-3.96 (m, 4H), 5.13-4.96 (m, 1H), 6.54 (dd, J = 3.61, 1.68 Hz, 1H), 7.39 (d, J = 3.66 Hz, 1H), 7.60 (d, J = 1.52 Hz, 1H) **^{13}C NMR** (100 MHz, CDCl$_3$) δ: 17.0, 40.2, 62.4, 66.8, 117.2, 120.4, 131.6, 158.4, 190.3 **HRMS** C$_{11}$H$_{16}$O$_6$P (MH$^+$): Calculated 277.0796, found 277.0828.

Optical rotation of the **BHP-9** was determined as $[\alpha]_D^{16}$= +1.64° (c = 4.9 x 10^{-2} g/mL, CHCl$_3$)

2.5 Synthesis of diethyl (2,2-diethoxyethyl) phosphonate

Three-necked round bottom flask fitted with a magnetic stirrer, dropping funnel and Ar inlet was filled 4 mL (1 mol) bromoacetaldehyde diethyl acetal. Gentle stream of Ar was then passed through the system. To the stirred solution phosphide 5.1 mL (1mol) was added dropwise over a period of 30 minutes at 100 °C. The mixture was then stirred for 8 hours at 170 °C (the elevated temperature required for the activation of the reaction). The product was obtained by vacuum distillation 0.33 mbar; thermocouple was fixed at 65 °C. Unreacted reactants were collected to one balloon and product was left in reaction flask. The Michaelis Arbuzov reaction is initiated with an S_N2 reaction of nucleophilic trialkylphosphite with aryl-methyl halides to give a phosphonium intermediate. The displaced halide anion upon a second S_N2 reaction with phosphonium intermediate led to the phosphonate ester along with an alkyl halide. Procedure was from the literature [55].
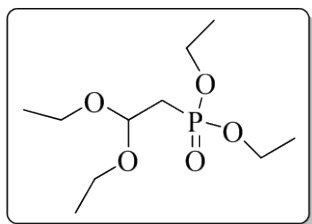
TLC EtOAc : Hexane = 1:1 Ninhidrin R_f; 0.24 **¹H NMR** (400 MHz, CDCl₃) δ: 1.18-1.11 (t, 6H), 1.30-1.21 (t, 6H), 2.13-2.09 (d, 1H), 2.18-2.14 (d, 1H), 3.67-3.42 (dq, 4H), 4.12-3.97 (dq, 4H), 4.87-4.80 (q, 1H)

2.6 Synthesis of diethyl (2-oxoethyl) phosphonate
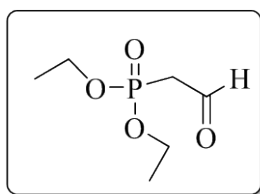
10 mmol solution of of diethyl (2,2-diethoxyethyl) phosphonate was added to the 40 mL acetone containing water (0.6 g). After addition of acid amberlyst-15 (0.4 g), the mixture was stirred for 24 hours. Finally, the mixture is filtered and evaporated to give the product [56]. TLC EtOAc : Hexane = 2:1 Ninhidrin R_f; 0.18.

¹H NMR (400 MHz, CDCl₃) δ: 1.33-1.22 (m, 6H), 2.99 (d, J = 3.24 Hz, 1H), 3.05 (d, J = 3.23 Hz, 1H), 4.19-3.98 (m, 4H), 9.60 (t, J = 3.23 Hz, 1H)

2.7 General procedures of cross acyloin condensation of aliphatic aldehyde reactions with thiazolium catalyst

Two procedures were applied with N-hetercyclic carbene catalyst (3-ethyl-5-(2-hydroxyethyl)-4-methylthiazol-3-ium bromide) and ThDP is shown in Figure 17.
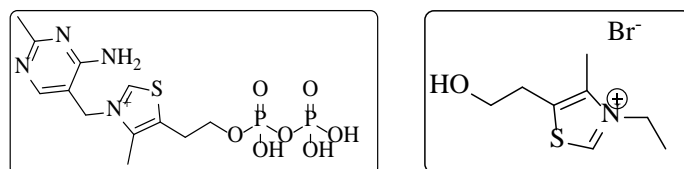
Figure 17. ThDP cofactor of BAL and thiazolium catalyst.

1. 0.5 mmol benzaldehyde and 5 mmol acetaldehyde were dissolved in 1mL dry THF. 0.05 mmol 3-ethyl-5-(2-hydroxyethyl)-4-methylthiazol-3-ium bromide catalyst was added with 0.05 mmol Cs_2CO_3. After stirring at room temperature for 15 hour, reaction mixture extracted with EtOAc (1mL x 3), dried with $MgSO_4$ and concentrated in vacuo. Pure hydroxy ketone was obtained after column chromatography (EtOAc/Hexane =1:10) as an oil.

Instead of acetaldehyde, diethyl (2-oxoethyl) phosphonate was used as an aliphatic aldehyde but in TLC we did not observe the product. This procedure was adapted from ref. [57].

2. 14 mg (0.05 mmol = 10 mol%) thiazolium bromide precatalyst (3-ethyl-5-(2-hydroxyethyl)-4-methylthiazol-3-ium bromide) was added to a flame-dried tube and placed under high vacuum. 1 hour later, 0.16 mL of EtOH was added and stirred for 5 min. After then, 0.5 mmol aromatic aldehyde and 1.5 mmol aliphatic aldehyde were added. After final addition of 0.3 mmol triethylamine, the reaction tube heated for 16 h at 90 °C under an N_2 atmosphere. This procedure was modified from Stetter [58].

The below Figure 18, listed aromatic aldehydes were tried with this procedure, monitored with TLC and different spots from reactants were observed but products were not observed.
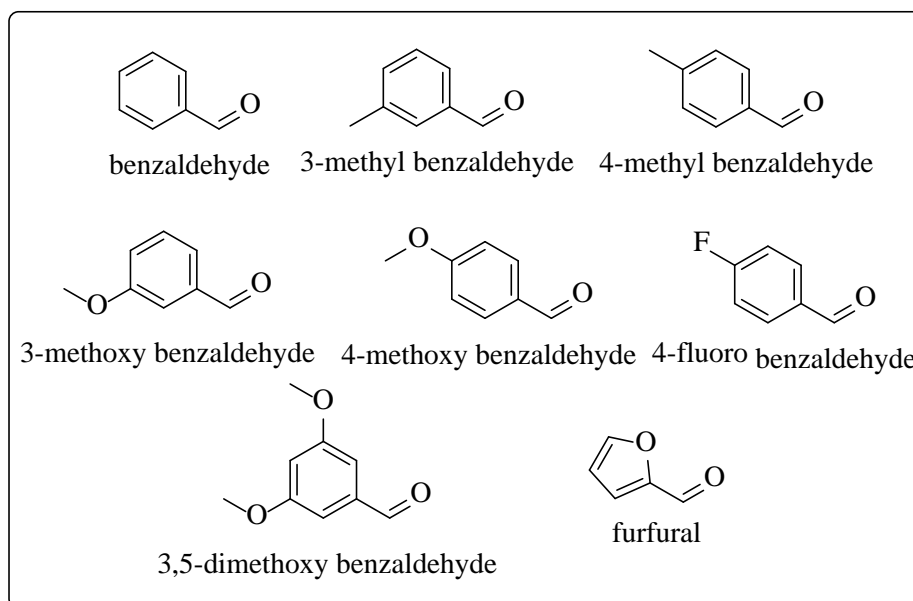
Figure 18. Listed aromatic aldehydes were tried with precatalyst (3-ethyl-5-(2-hydroxyethyl)-4-methylthiazol-3-ium bromide) in procedure 2.

3. 23 mg (0.05 mmol = 10 mol%) ThDP was dissolved in 1mL dry THF. After stirring, 0.5 mmol aromatic aldehyde and 1.5 mmol aliphatic aldehyde were added with 0.05 mmol (16.3 mg) Cs_2CO_3. After 15 hour at room temperature, reaction mixture extracted with EtOAc (1mL x 3), dried with $MgSO_4$ and concentrated in vacuo. Instead of acetaldehyde, diethyl (2-oxoethyl) phosphonate was used as an aliphatic aldehyde but in TLC we did not observe the product.

2.8 HPLC conditions of chiral hydroxyphosphonates

2.8.1 (2-Hydroxy-3-oxo-3-phenylpropyl) phosphonate (BHP-1)

Enantiomerically enriched chiral (2-hydroxy-3-oxo-3-phenylpropyl) phosphonate was obtained in 79% ee. Enantiomeric purity was determined by chiral HPLC analysis (AD-H column, hexane/i-PrOH gradient from 95:5 up to 70:30, flow rate 0.5 mL/min, λ= 254 nm) t_R = 33.5 min (major enantiomer), t_R = 27.3 min (minor enantiomer).

2.8.2 (2-Hydroxy-3-oxo-3-(*m*-tolyl)propyl) phosphonate (BHP-2)

Enantiomerically enriched chiral (2-hydroxy-3-oxo-3-(*m*-tolyl)propyl)phosphonate was obtained in 94% ee. Enantiomeric purity was determined by chiral HPLC analysis (AD-H column, hexane/*i*-PrOH gradient from 95:5 up to 70:30, flow rate 0.5 mL/min, λ= 254 nm) t_R = 34.3 min (major enantiomer), t_R = 32.3 min (minor enantiomer).

2.8.3 (2-Hydroxy-3-oxo-3-(*p*-tolyl)propyl) phosphonate (BHP-3)

Enantiomerically enriched chiral (2-hydroxy-3-oxo-3-(*p*-tolyl)propyl) phosphonate was obtained in 98% ee. Enantiomeric purity was determined by chiral HPLC analysis (AD-H column, hexane/*i*-PrOH gradient from 95:5 up to 70:30, flow rate 0.5 mL/min, λ= 254 nm) t_R = 32.6 min (major enantiomer), t_R = 29.9 min (minor enantiomer).

2.8.4 (2-Hydroxy-3-(3-methoxyphenyl)-3-oxopropyl) phosphonate (BHP-4)

Enantiomerically enriched chiral (2-hydroxy-3-(3-methoxyphenyl)-3-oxopropyl) phosphonate obtained in 80% ee. Enantiomeric purity was determined by chiral HPLC analysis (AD-H column, hexane/*i*-PrOH gradient from 95:5 up to 70:30, flow rate 0.5 mL/min, λ= 254 nm) t_R = 32.4 min (major enantiomer), t_R = 29.8 min (minor enantiomer).

2.8.5 (2-Hydroxy-3-(4-methoxyphenyl)-3-oxopropyl) phosphonate (BHP-5)

Enantiomerically enriched chiral (2-hydroxy-3-(4-methoxyphenyl)-3-oxopropyl) phosphonate obtained in 86% ee. Enantiomeric purity was determined by chiral HPLC analysis (AD-H column, hexane/*i*-PrOH gradient from 95:5 up to 70:30, flow rate 0.5 mL/min, λ= 254 nm) t_R = 35.8 min (major enantiomer), t_R = 27.9 min (minor enantiomer).

2.8.6 (3-(3,5-Dimethoxyphenyl)-2-hydroxy-3-oxopropyl) phosphonate (BHP-6)

Enantiomerically enriched chiral (3-(3,5-dimethoxyphenyl)-2-hydroxy-3-oxopropyl) phosphonate obtained in 87% ee. Enantiomeric purity was determined by chiral HPLC analysis (AD-H column, hexane/i-PrOH gradient from 95:5 up to 70:30, flow rate 0.5 mL/min, λ= 254 nm) t_R = 34.2 min (major enantiomer), t_R = 30.9 min (minor enantiomer).

2.8.7 (2-Hydroxy-3-(4-hydroxyphenyl)-3-oxopropyl) phosphonate (BHP-7)

Enantiomerically enriched chiral ((2-hydroxy-3-(4-hydroxyphenyl)-3-oxopropyl) phosphonate obtained in 98% ee. Enantiomeric purity was determined by chiral HPLC analysis (AD-H column, hexane/i-PrOH gradient from 95:5 up to 70:30, flow rate 0.5 mL/min, λ= 254 nm) t_R = 28.2 min (major enantiomer), t_R = 26.5 min (minor enantiomer).

2.8.8 (3-(4-Fluorophenyl)-2-hydroxy-3-oxopropyl) phosphonate (BHP-8)

Enantiomerically enriched chiral (3-(4-fluorophenyl)-2-hydroxy-3-oxopropyl) phosphonate obtained in 95% ee. Enantiomeric purity was determined by chiral HPLC analysis (AD-H column, hexane/i-PrOH gradient from 95:5 up to 70:30, flow rate 0.5 mL/min, λ= 254 nm) t_R = 27.9 min (major enantiomer), t_R = 24.7 min (minor enantiomer).

2.8.9 (3-(Furan-2-yl)-2-hydroxy-3-oxopropyl) phosphonate (BHP-9)

Enantiomerically enriched chiral (3-(4-fluorophenyl)-2-hydroxy-3-oxopropyl) phosphonate obtained in 94% ee. Enantiomeric purity was determined by chiral HPLC analysis (AD-H column, hexane/i-PrOH gradient from 95:5 up to 70:30, flow rate 0.5 mL/min, λ= 254 nm) t_R = 30.9 min (major enantiomer), t_R = 30.1 min (minor enantiomer).

2.8.10 Diethyl (2,2-diethoxyethyl) phosphonate

Diethyl (2,2-diethoxyethyl) phosphonate HPLC analysis was obtained with (AD-H column, hexane/i-PrOH gradient from 95:5 up to 70:30, flow rate 0.5 mL/min, λ= 254 nm) t_R = 5.6, 7.0, 8.6 min

2.8.11 Diethyl (2-oxoethyl) phosphonate

Diethyl (2-oxoethyl) phosphonate HPLC analysis was obtained with (AD-H column, hexane/i-PrOH gradient from 95:5 up to 70:30, flow rate 0.5 mL/min, λ= 254 nm) t_R = 7.2, 7.9, 8.6 min

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Asymmetric synthesis of β-hydroxy phosphonates via benzaldehyde lyase

According to literature, syntheses of asymmetric hydroxy phosphonates have not been performed by enzymatic C-C bond formation. Therefore, this study both widens the scope of hydroxy phosphonate synthesis methodologies (which are biocatalytic reduction of keto phosphonates, enantioselective acylation of rasemic hydroxy phosphonates, enantioselective hydrolysis of epoxyethylphosphonates) and also makes addition to the diversity of aliphatic substrates that benzaldehyde lyase catalyzes. The listed type of stereoselective 2-hydroxyketones have not been synthesized until now. In this study, chiral beta hydroxy phosphonates were catalyzed by benzaldehyde lyase which is shown in Figure 17.
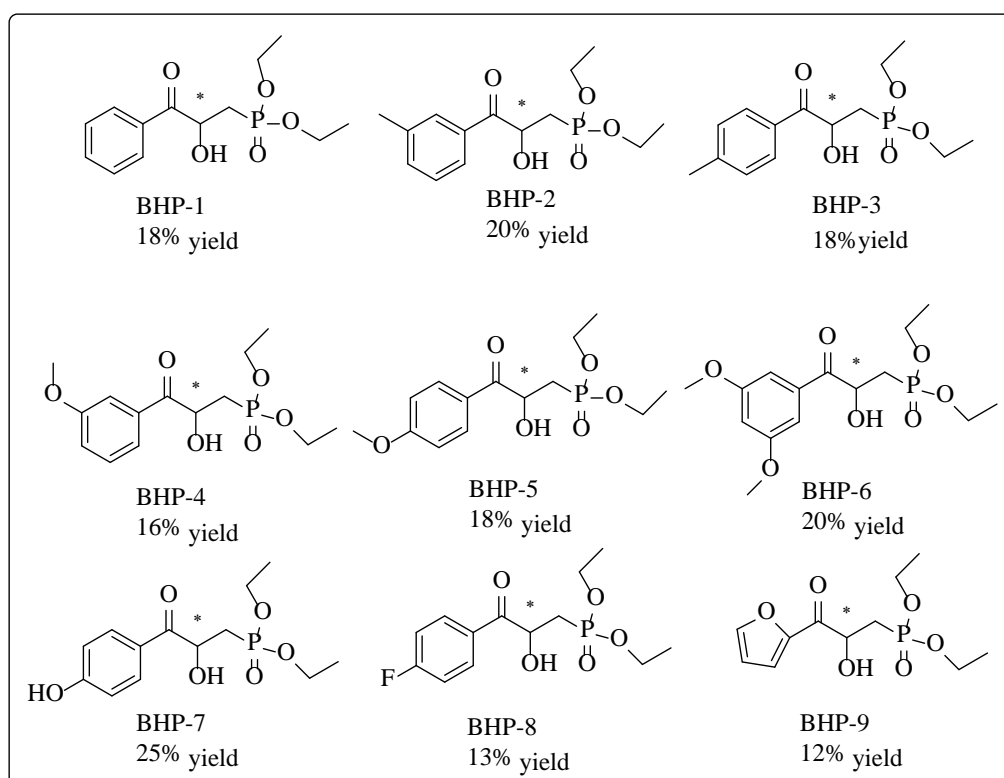
Figure 17. Synthesized β-hydroxy phosphonates.

As it was shown in Figure 17, β-hydroxy phosphonates were obtained in low yields (12-25% yield) because these products are side products of ligation reactions of benzaldehyde lyase. According to literature, the basic carboligation of an aromatic and an aliphatic aldehyde, which is starting from benzaldehyde and acetaldehyde, four different products was investigated, each of them stereoselective in the (R)- or (S)-configuration [45]. Following the procedure, carboligation of benzaldehyde derivatives with diethyl (2-oxoethyl) phosphonate, given in experimental part, we assumed that four different products can occur, each in the R- or S- configuration. (Scheme 25 shows the possible products of the aromatic-aliphatic cross coupling reaction).
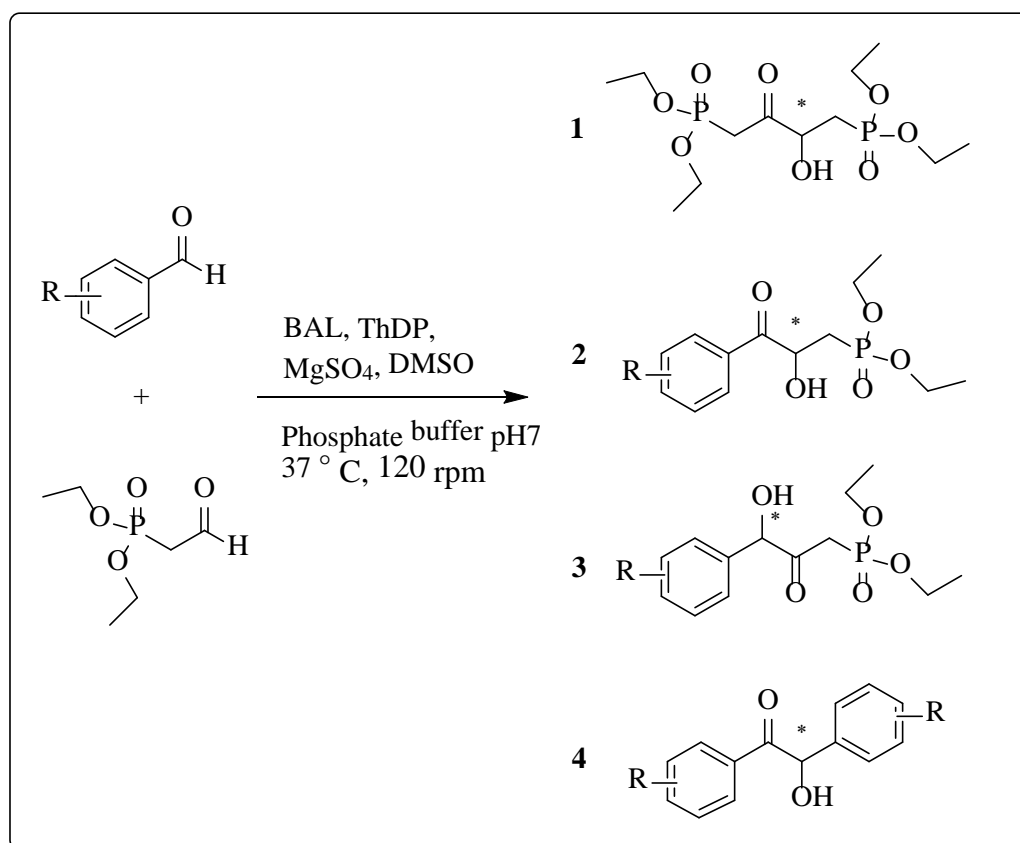
Scheme 25. The four possible different enantiomeric products derived from the carboligation of diethyl (2-oxoethyl) phosphonate and benzaldehyde derivatives.

Product **1** is obtained by the self-ligation of diethyl (2-oxoethyl) phosphonate while benzoin derivatives, product **4**, results from the coupling of two benzaldehyde molecules.

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If a mixed product can appear it can be obtained from diethyl (2-oxoethyl) phosphonate as the donor and benzaldehyde derivatives as the acceptor product **3**, or product **2** resulting from benzaldehyde as the donor and diethyl (2-oxoethyl) phosphonate as the acceptor. The chemoselectivity of ThDP-enzymes depends on the binding order of the substrates in the active site was mentioned in the introduction part. If the benzaldehyde is bound to the C2 atom of ThDP, it functions as the donor aldehyde and the aliphatic acetaldehyde as the acceptor aldehyde yielding according to this, benzaldehyde lyase shows parallel type of chemoselectivity and product **2** (minor product) and **4** (major product) were shown. Since benzaldehyde derivatives are more electrophilic due to their π excessive aromatic rings, product **4** was obtained as a major and in high yield (~60%).
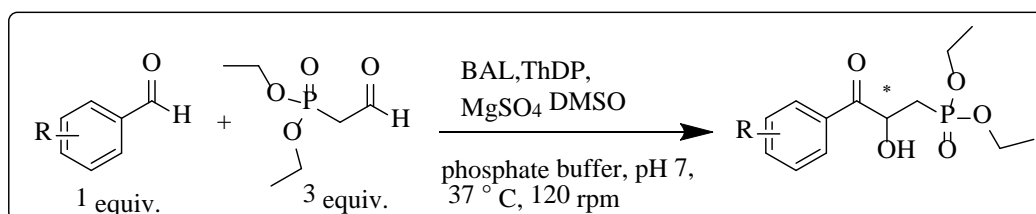

Scheme 26. General procedure for the aromatic-aliphatic cross coupling of aldehydes with benzaldehyde.

According to benzaldehyde lyase catalyzed carboligation reaction general procedure, dimethyl sulfoxide (DMSO) as a solvent was shown to be extremely useful; it provides solubility of the aromatic compounds and the stability of BAL during biotransformations. Alternatively, diisopropylether was also used as a solvent to permit a simplified work-up procedure but it decreases the yield of product. Additionally, temperature, pH, buffer salt and cofactors have been studied; according to literature, enzyme is stable between pH 6 and 8. 37 °C is the optimum reaction temperature. Potassium phosphate was determinated as optimal reaction buffers. For stabilization of BAL, Mg^{2+} as a cofactor is bound non-covalently in the active site (hydrophobic and ionic interactions between protein side chains and the thiazol- and pyrimidine ring of ThDP). Aromatic donor substrates of this ligation reactions were tried very large range substituted in ortho-, meta- and para- position.

Besides this, aliphatic ones were tried from mono-acetaldehyde, dimethoxyacetaldehyde to formaldehyde. The presence of additional methyl groups

nearby aldehyde group is noted as a higher steric hindrance for the enzymes and decreases the reaction rate. However aliphatic aldehydes are both suitable donors and acceptors as far as the steric hindrance is not too high [59]. Until now, benzoin (benzaldehyde derivatives are donor and acceptor) and hydroxypropiophenone derivatives (benzaldehyde donor, aliphatic aldehyde acceptor) are the products of the chiral acyloins were produced by BAL catalysis. However, by using diethyl (2-oxoethyl) phosphonate we have been able to enlarge the product range towards highly substituted hydroxyphosphonates.

According to proposed mechanism for BAL-catalyzed acyloin formation and cleavage [45], Scheme 26 is suggested mechanism for synthesis of β-hydroxy phosphonates.
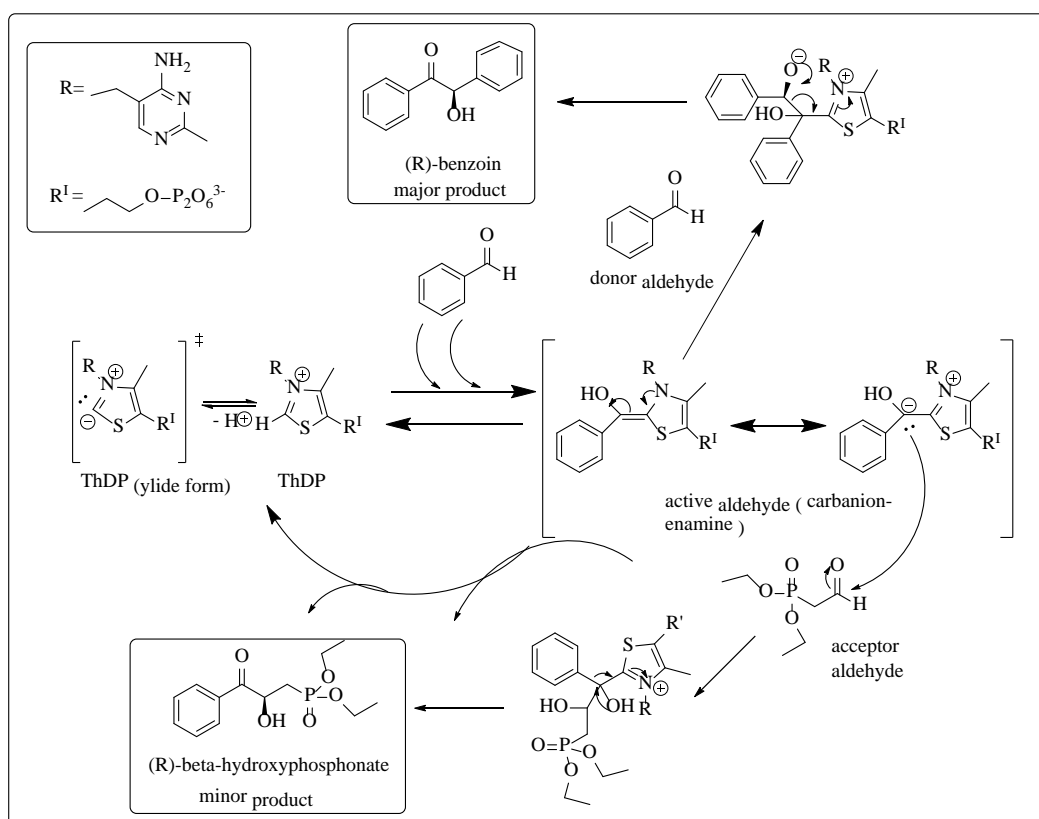

Scheme 27. Suggested mechanism for the synthesis of hydroxyphosphonates.

C2-carbon of the thiazolium ring ThDP is activated by deprotonation to form nucleophilic ylide. Glutamate residue present in ThDP dependent enzymes which supports the formation of a 1,4-imino tautomer in the pyrimidine ring. Nitrogen part

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of the pyrimidine ring is highly electrophilic and carboligation reaction occured by attacking ylide to benzaldehyde. As a result of carbanion-enamine intermeditate which is active aldehyde attacks to the acceptor aldehyde and forms hydroxy phosphonates. Catalytic cycle is completed with the release of formed acyloin, hydroxy phosphonate and renewal of ylide.

^{1}H and ^{13}C NMR spectra of synthesized β-hydroxy phosphonates which are not known in the literature are shown in Appendix A. To be sure our NMR, hydrogen atom **6** in beta position of hydroxy phosphonate reveals a specialty at 5.3 ppm as a multiplet which is shown in Figure 20.
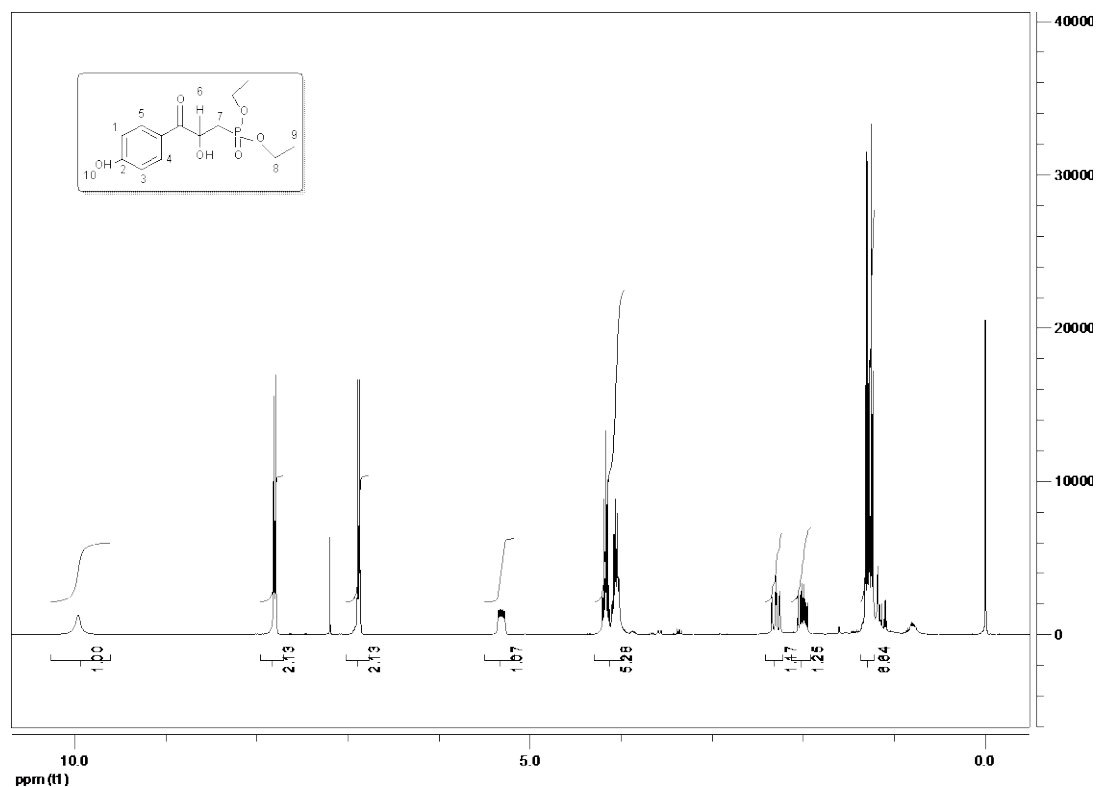
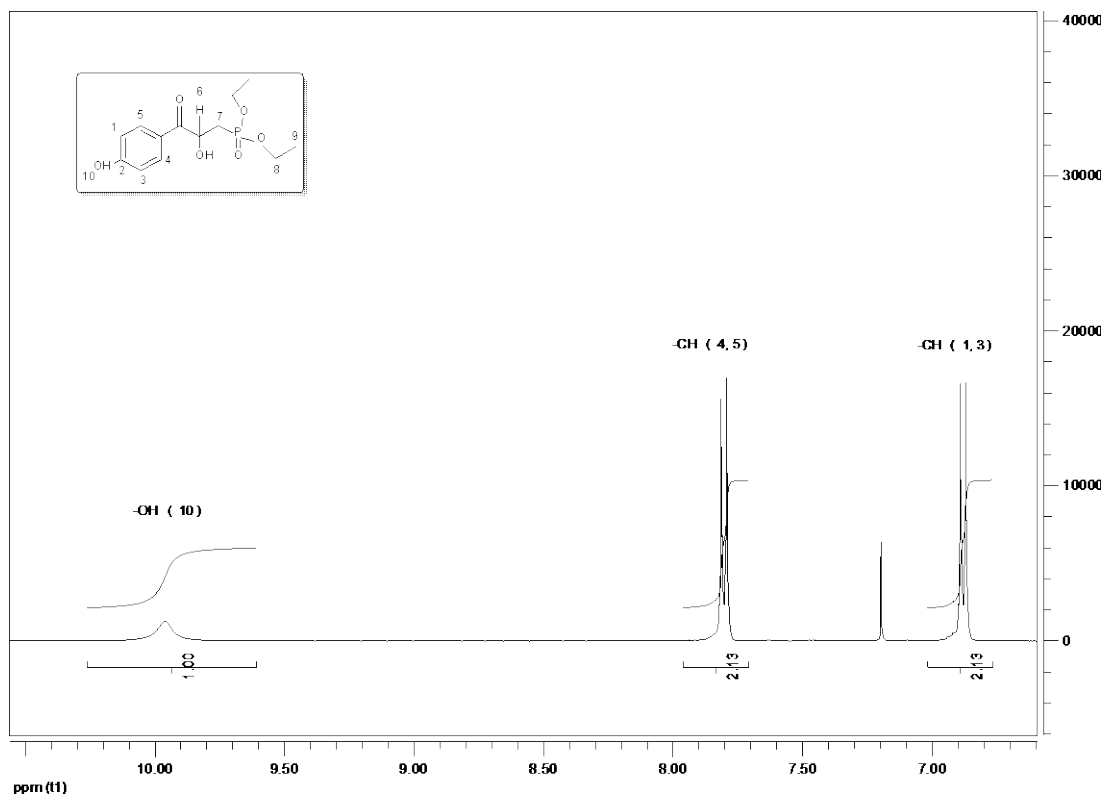**Figure 18.** ^{1}H NMR of the 4-hydroxy benzaldehyde linked beta hydroxyphosphonates (**BHP-7**).

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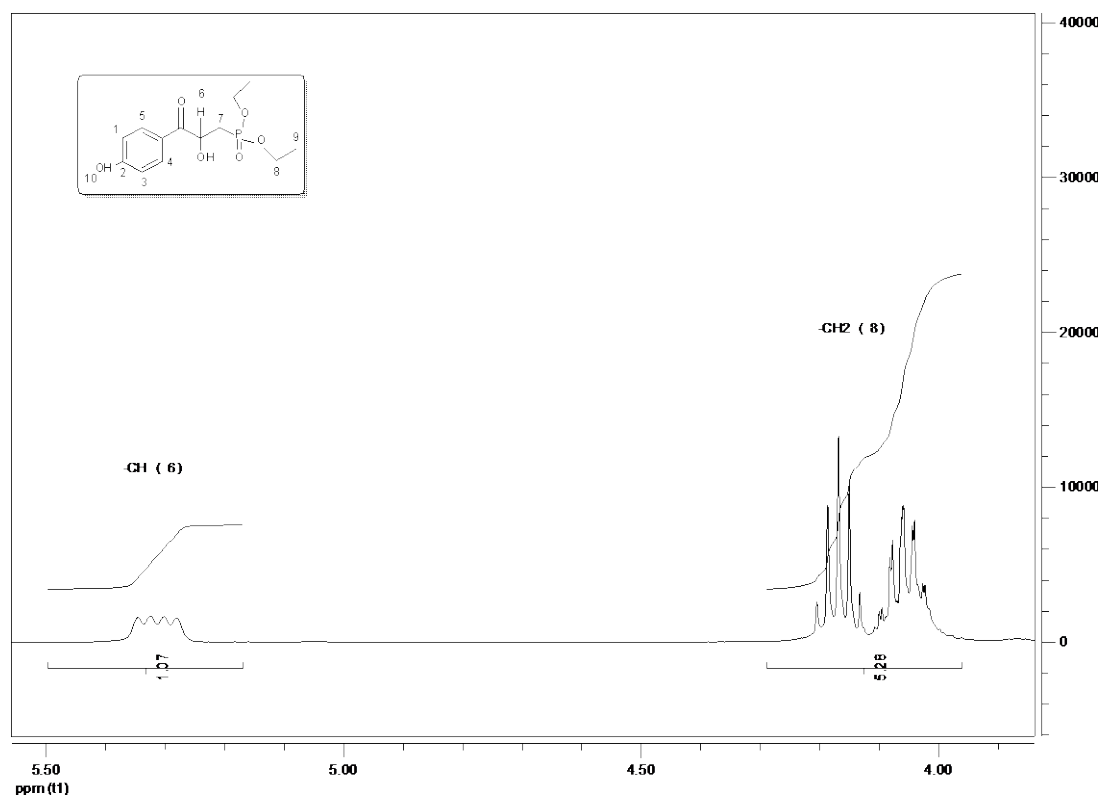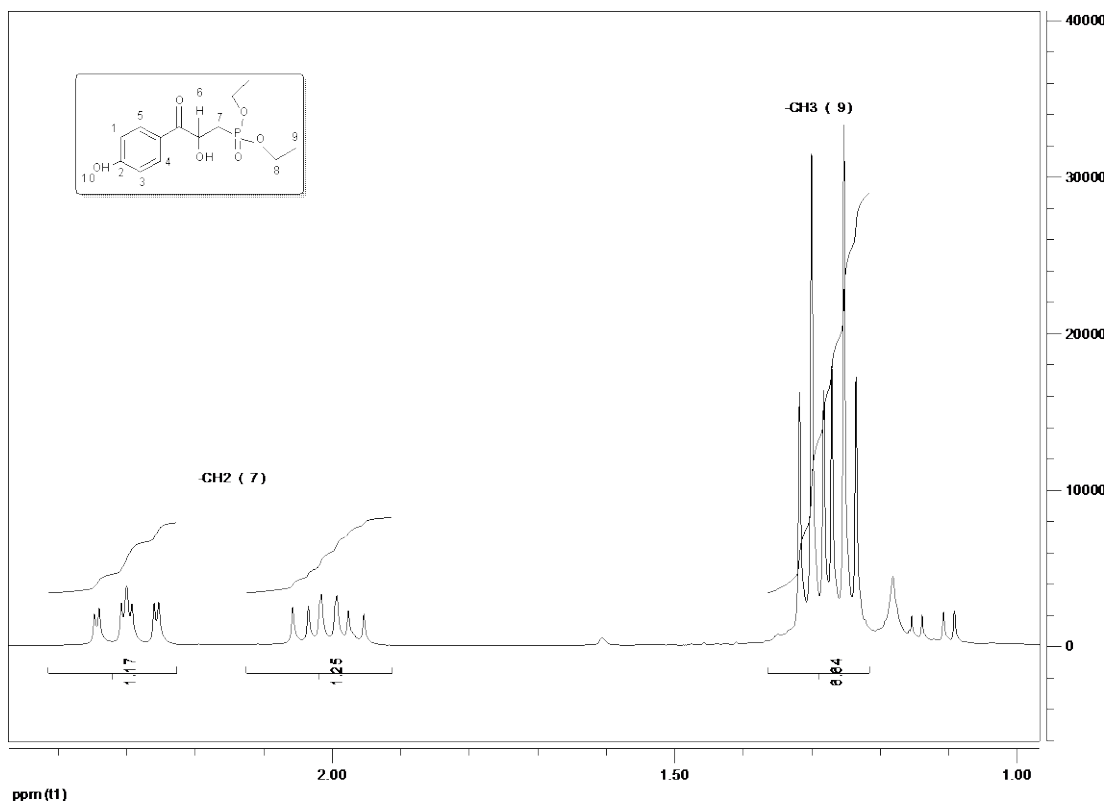**Figure 19.** Part of ^{1}H NMR for the beta-hydroxy phosphonate (**BHP-7**).

Figure 20. Part of ^{1}H NMR for the beta-hydroxy phosphonate (**BHP-7**).

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Labeled all hydrogen atoms (**7-8-9**) inside phosphonoacetaldehyde have splited different multiplet due to their diastereotopic properties and since P^{31} chemistry, aliphatic part splited multiplet which are shown in Appendix A.


Figure 21. Part of ^{1}H NMR for the beta-hydroxy phosphonates (**BHP-7**)

For instance, hydrogen protons labelled as 7, (-CH$_2$), shows different coupling at 2.1 ppm and 2.3 ppm as a multiplet. Various derivatives of benzaldehyde with electron-donating and withdrawing groups on meta- and para- positions were used to synthesize hydroxyphoshponates at optimized conditions. The conversions were very low (12%-25%). Beside of minor productivity, lower conversion may be the cause of electronic effect of methoxy group at para- position which decreases the acidity of α-proton more than meta- position.

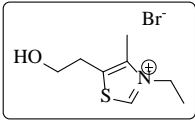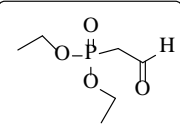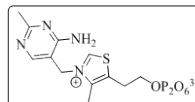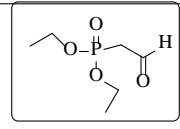## 3.2 Rasemic synthesis of beta hydroxy phosphonates via chemical catalyst

According to literature, Stetter and co-workers pioneered to crossed acyloin condensation in which an aromatic aldehyde was treated with an excess of an aliphatic aldehyde in the presence of triethylamine and a catalytic amount of the thiamine analogue 3-benzyl-5-(2-hydroxyethyl)-4-methylthiazolium chloride [58]. Another example from literature is the crossed coupling of aromatic aldehyde with acetaldehyde in presence of cesium carbonate and a catalytic amount of 3-ethyl-5-(2-hydroxyethyl)-4-methyl-thiazol-3-ium bromide [57]. Two procedures were performed with our aldehydes by changing temperature, amount and type of catalysts and bases. These reactions are listed in the Table 4.
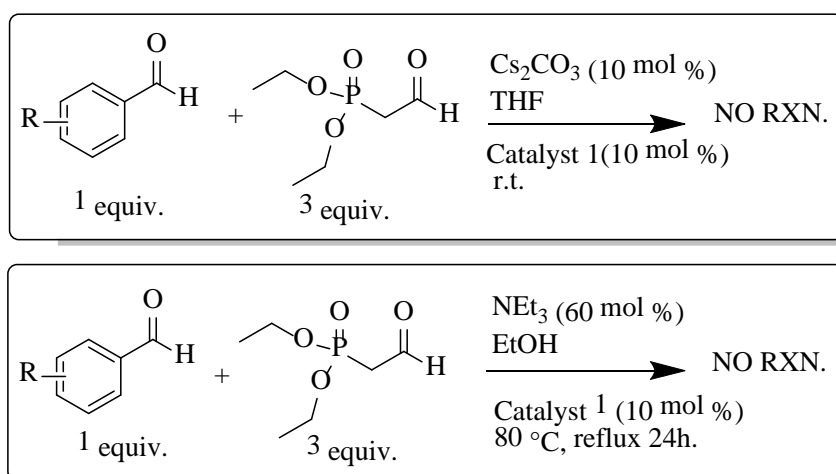

Scheme 28. General scheme for the aromatic-aliphatic acyloin reactions are adapted from literature which are mentioned above.

Table 4. Aromatic-aliphatic coupling reactions are adapted from literature.

| Catalysts	Aromatic Aldehyde (1eqv.)	Aliphatic aldehyde (3eqv.)	Solvent	Base	Temperature
Catalyst 1 (3-ethyl-5-(2-hydroxyethyl)-4-methylthiazol-3-ium bromide)	benzaldehyde		THF	Cs$_2$CO$_3$ 10 mol% 20 mol%	R.T. 40°C 60°C 80°C
	m-tolylaldehyde				
	p-tolylaldehyde				
	m-anisaldehyde	acetaldehyde	EtOH DMSO	NEt$_3$	40°C 60°C 80°C
	p-anisaldehyde				
	p-fluorobenzaldehyde				
Catalyst 2 ThDP	benzaldehyde		THF DMSO	Cs$_2$CO$_3$ 10 mol% 20 mol%	R.T. 40°C 60°C 80°C
	m-tolylaldehyde				
	p-tolylaldehyde	acetaldehyde			

All these acyloin type of reactions were not resulted except when aliphatic part was acetaldehyde we obtained cross coupling product. Aliphatic part (diethyl (2-oxoethyl) phosphonates) of our reaction can be reason of our results. To be sure for aliphatic part, aliphatic-aliphatic coupling (diethyl (2-oxoethyl) phosphonates with acetaldehyde) was performed with catalyst 1 according to reference [57]. As a result of this reaction products were not observed or monitored by TLC (Scheme 29).
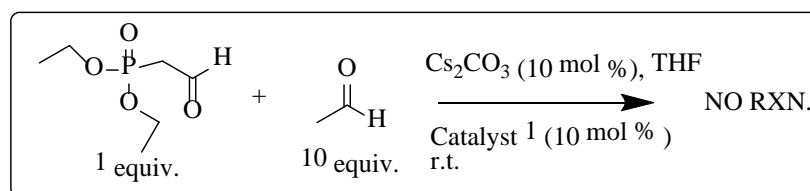
Scheme 29. Aliphatic-aliphatic cross coupling reactions with catalyst 1.

3.3 Determination of HPLC chromatography

High enantiomeric excesses were obtained by HPLC which are listed below Figure 22. Since it was not possible to synthesize racemic products of β-hydroxy phosphonates, enantiomeric excesses were calculated from pure chiral products peaks which were separated with chiral HPLC. Therefore, to obtain enantiomeric excesses, gradient system was used to get a better resolution of peaks with AD-H column, with a 0.5 mL/min flow rate, hexane:isopropanal solvent system from 95:5 to 70:30 in 50 min.
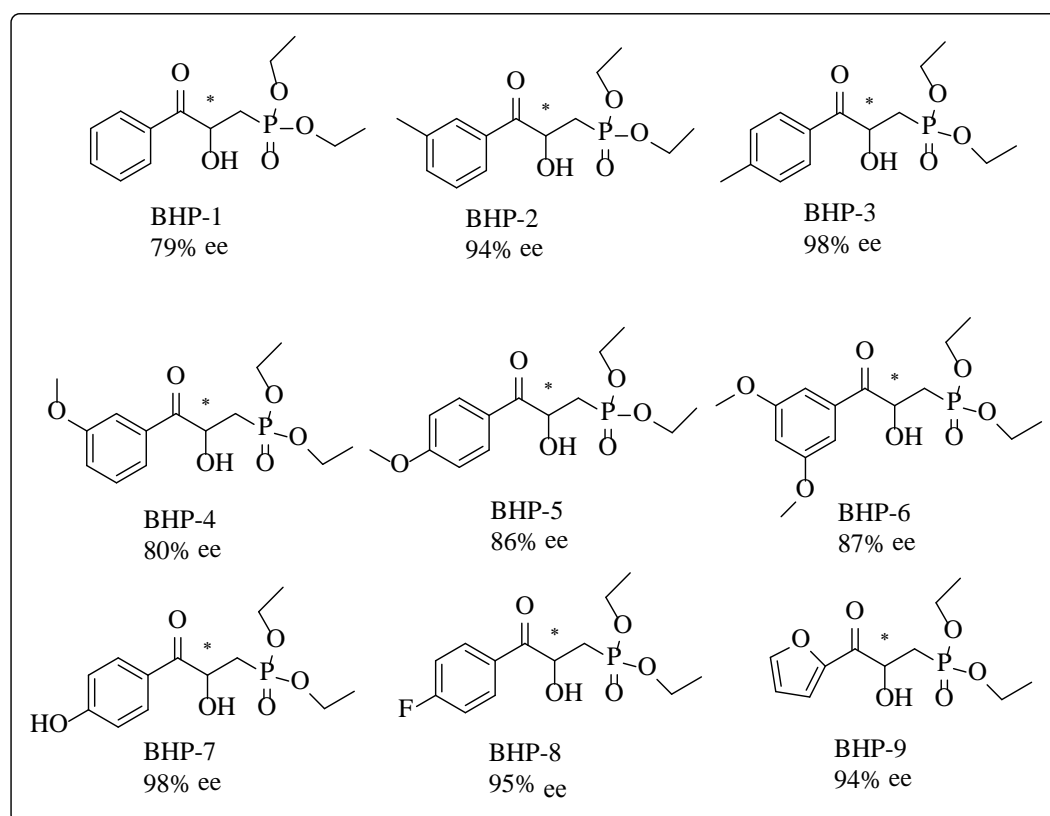
Figure 22. Enantiomeric excesses results monitored by HPLC.

For instance, if we take (2-hydroxy-3-oxo-3-(*m*-tolyl)propyl) phosphonate HPLC chromatogram, we see that without our chiral (2-hydroxy-3-oxo-3-(*m*-tolyl)propyl) phosphonate (94% ee) there are peaks in 10 minutes due to acetal (diethyl (2,2-diethoxyethyl) phosphonates), aldehyde (diethyl (2-oxoethyl) phosphonates) which are shown in Figure 25.
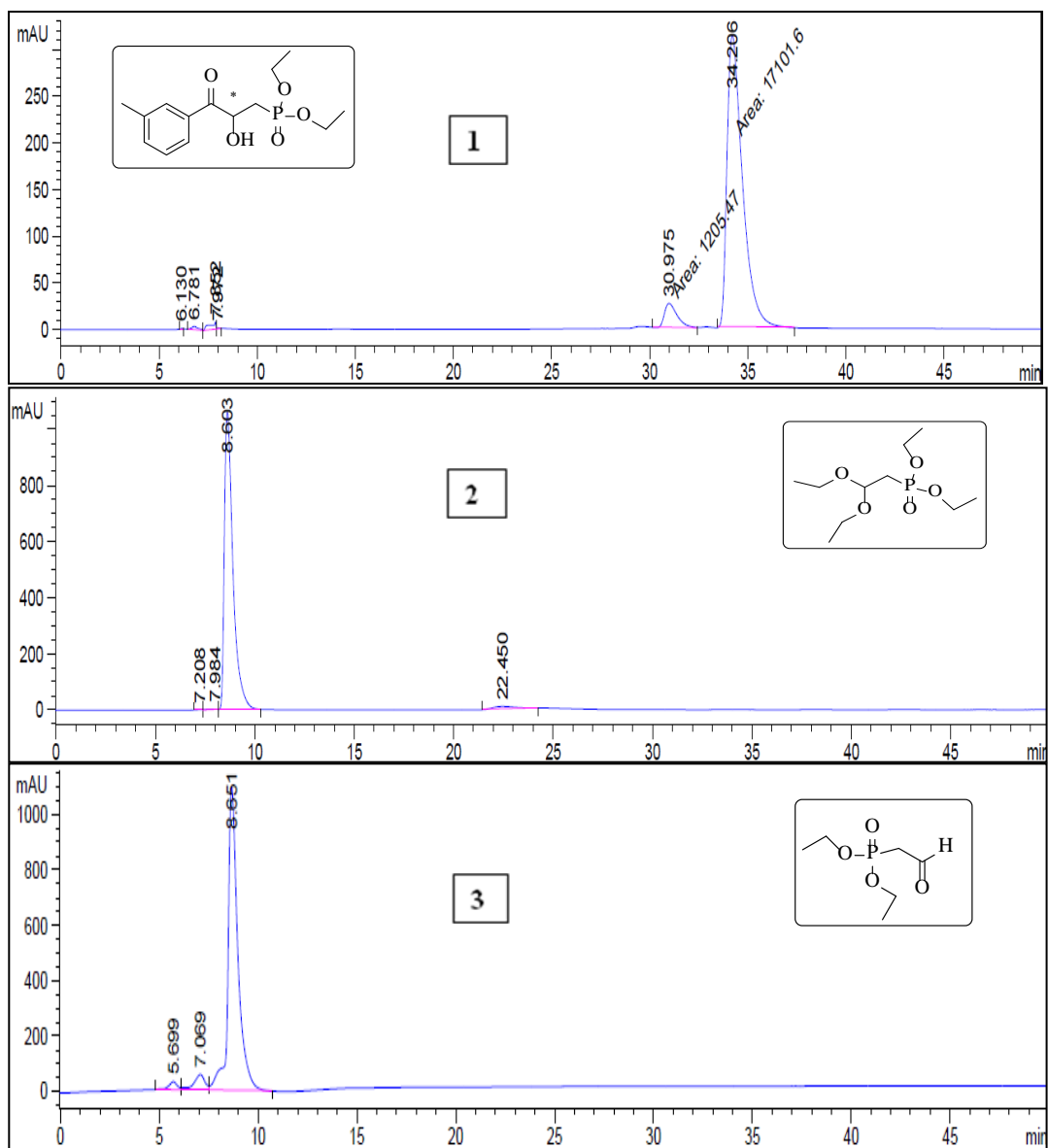
Figure 23. HPLC chromatogram of (2-hydroxy-3-oxo-3-(*m*-tolyl)propyl) phosphonate **1** **(BHP-2)**, diethyl (2,2-diethoxyethyl) phosphonate **2**,(diethyl (2-oxoethyl) phosphonate **3.**

CHAPTER 4

CONCLUSION

In this study asymmetric synthesis of novel β-hydroxy phosphonates with cross acyloin reactions was achieved via benzaldehyde lyase (BAL). Besides, high enantiomeric excesses were obtained up to 98% that many of these hydroxy phosphonates interest attention due to their remarkable biological properties as antibacterial, antiviral and antitumour agents, antibiotics, enzyme inhibitors, amino acid mimetics and pesticides. Therefore, study of the biological activities of hydroxy phosphonates appears favorable for the development of new bioregulators and drugs [51].

Therefore, this study made two different contributions to hydroxy phosphonate synthesis. Firstly, this application can be a preference to chemoenzymatic methods for the synthesis of hydroxy phosphonates. Secondly, these results widen the aliphatic substrate range of benzaldehyde lyase catalyzed reactions.

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NMR DATA
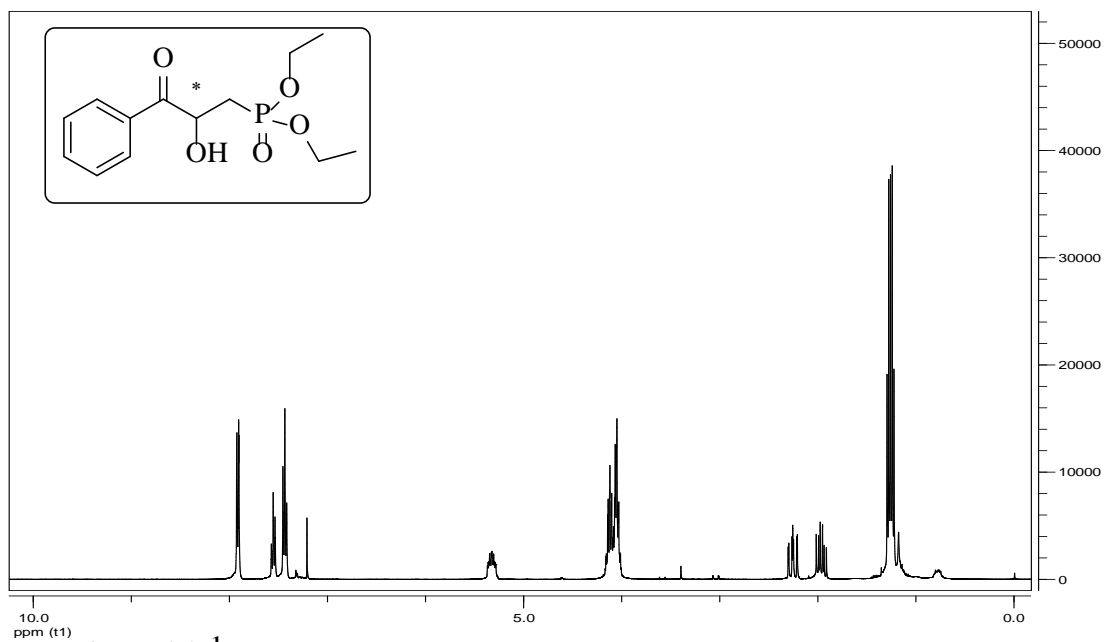
Figure A1. ^{1}H NMR spectrum of (2-hydroxy-3-oxo-3-phenylpropyl) phosphonate (BHP-1).
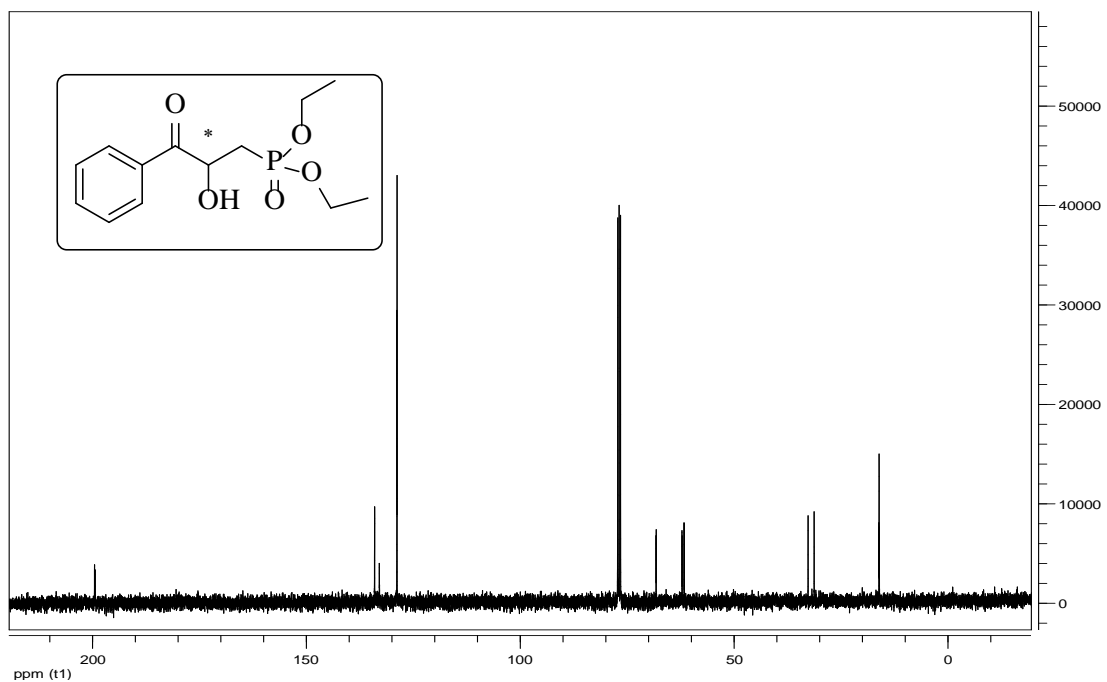
Figure A2. ^{13}C NMR spectrum of (2-hydroxy-3-oxo-3-phenylpropyl) phosphonate (BHP-1)
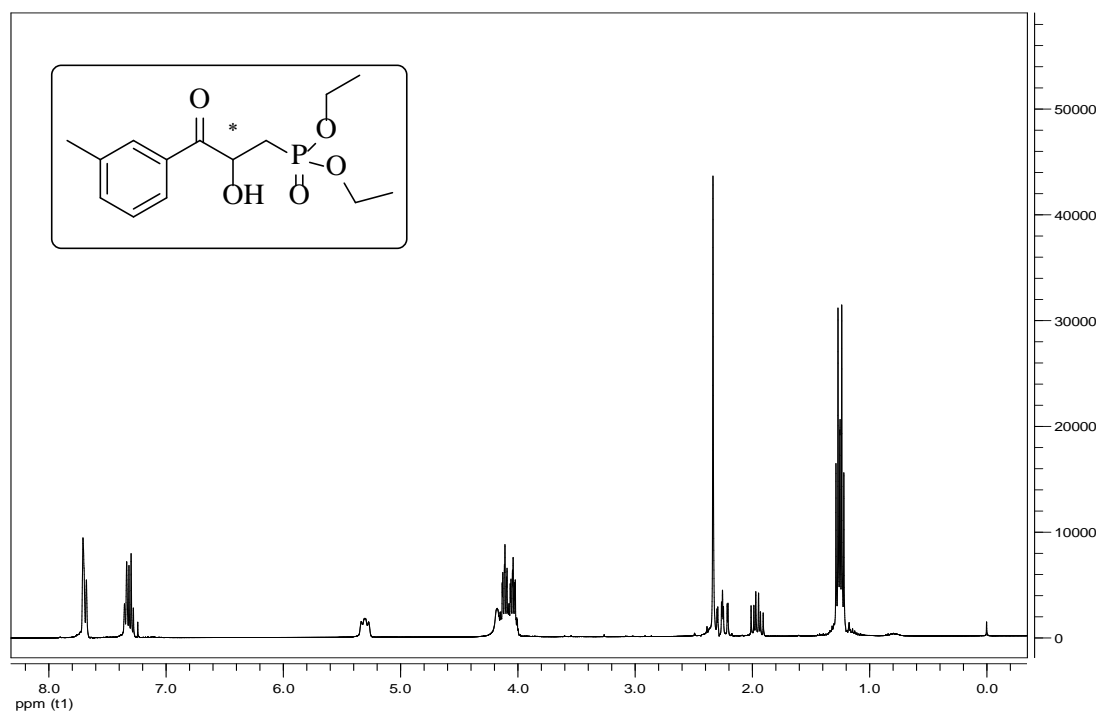
Figure A3. ^{1}H NMR spectrum of (2-hydroxy-3-oxo-3-(m-tolyl)propyl)phosphonate (BHP-2).
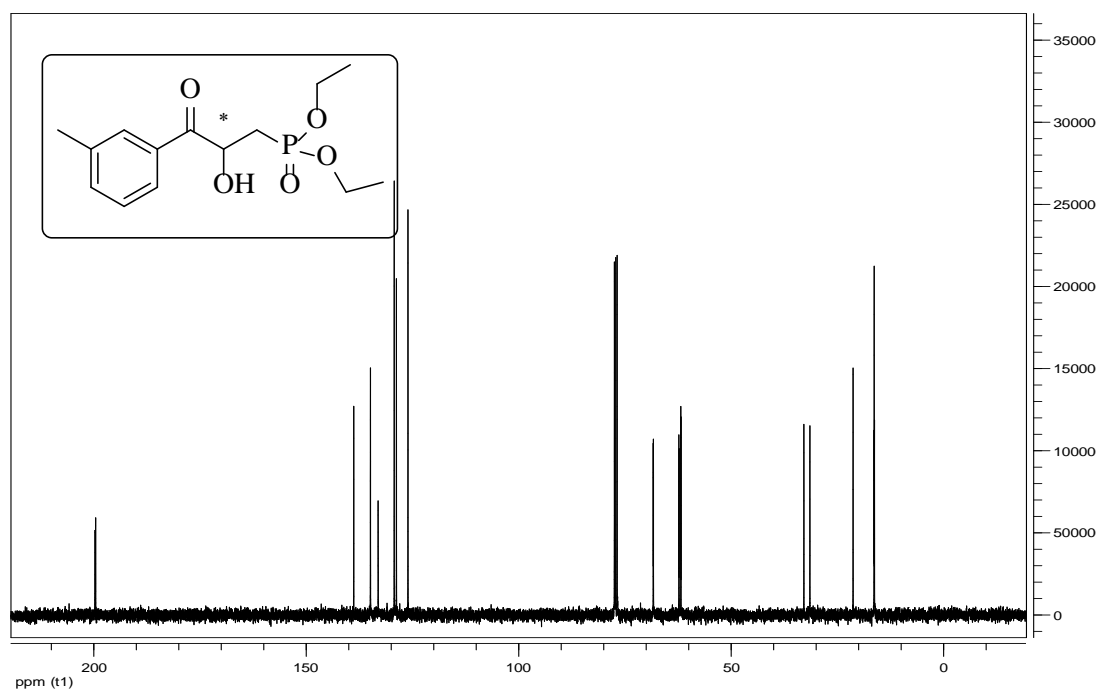
Figure A4. ^{13}C NMR spectrum of (2-hydroxy-3-oxo-3-(m-tolyl)propyl)phosphonate (BHP-2).
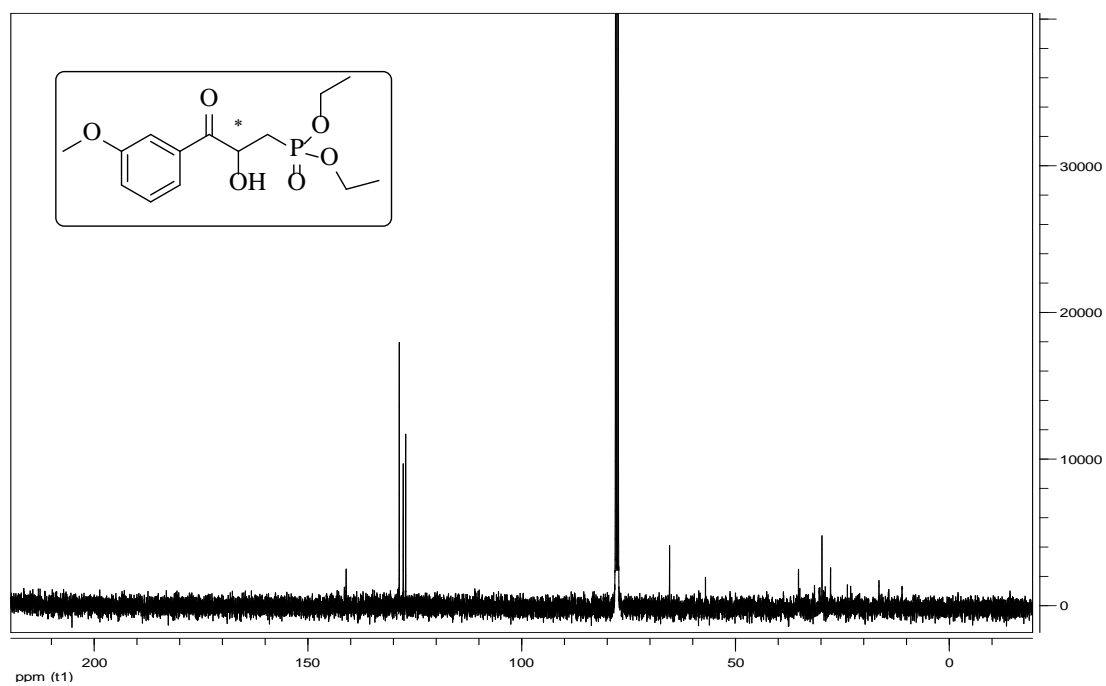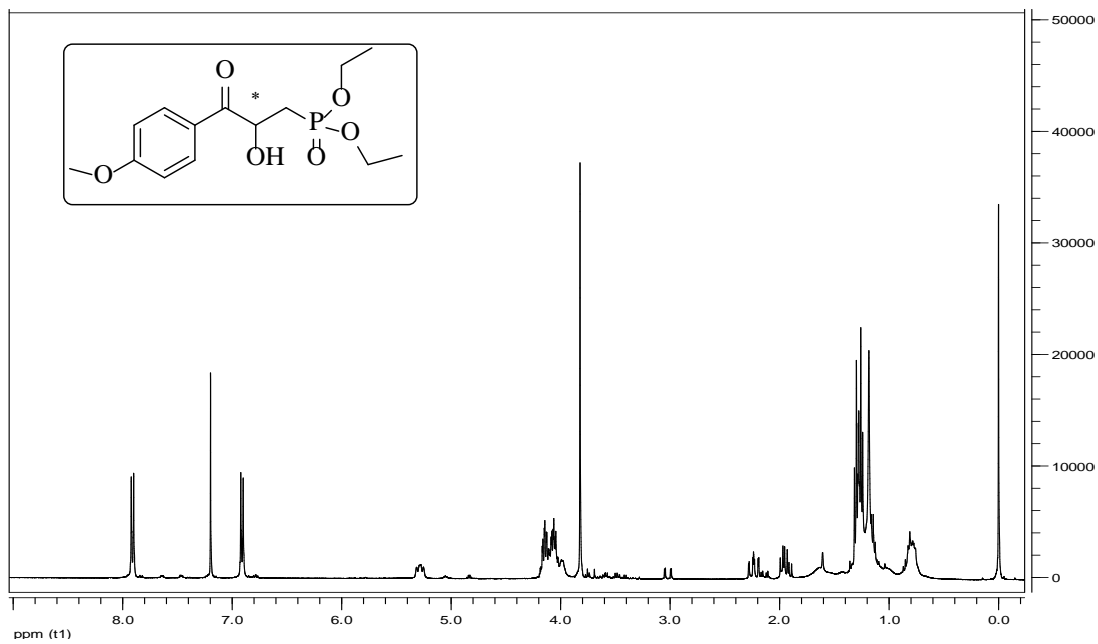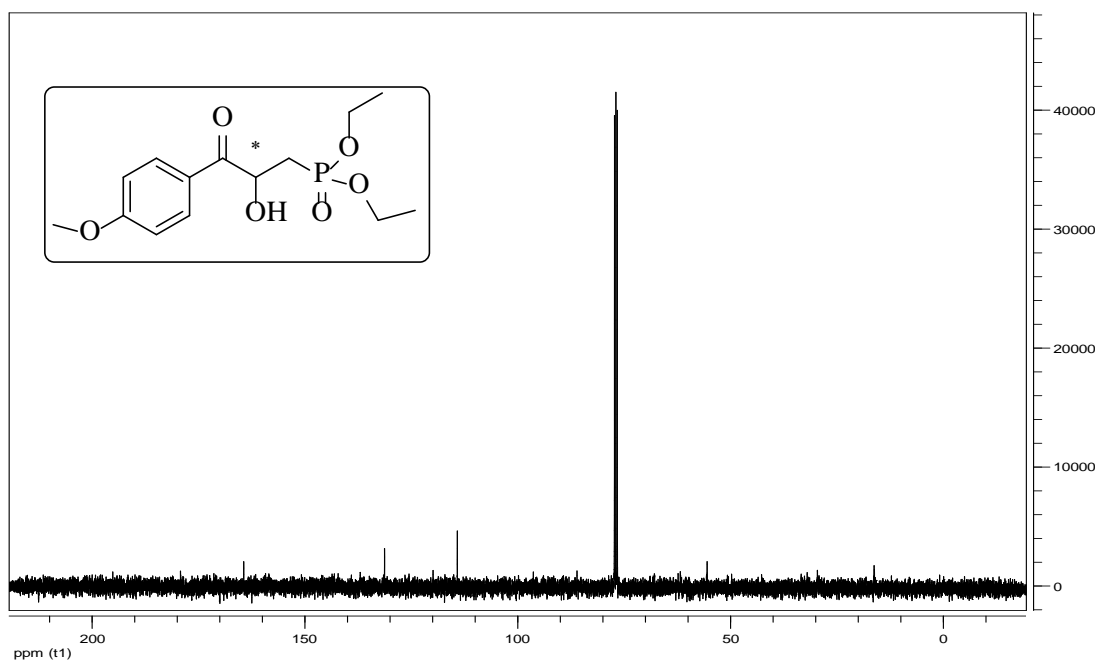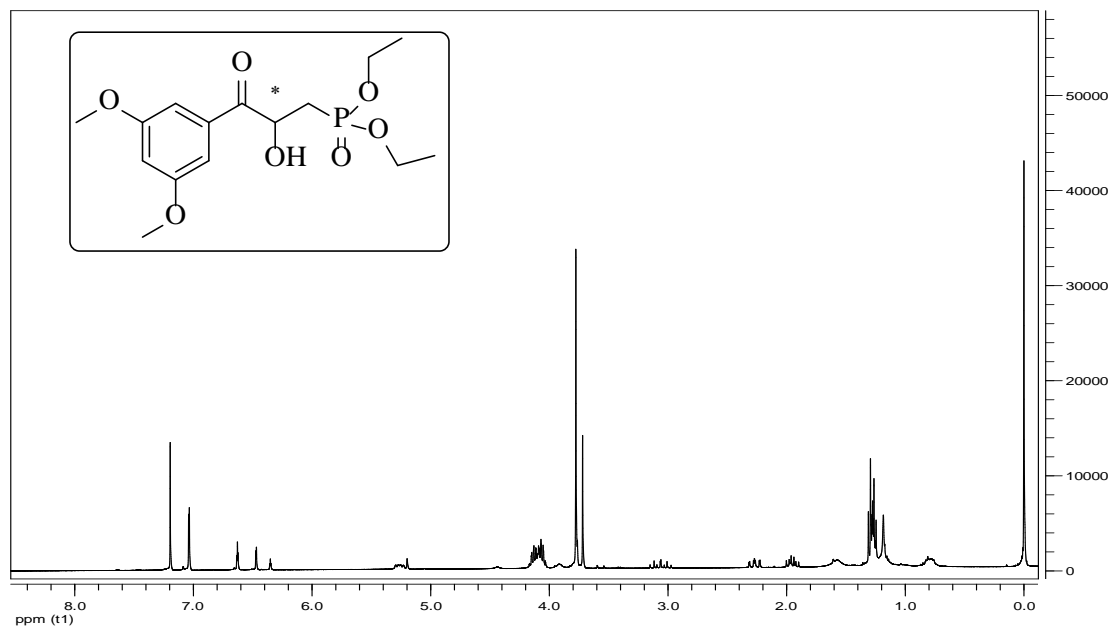
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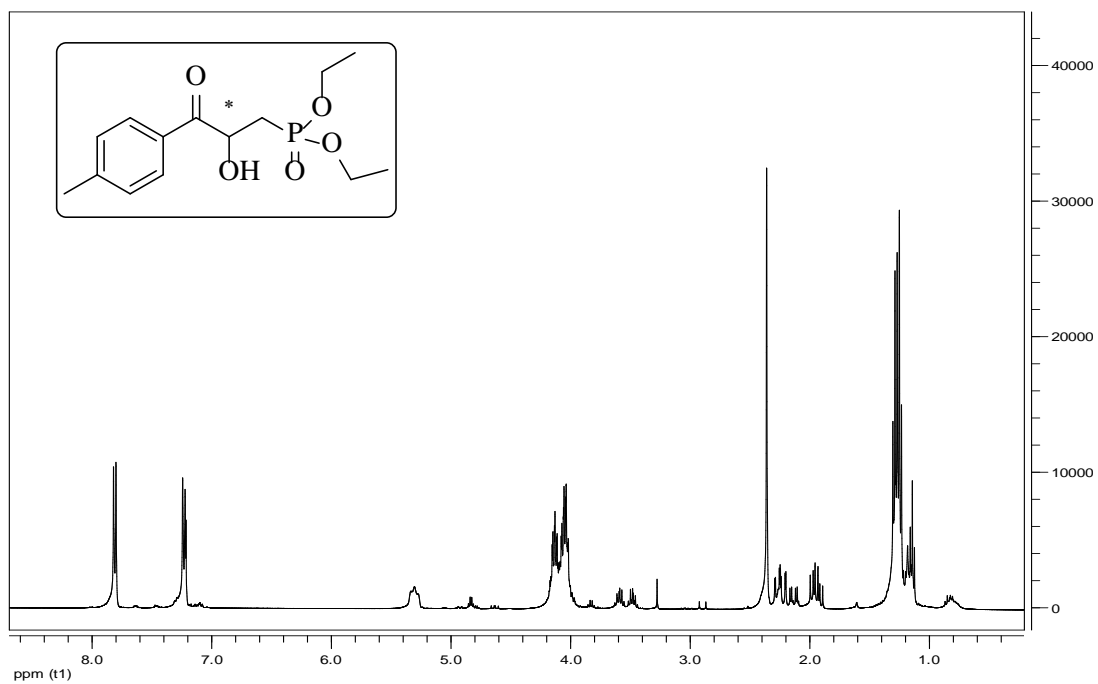
Figure A5. ^{1}H NMR spectrum of (2-hydroxy-3-oxo-3-(*p*-tolyl)propyl) phosphonate (BHP-3).
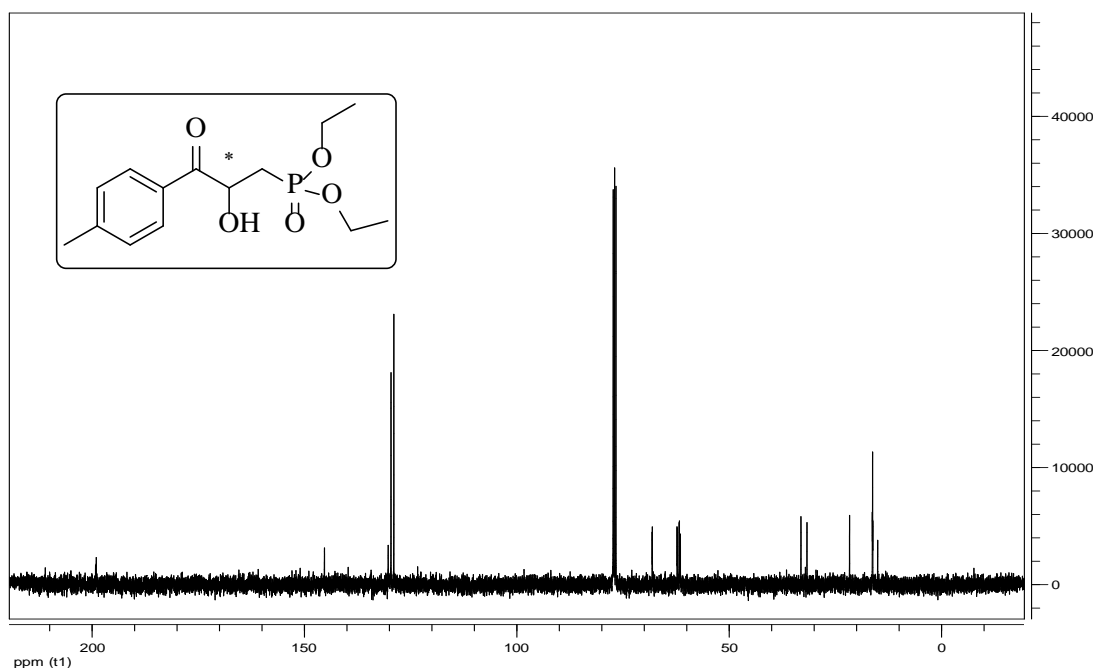
Figure A6. ^{13}C NMR spectrum of (2-hydroxy-3-oxo-3-(*p*-tolyl)propyl) phosphonate (BHP-3).
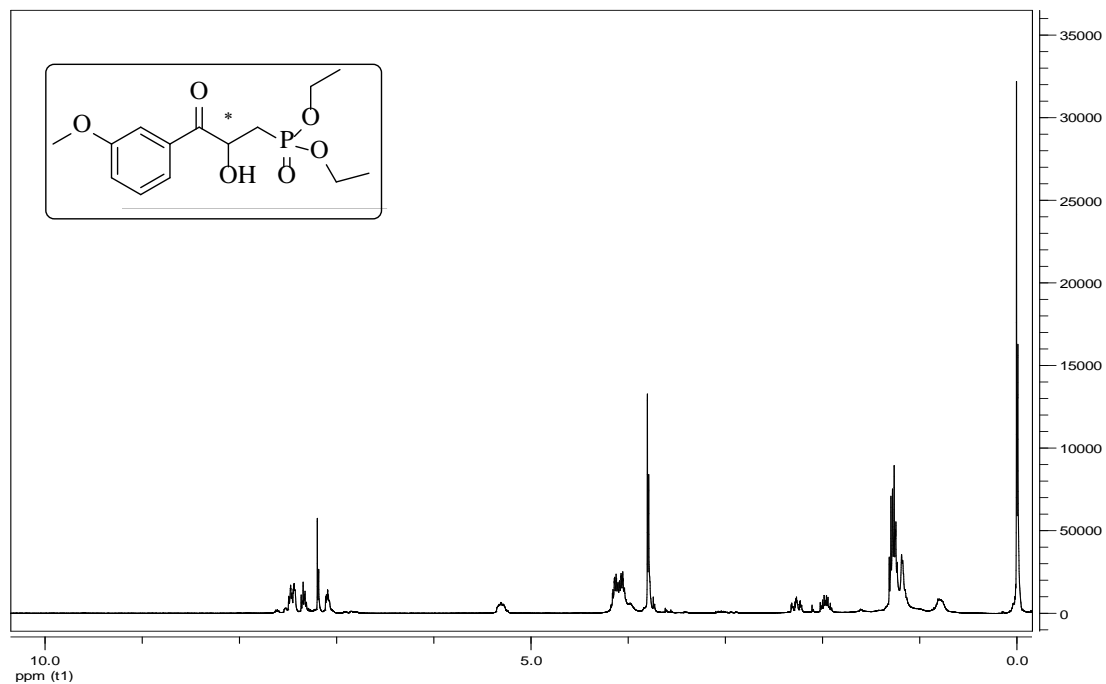
Figure A7. ¹H NMR spectrum of (2-hydroxy-3-(3-methoxyphenyl)-3-oxopropyl) phosphonate (BHP-4).

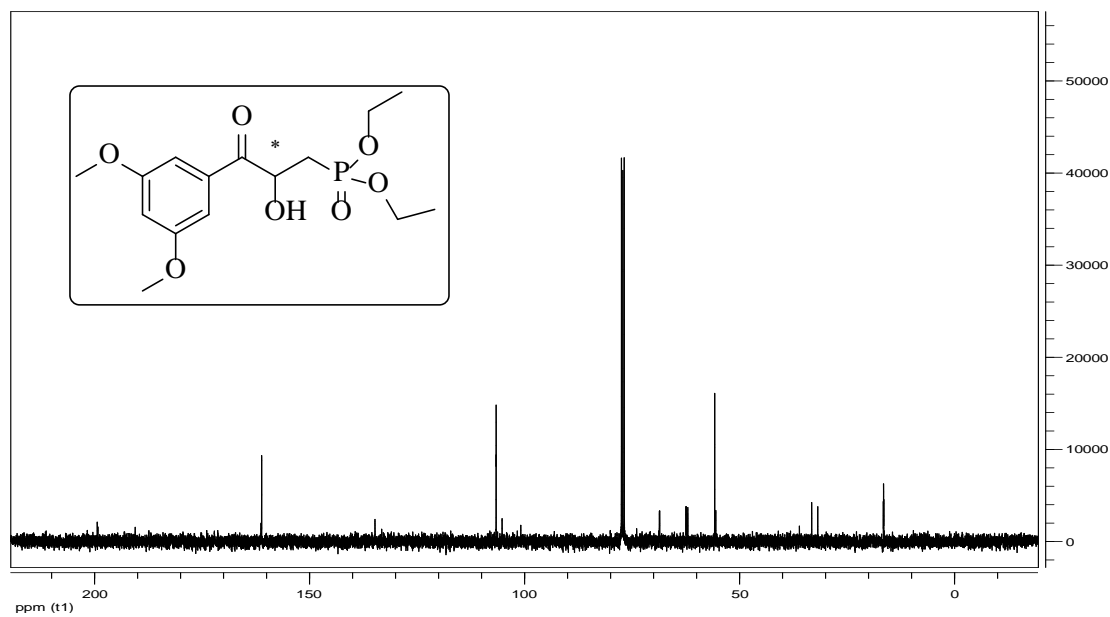**Figure A8.** ¹³C NMR spectrum of (2-hydroxy-3-(3-methoxyphenyl)-3-oxopropyl) phosphonates (BHP-4).

Figure A9. ^{1}H NMR spectrum of (2-hydroxy-3-(4-methoxyphenyl)-3-oxopropyl)
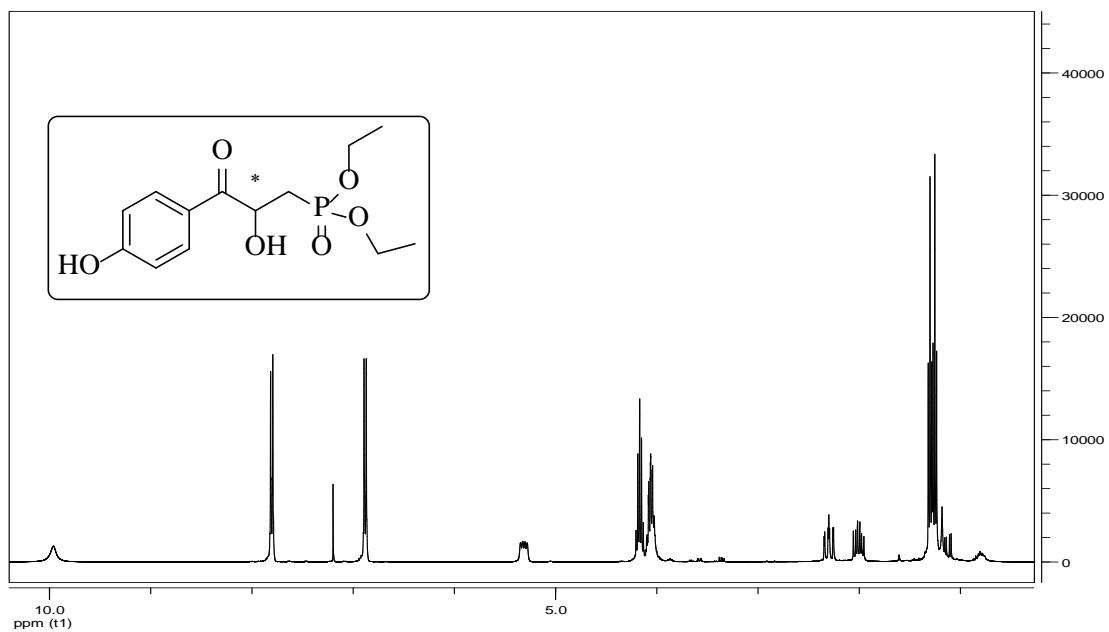phosphonate (BHP-5).

Figure A10. ^{13}C NMR spectrum of (2-hydroxy-3-(4-methoxyphenyl)-3-oxopropyl)
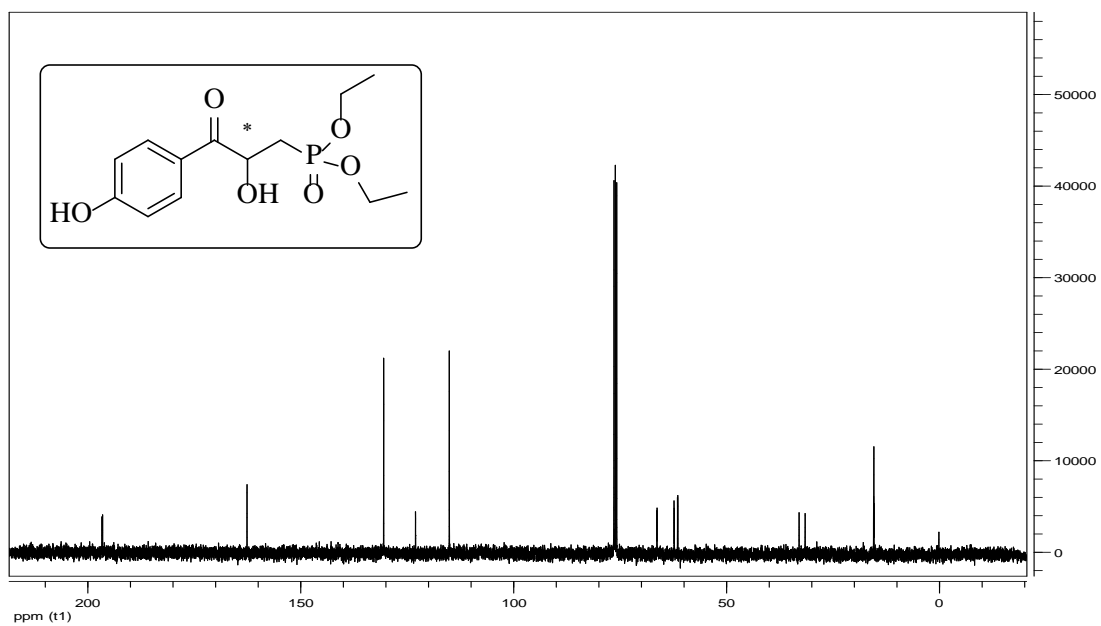phosphonate (BHP-5).

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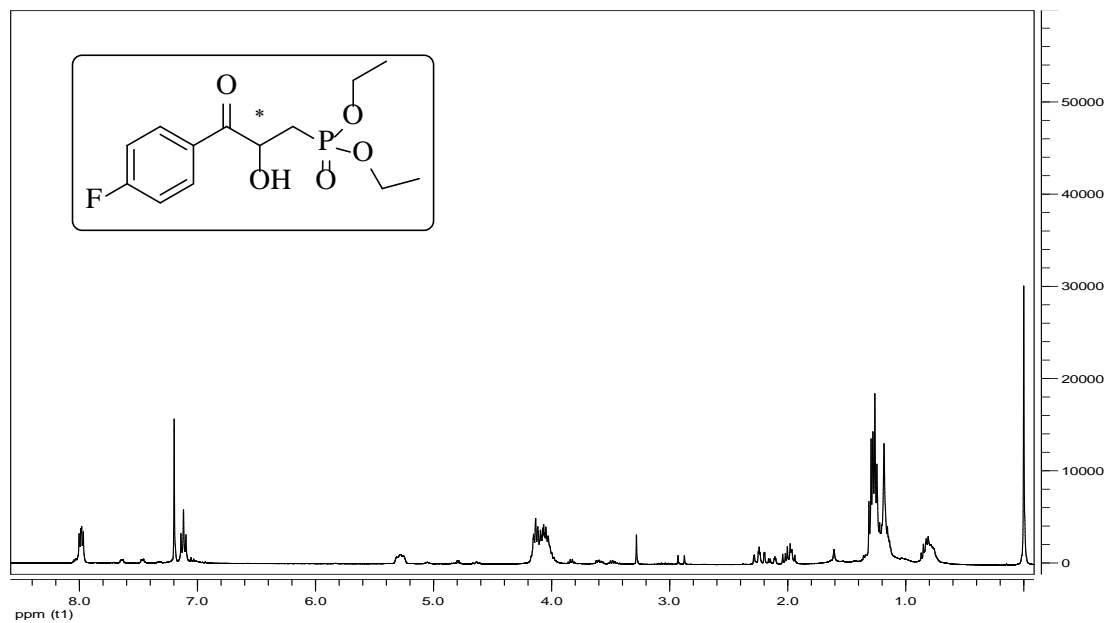**Figure A11.** ^{1}H NMR spectrum of (3-(3,5-dimethoxyphenyl)-2-hydroxy-3-oxopropyl) phosphonate (BHP-6).

Figure A12. ^{13}C NMR spectrum of (3-(3,5-dimethoxyphenyl)-2-hydroxy-3-oxopropyl) phosphonate (BHP-6).
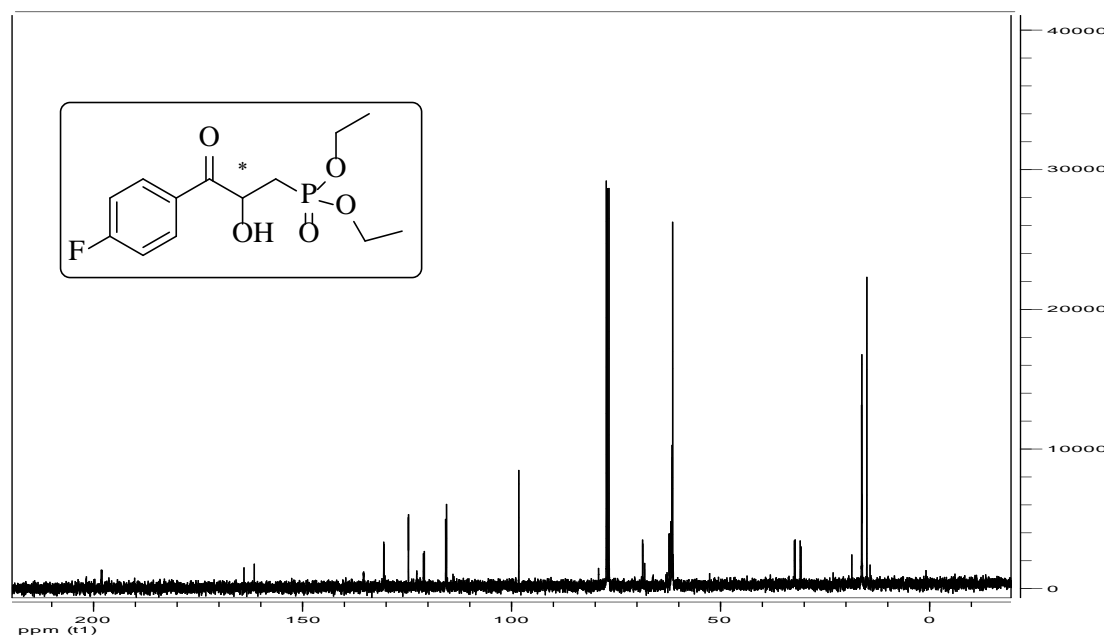
Figure A13. ^{1}H NMR spectrum of (2-hydroxy-3-(4-hydroxyphenyl)-3-oxopropyl) phosphonate (BHP-7).

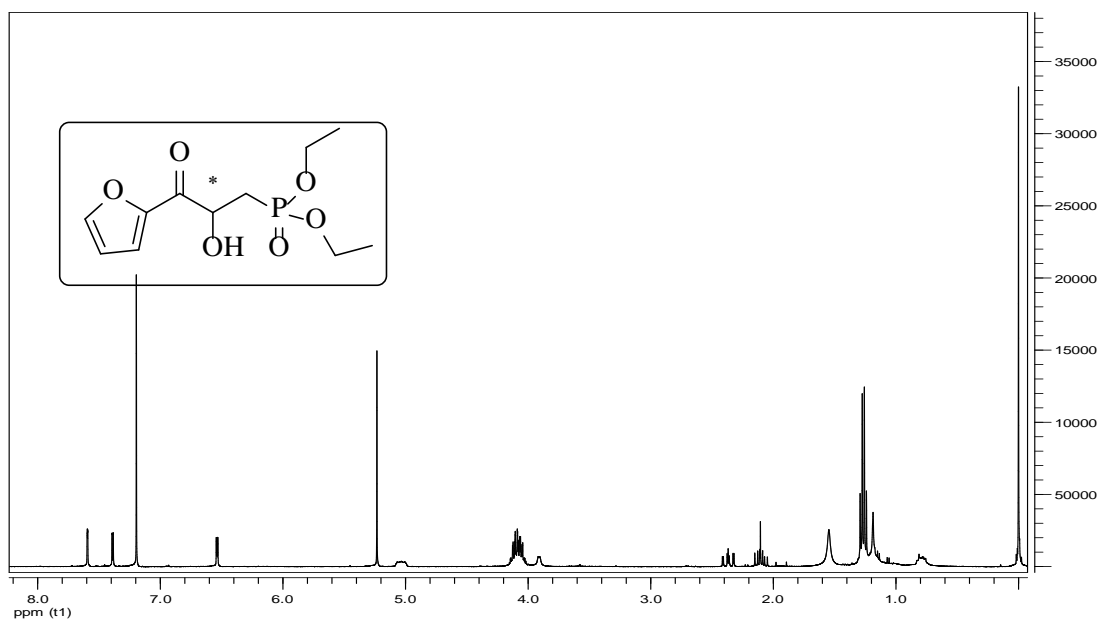**Figure A14.** ^{13}C NMR spectrum of (2-hydroxy-3-(4-hydroxyphenyl)-3-oxopropyl) phosphonate (BHP-7).
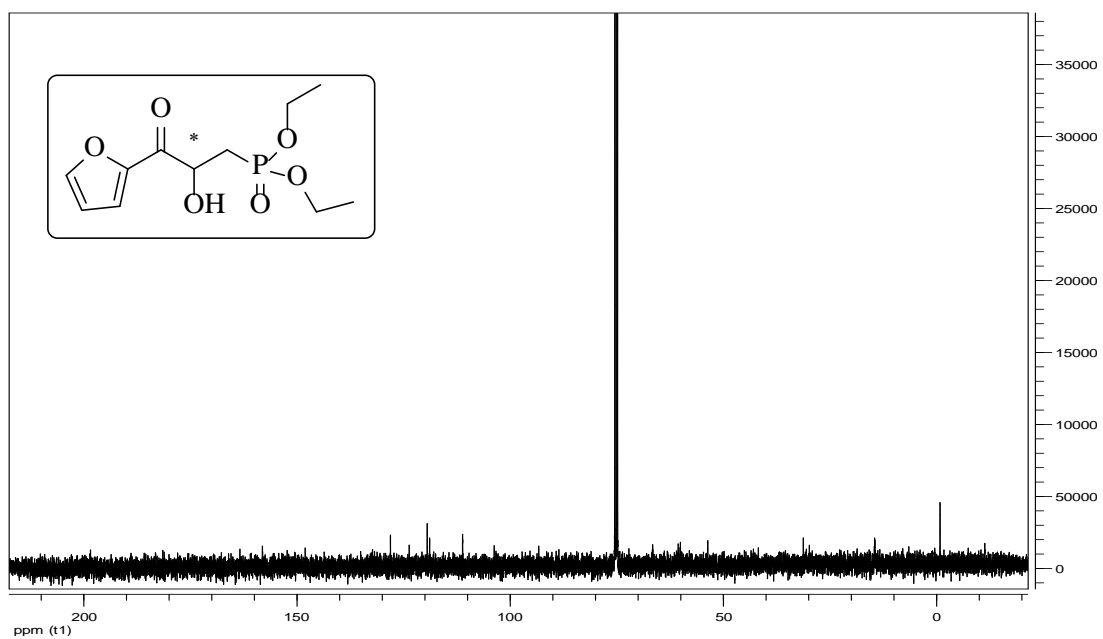
Figure A15. ^{1}H NMR spectrum of (3-(4-fluorophenyl)-2-hydroxy-3-oxopropyl) phosphonate (BHP-8).
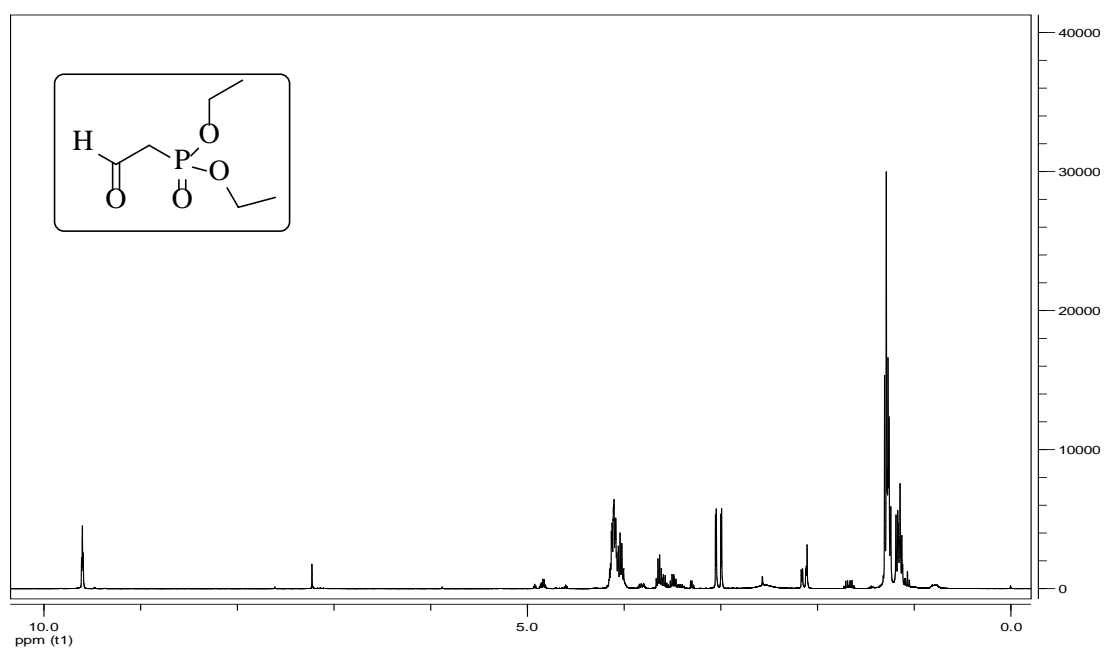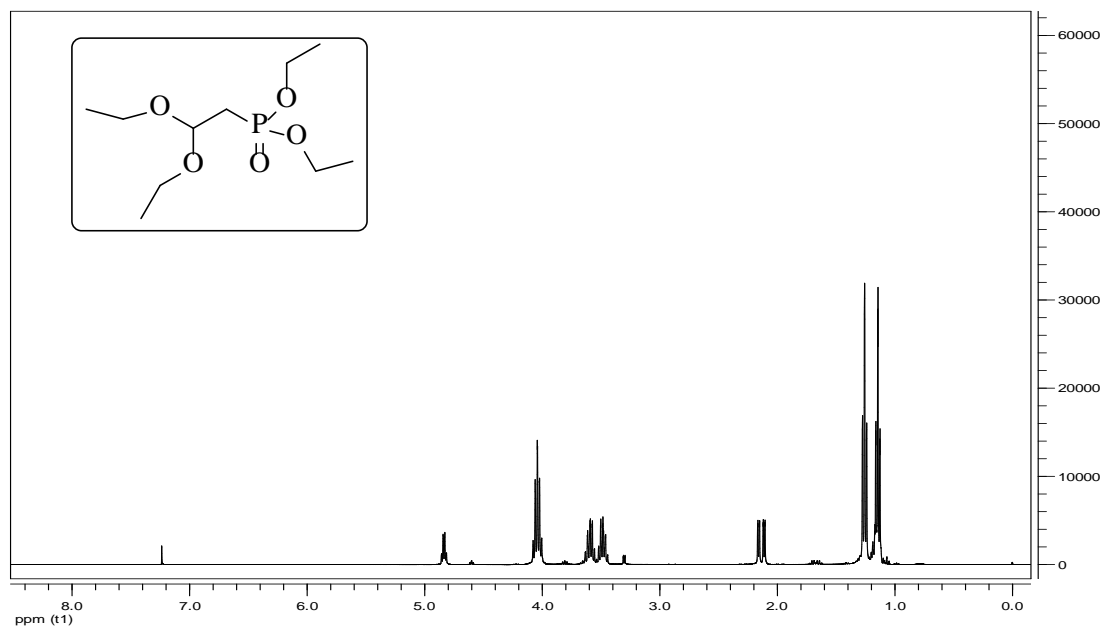

Figure A16. ^{13}C NMR spectrum of (3-(4-fluorophenyl)-2-hydroxy-3-oxopropyl) phosphonate (BHP-8).

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Figure A17. ^{1}H NMR spectrum of (3-(furan-2-yl)-2-hydroxy-3-oxopropyl) phosphonate
(BHP-9).

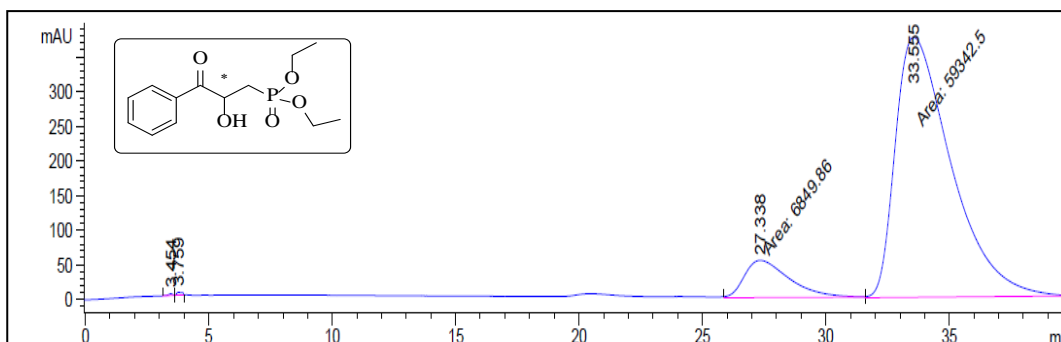**Figure A18.** ^{13}C NMR spectrum of (3-(furan-2-yl)-2-hydroxy-3-oxopropyl) phosphonate
(BHP-9).

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Figure A19. ^{1}H NMR spectrum of diethyl (2,2-diethoxyethyl) phosphonate.

Figure A20. Crude ^{1}H NMR spectrum of diethyl (2-oxoethyl) phosphonate.
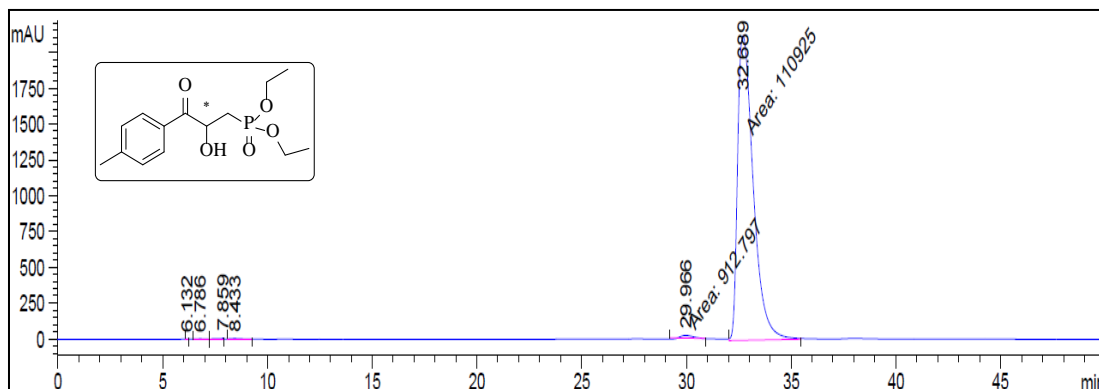
HPLC DATA

Figure B1. HPLC chromatogram of (2-hydroxy-3-oxo-3-phenylpropyl) phosphonate (BHP-1).

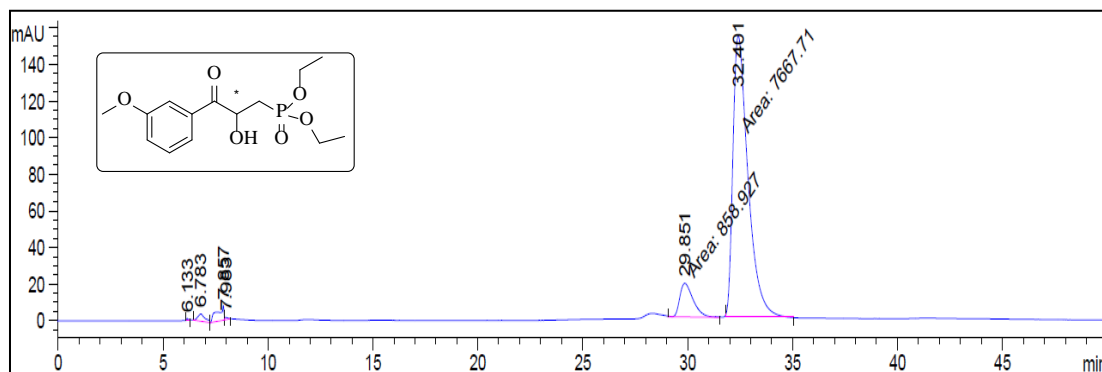| Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	3.454	BV	0.2045	44.52459	3.04657	0.0671
2	3.759	VV	0.2254	79.98338	4.77651	0.1206
3	27.338	MM	2.1159	6849.85742	53.95621	10.3290
4	33.555	MM	2.6319	5.93425e4	375.78949	89.4833
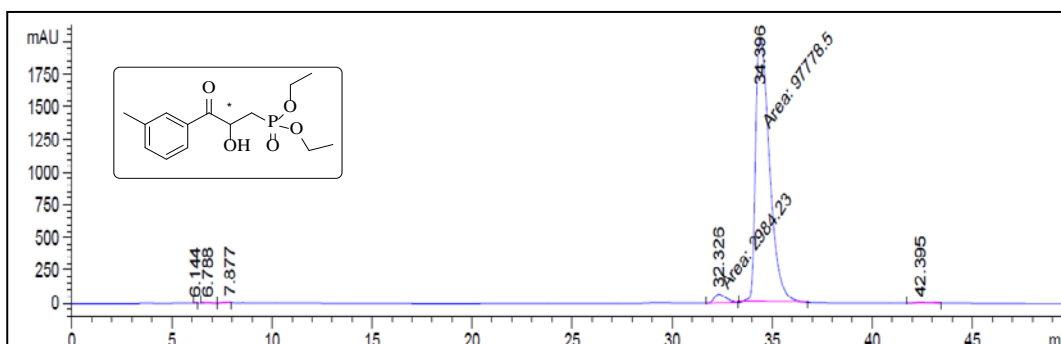
Figure B2. HPLC chromatogram of (2-hydroxy-3-oxo-3-(tolyl)propyl) phosphonate (BHP-2).

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	6.144	BB	0.0794	7.22447	1.28786	7.132e-3
2	6.788	BV	0.3468	110.11663	4.35805	0.1087
3	7.877	VV	0.2607	173.97659	8.29485	0.1718
4	32.326	MM	0.7181	2984.22729	69.26066	2.9461
5	34.396	MM	0.8085	9.77785e4	2015.61658	96.5307
6	42.395	BB	0.6371	238.55635	4.46650	0.2355

Figure B3. HPLC chromatogram of (2-hydroxy-3-oxo-3-(*p*-tolyl)propyl) phosphonate (BHP-3).
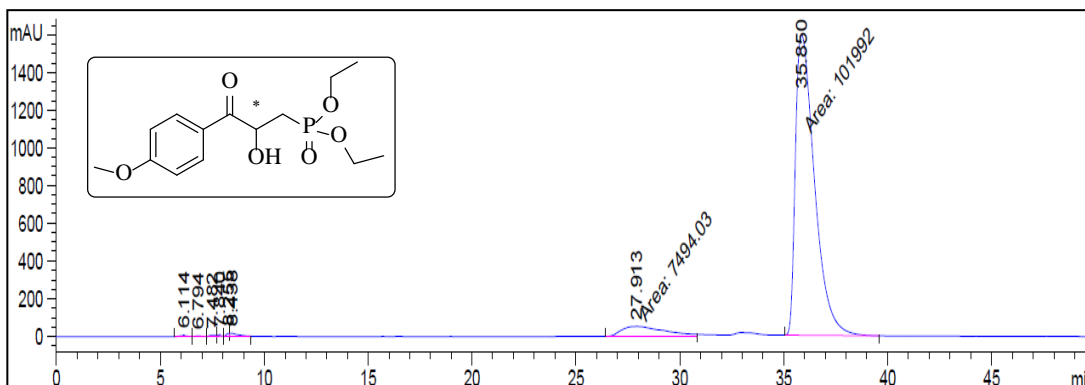
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	6.132	BB	0.0702	6.78547	1.40900	6.039e-3
2	6.786	BV	0.3339	88.35896	3.53209	0.0786
3	7.859	VV	0.2696	205.53104	9.38041	0.1829
4	8.433	BB	0.5067	217.27589	5.50214	0.1934
5	29.966	MM	0.7348	912.79700	20.70382	0.8124
6	32.689	MM	0.8707	1.10925e5	2123.27344	98.7266

Figure B4. HPLC chromatogram of (2-hydroxy-3-(3-methoxyphenyl)-3-oxopropyl) phosphonate (BHP-4).
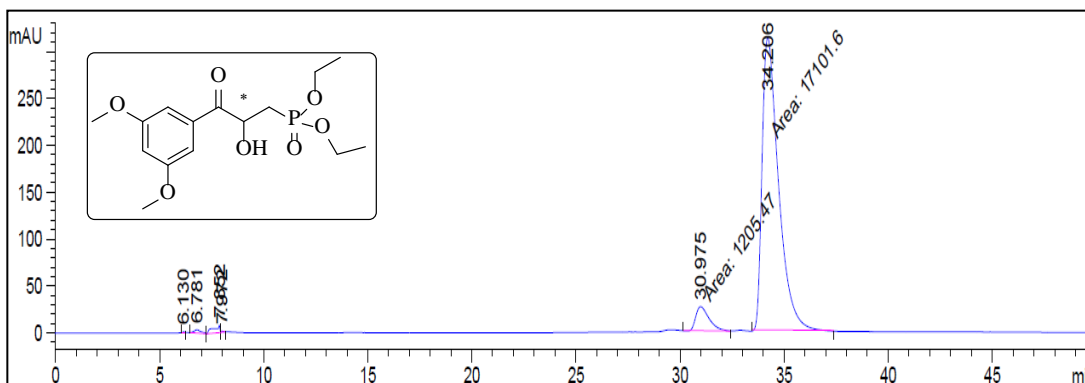
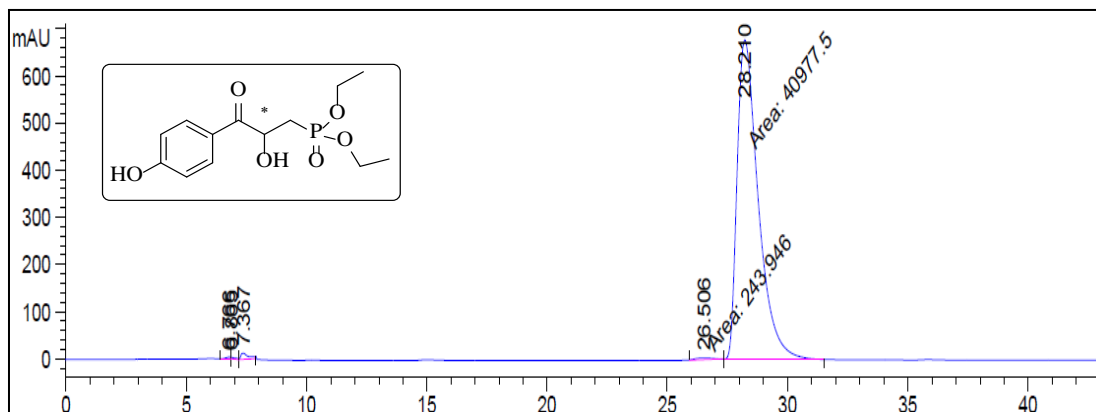| Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	6.133	BB	0.0768	6.19364	1.15101	0.0701
2	6.783	BV	0.3328	97.70041	4.03058	1.1061
3	7.857	VV	0.2867	187.44414	8.07827	2.1220
4	7.983	VB	0.1506	15.25089	1.47556	0.1727
5	29.851	MM	0.7678	858.92651	18.64413	9.7238
6	32.401	MM	0.8310	7667.71240	153.78482	86.8053

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Figure B5. HPLC chromatogram of (2-hydroxy-3-(4-methoxyphenyl)-3-oxopropyl) phosphonate (BHP-5).

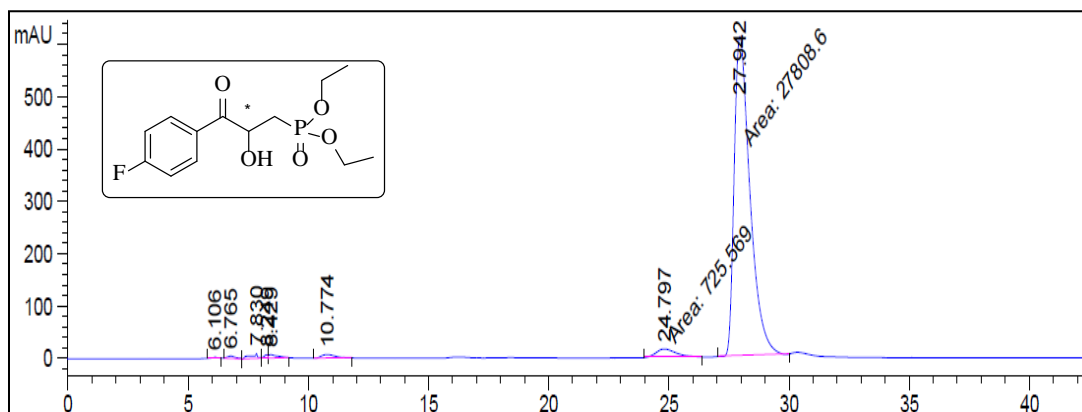| Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	6.114	BB	0.2258	164.02222	9.12008	0.1483
2	6.794	BV	0.3765	84.82320	2.92317	0.0767
3	7.482	VV	0.3387	151.82996	6.84734	0.1372
4	7.840	VB	0.1162	103.67303	11.83595	0.0937
5	8.255	BV	0.1618	166.43478	15.43162	0.1504
6	8.438	VB	0.3706	477.39932	17.05470	0.4315
7	27.913	MM	2.4087	7494.02539	51.85369	6.7737
8	35.850	MM	1.0673	1.01992e5	1592.66443	92.1885

Figure B6. HPLC chromatogram of (3-(3,5-dimethoxyphenyl)-2-hydroxy-3-oxopropyl) phosphonate (BHP-6).

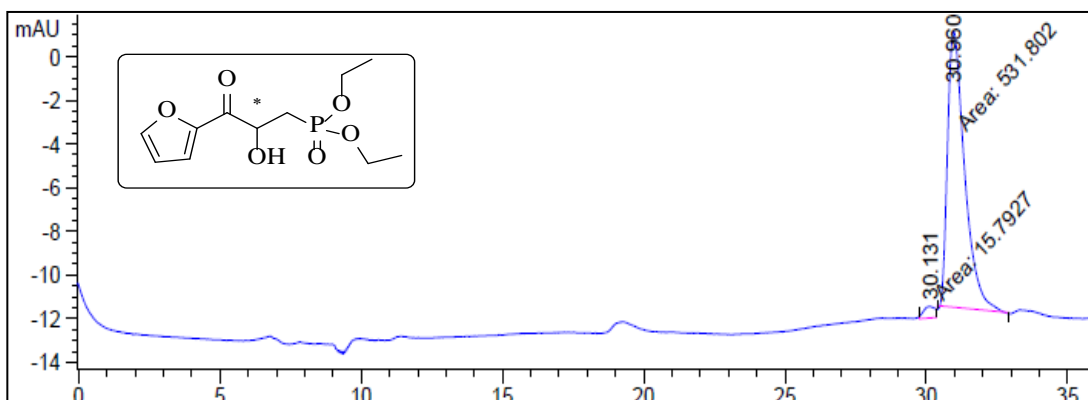| Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	6.130	BB	0.0781	6.65485	1.21128	0.0358
2	6.781	BV	0.3300	91.60638	3.76319	0.4925
3	7.852	VV	0.2829	182.31299	7.90943	0.9802
4	7.972	VB	0.1392	12.63180	1.34924	0.0679
5	30.975	MM	0.7696	1205.47095	26.10679	6.4809
6	34.206	MM	0.9129	1.71016e4	312.20972	91.9427

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Figure B7. HPLC chromatogram of (2-hydroxy-3-(4-hydroxyphenyl)-3-oxopropyl) phosphonate (BHP-7).

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	6.766	BV	0.1970	61.51928	4.57235	0.1477
2	6.865	VV	0.1777	62.64341	4.51859	0.1504
3	7.367	VB	0.3057	314.59427	13.31292	0.7551
4	26.506	MM	0.8871	243.94553	4.58337	0.5856
5	28.210	MM	1.0110	4.09775e4	675.52313	98.3613

**Figure B8.** HPLC chromatogram of (3-(4-fluorophenyl)-2-hydroxy-3-oxopropyl) phosphonate (BHP-8).

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	6.106	BB	0.2177	60.16004	3.44495	0.2037
2	6.765	BV	0.3558	151.58784	5.67141	0.5133
3	7.830	VV	0.2810	224.21355	9.79747	0.7592
4	8.249	VV	0.1806	86.71447	7.09663	0.2936
5	8.429	VB	0.3586	197.37506	7.13706	0.6683
6	10.774	BB	0.5435	280.14923	6.96081	0.9486
7	24.797	MM	0.8513	725.56885	14.20497	2.4567
8	27.942	MM	0.7610	2.78086e4	609.05786	94.1567

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Figure B9. HPLC chromatogram of (3-(furan-2-yl)-2-hydroxy-3-oxopropyl) phosphonate (BHP-9).

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Peak  RetTime Type  Width     Area       Height     Area
 #     [min]        [min]    [mAU*s]     [mAU]        %
----|-------|----|-------|----------|----------|--------|
  1   30.131 MM   0.4709   15.79266 5.59002e-1   2.8840
  2   30.960 MM   0.7008  531.80212  12.64779    97.1160
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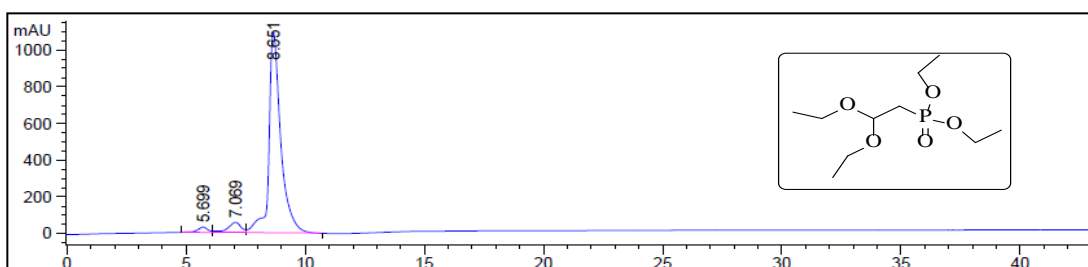

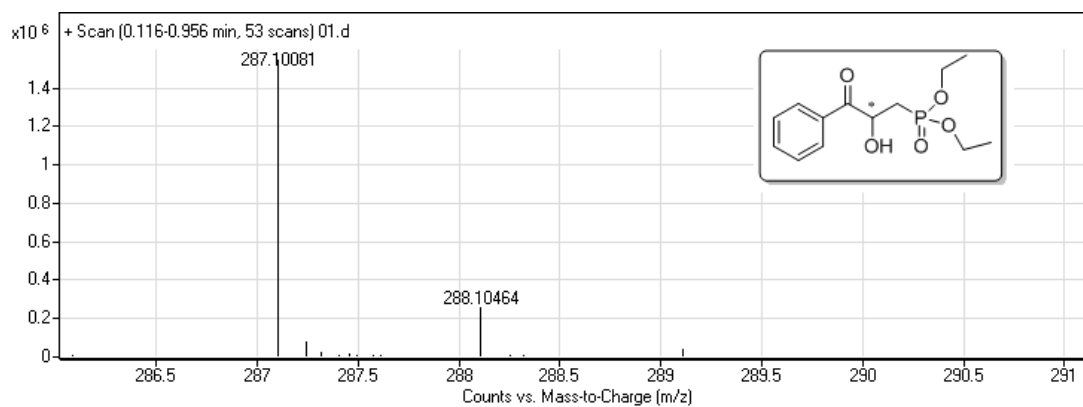**Figure B10.** HPLC chromatogram of diethyl (2,2-diethoxyethyl) phosphonate.
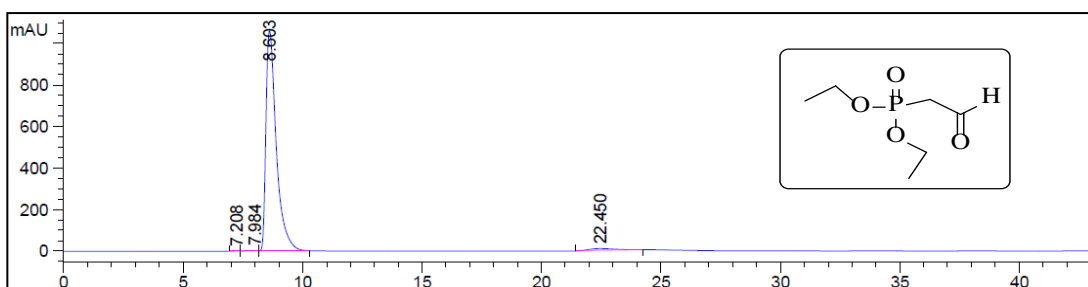
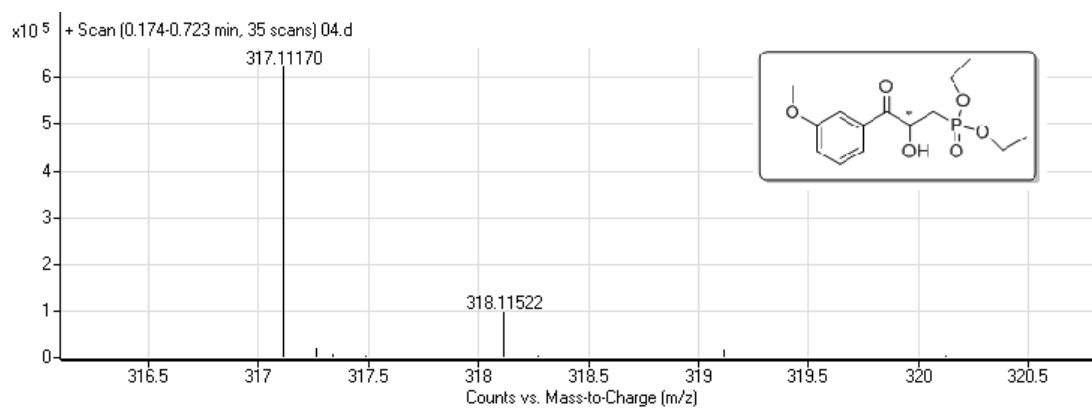**Figure B11.** HPLC chromatogram of diethyl (2-oxoethyl) phosphonate.

HRMS

Figure C1. HRMS chromatogram of diethyl (2-hydroxy-3-oxo-3-phenylpropyl) phosphonate (BHP-1).
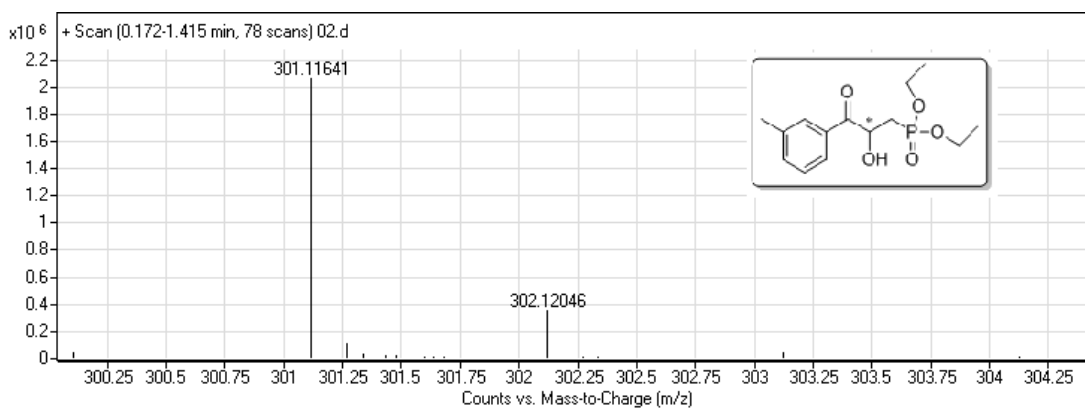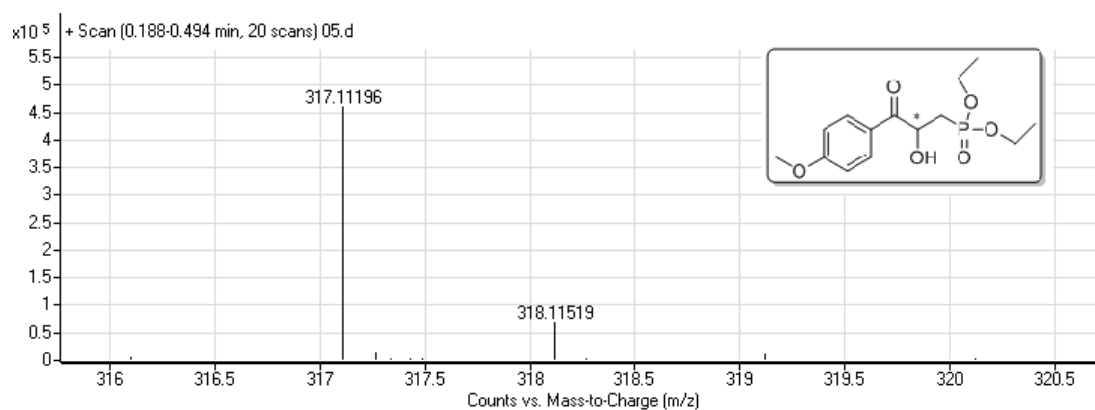

Figure C2. HRMS chromatogram of diethyl (2-hydroxy-3-oxo-3-(*m*-tolyl)propyl) phosphonate (BHP-2).
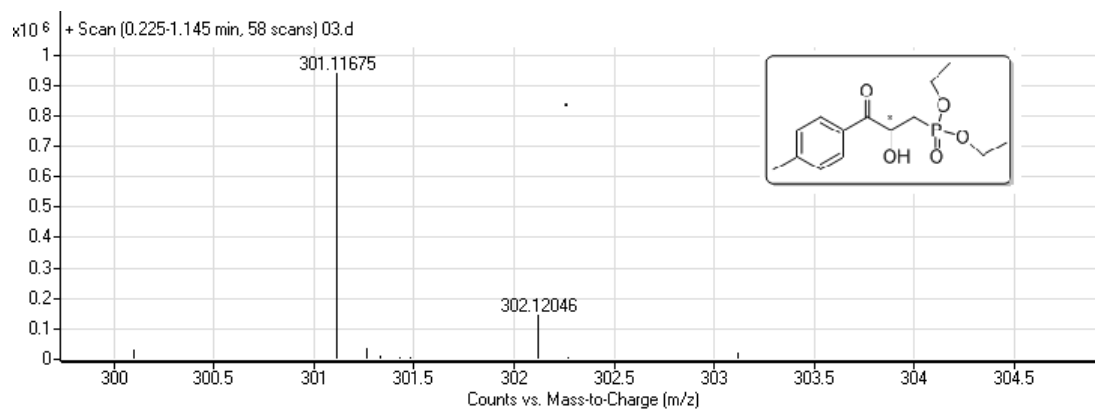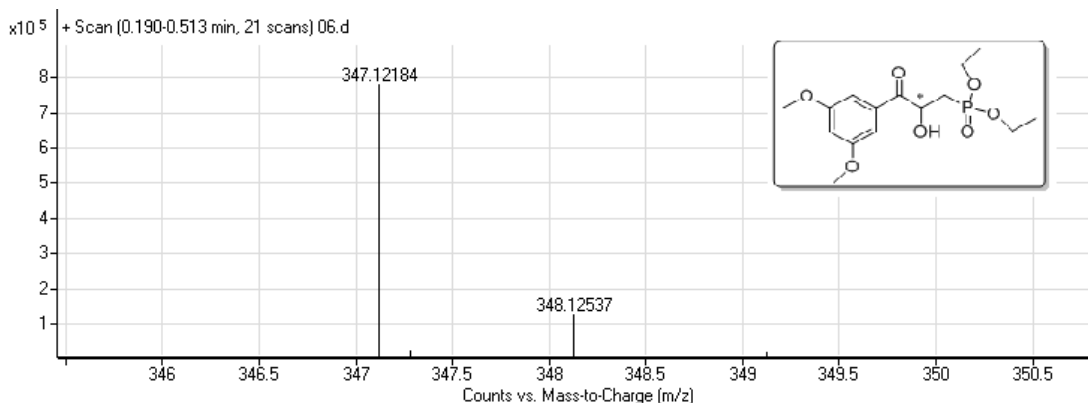
Figure C3. HRMS chromatogram of diethyl (2-hydroxy-3-oxo-3-(*p*-tolyl)propyl) phosphonate (BHP-3).
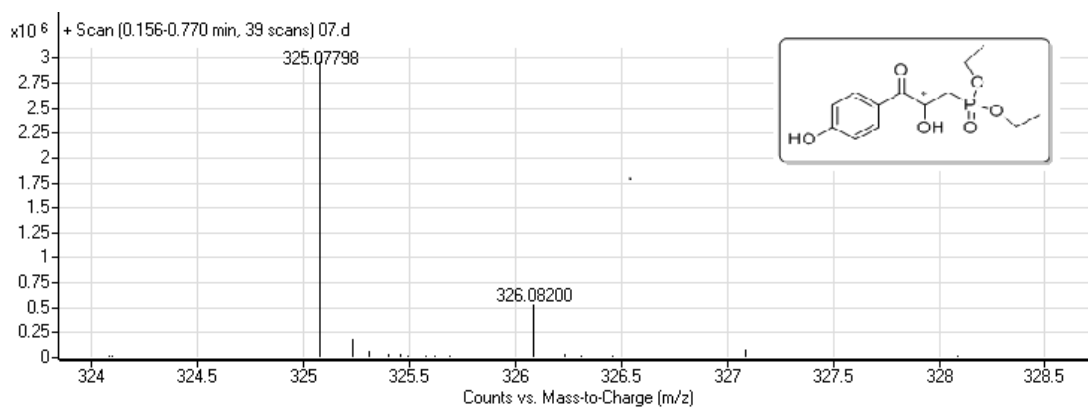

Figure C4. HRMS chromatogram of diethyl (2-hydroxy-3-(3-methoxyphenyl)-3-oxopropyl) phosphonate (BHP-4).
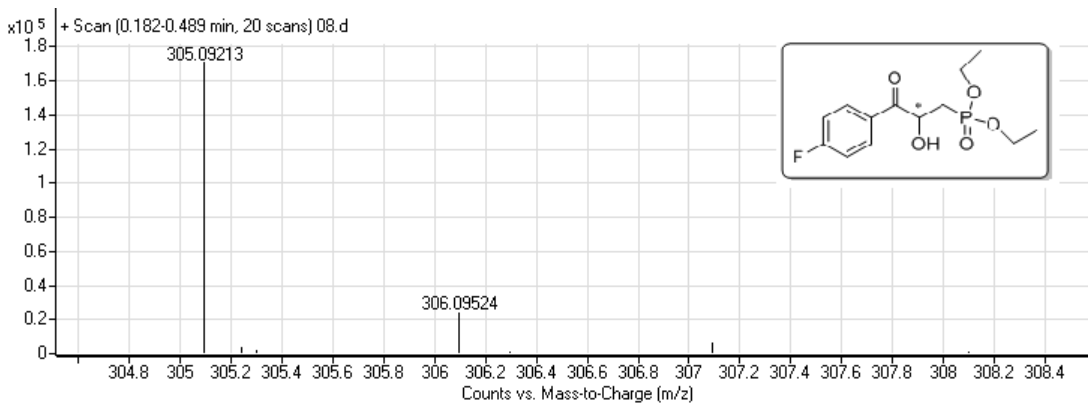

Figure C5. HRMS chromatogram of diethyl (2-hydroxy-3-(4-methoxyphenyl)-3-oxopropyl) phosphonate (BHP-5).
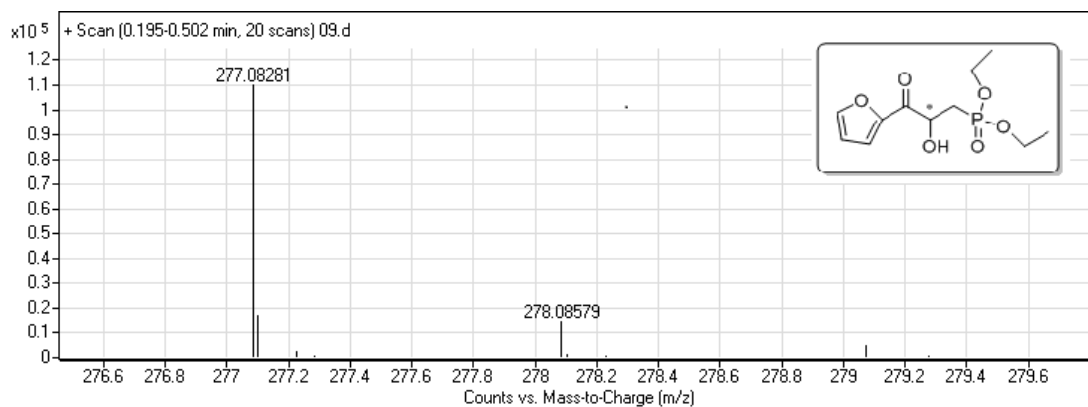
Figure C6. HRMS chromatogram of diethyl (3-(3,5-dimethoxyphenyl)-2-hydroxy-3-oxopropyl) phosphonate (BHP-6).

Figure C7. HRMS chromatogram of diethyl (2-hydroxy-3-(4-hydroxyphenyl)-3-oxopropyl) phosphonate (BHP-7).

Figure C8. HRMS chromatogram of diethyl (3-(4-fluorophenyl)-2-hydroxy-3-oxopropyl) phosphonate (BHP-8).

Figure C9. HRMS chromatogram of diethyl (3-(furan-2-yl)-2-hydroxy-3-oxopropyl) phosphonate (BHP-9).