RESEARCH ARTICLE

International Journal of Agriculture, Environment and Food Sciences 2023; 7(2): 275-283
DOI: https://doi.org/10.31015/jaefs.2023.2.4

Chemo-enzymatic synthesis of chiral precursor molecules with chiral ring hydroxyenone and acetoxyenone structures

Hatice Becerekli[1] • **S. Betül Sopacı**[2]

[1] Food Business Department, Vocational School of Technical Science, Batman University, Batman, Turkiye

[2] Chemistry Department, Faculty of Science and Literature, Kırşehir Ahi Evran University, Kırşehir, Turkiye

Citation: Becerekli, H., Sopacı, S.B. (2023). Chemo-enzymatic synthesis of chiral precursor molecules with chiral ring hydroxyenone and acetoxyenone structures. International Journal of Agriculture, Environment and Food Sciences, 7 (2), 275-283

Received: 06 December 2022
Revised: 09 February 2023
Accepted: 11 February 2023
Published Online: 07 June 2023

Corresponding Author: Hatice Becerekli
E-mail: hatice.becerekli@batman.edu.tr

Available online at
https://jaefs.com/
https://dergipark.org.tr/jaefs

Abstract

A biocatalytic transformation has the potential to perform organic reactions that are quite challenging to achieve with synthetic organic chemistry. They also catalyze these reactions with a chemo and enantio selective manner. The discovery and development of new chemoenzymatic methods for the synthesis of these chiral structures is essential to the production of a wide range of bioactive compounds. In this study, two important pharmaceutical precursors were synthesized chemoenzymatically and subjected to biocatalytic conversions with different dehydrogenases. One of these compound is an α-acetoxy enone structure 4-methoxy-2-oxacyclohex-3-enyl acetate and the other is an α-hydroxy ketone 6-hydroxy-3-methoxycyclohex-2-enone. To obtain these pharmaceutical precursors, 3-methoxy-cyclohex-2-enone was prepared using 1,3-diketone as a starting material. After obtaining this material, α-acetoxy enone was synthesized by chemical acetylation and α-hydroxy ketone prepared by enzymatic deacetylation. The structure of these products was elucidated by NMR analysis. In addition, biocatalytic reduction reactions involving the enzymes galactitol dehydrogenase (GatDH), shikimate dehydrogenase (SDH) and diaphorase were carried out with these products.

Keywords: Acetoxy enone, Biotransformation, Dehydrogenase, Lipase, α-hydroxyketon

INTRODUCTION

Drug discovery and development are among the most important translational science activities that contribute to human health and well-being. However, the development of a new drug is a complex, expensive and lengthy process (Çelik *et. al.* 2021). The sequence of biologically active molecules alters their physiological properties, which highlights the importance of preparing stereoisomers of the precursor molecule (Zhuozhuo, *et. al.* 2022). Since enzymes and biological catalysts have asymmetric structures, they can perform selective and targeted biological functions in nature. Moreover, special attention is paid to the currently available methods for their asymmetric synthesis (Ari, 2022). They are used in the synthesis of biologically active molecules, such as pharmaceutical molecules and agricultural control agents, where optical purity (% ee) is the most important criterion for such structures (Csuk, *et. al.* 1990). The human body is also enantioselective and consequently enantiopure drugs are essential for the treatment of disease. Therefore approximately 50 % of drugs produced in pharmaceutical industry are chiral (Mane, 2016).

One of the most demanded chiral structures in pharmaceutical industry is chiral alcohols. They are also found in the structure of many bioactive compounds.

α-hydoxy ketones are the versatile chemical structure that contain a functional group at the α carbon attached to a chiral carbon atom. Asymmetric catalysis has emerged as a general and powerful approach to prepare chiral compounds. Developing new chiral ligands and catalysts that can effectively induce asymmetry in reactions is crucial in modern chemical synthesis (Huang, 2022). Since the carbonyl group can be easily converted into other functional structures such as diols, halo or amino derivatives, these structures are important bioactive building blocks (Kataoka, *et. al.* 2003). Reduction products of alpha hydroxy ketones are diol structures (Faber, 1996). Diols are likewise as important structures as α hydroxy ketones considering the chiral information they carry and the functionality of the alcohol groups. Vicinal diols are used in the synthesis of aldehydes and ketones (Noyori, *et. al.* 2006). They are also known to be used in the synthesis of 1,2-diketones and the formation of α-ketoalcohols (Demir, *et. al.* 2007). Biocatalytic methods can be used effectively in the synthesis of the chiral structures such as enzymatic oxidation and reduction, deacetylation and kinetic decomposition reactions (Heiba, *et. al.* 1974).

In this study, the reactions of two chiral α-hydroxy ketone formed by biocatalytic deacetylation of α-acetoxy enone and α-acetoxyenone structures to be used as substrate in biocatalytic reduction with dehydrogenase enzymes. The first structure, 4-methoxy-2-oxacyclohex-3-enyl acetate, is a 2-ring polyoxo ketone, and this type of substances are important structural units of many bioactive molecules as shown in Figure 1 (Noyori, 2006). Among efficient asymmetric synthesis methods, metal-acting radical reactions using $Mn(OAc)_3$ are one of the most widely used approach (Faber, 1996). Reactions using $Mn(OAc)_3$ have been used in the development of many chemo- and stereoselective synthesis methods (Demir, *et. al.* 2007). The use of *in situ* preparations of $Mn(OAc)_3$ from KMnO4/$Mn(OAc)_2$ is also available in the literature (Heiba, *et. al.* 1974).

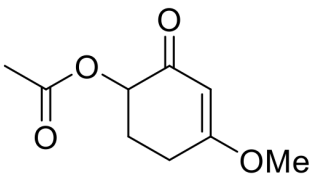

Figure 1. Structure of 4-methoxy-2-oxacyclohex-3-enyl acetate.

6-Hydroxy-3-methoxycycyclohex-2-enone structure can be obtained by biocatalytic deacetylation of the acetoxy enone molecule with lipase or esterase enzymes. Among enzymatic methods, lipase enzymes are particularly used to obtain optically pure compounds by kinetic dissociation reactions. In this study, the experimental design was to obtain optically pure product by subjecting the racemic starting material to kinetic dissociation. By taking the advantage of enantioselective deacetylation property, the enzyme lipase is planned to employ for this kinetic resolution reaction. Due to the nature of kinetic resolution reactions, the product formed with 50% yield at the best resolution level and the untransformed starting material can be obtained with high optical purity as well.

The other group of biocatalysts used in this study are dehydrogenase enzymes. Galactitol dehydrogenase (EC 1.1.1.16) and shikimate dehydrogenase (EC 1.1.1.24) and diaphorase are the enzymes that we selected to carry out for the biocatalytic reduction reactions . As a result of these catalytic reductions, it was our aim to obtain a cyclic chiral alcohol or diol structures. Galactitol dehydrogenase (galactitol:NAD^+oxidoreductase; GatDH) was first isolated from the galactitol-utilizing mutant bacterium *Rhodobacter sphaeroides* (Carius *et.al*. 2010). This homotetrameric enzyme reduces galactitol and catalyses its conversion to L-tagatose (Demir, *et. al.* 2004). This enzyme has also been used in biotransformation studies for the reduction reactions of molecules such as hydroxyacetone and hydroxyketone. The other enzyme used in this study for bioreduction conversion shikimate dehydrogenase (SDH) was isolated from *Corynebacterium glutamicum* (Schoepe, *et.al.,* 2006). *Corynebacterium glutamicum* is a microorganism often used in industry for bioconversion of aromatic structures. It also appears to provide an attractive route for sustainable indole production from tryptophan in *Corynebacterium glutamicum* in the bioconversion production process (Mindt M. *et. al*. 2021). As for diaphorase a commercially available enzyme that is frequently reported in biotransformation studies, especially in the reduction of double bond structures (Fronza *et.al* 1996)

Here we aim to identify new enzymes that can be used in the synthesis of chiral molecules with pharmaceutical value. To this end, it is essential to screen enzyme candidates applicable to chemoenzymatic methods. The starting materials we have chosen here to create chiral centres by reduction reactions are 4-Methoxy-2-oxacyclohex-3-enylacetate and the esterase reaction product 6-Hydroxy-3-methoxycycyclohex-2-enone. While the enzymes we selected have the catalytic properties to give the candidate reactions, the range of non-specific substrates is unknown. An important factor in the selection of these enzymes is that they have been isolated, characterized and produced in quantities that can be used in chemoenzymatic studies. As a result of the optimization studies performed with these enzymes, 4-Methoxy-2-oxacyclohex-3-enylacetate, 6-Hydroxy-3-methoxycycyclohex-2-enone did not form any reduction product as a result of the analysis of the products isolated from the enzymatic reaction medium. The deacetylation product obtained by metal-activated radical reactions using $Mn(OAc)_3$ and lipase enzyme reactions was previously developed but was successfully applied again

with some modifications. This was followed by screening of the products with some of the new and previously unapplied dehydrogenase enzymes in the literature that may enable the synthesis of more advanced chiral structures.

MATERIALS AND METHODS

Synthesis of Substrates Used in Enzymatic Reduction Reactions

Two substrates were prepared for the bio-reduction reactions catalysed by galactitol dehydrogenase, shikimate dehydrogenase and diaphorase. First one is acetoxyenone (4-methoxy-2-oxocyclohex-3-enlylacetate) and deacetylation product of this structure alphahydroxyketone (6-hydroxy-3-methoxycycyclohex-2-enone). First, 3-methoxycyclohex-2-enone was synthesized from cyclohexane-1,3-dione by methylation method. This substance was then acetylated with manganese-III acetate. The resulting 4-methoxy-2-oxocyclohex-3-enlyacetate was converted to 6-hydroxy-3-methoxycycyclohex-2-enone by lipase and esterase enzymes. This material was used as starting material for enzymatic reduction reactions.

Synthesis of 3-Methoxycyclohexan-2-enone

4.500 g of 1,3-cyclohexanedione was taken into a 100 ml flask and 1 mL of CH_3COOH was added and stirred for a while in the heating mantle to dissolve. Then 50 ml of methanol was added to this medium and the back cooling process was started. The formation of the product was monitored by TLC (hexane: ethyl acetate 1:1). After the reaction was completed (approximately 12 hours), the product was extracted twice with ethyl acetate at a volume ratio of 1:1. The pH was adjusted to 7-8 with saturated $NaHCO_3$ solution. A silica-filled column was used for purification of the product by column chromatography. The column was conditioned at (2:1) Hexane:EtOAc and the product was fractionated at (1:1) Hexane: EtOAc. The structure of the product was determined by ^{1}H-NMR analysis.

Synthesis of 4-Methoxy-2-Oxocyclohex-3-Enyl Acetate

Synthesis of 4-Methoxy-2-Oxocyclohex-3-Enyl Acetate was carried out following the method described in Demir et al. (2004) with some modifications. As organic solvent benzene was used for this reaction. For this first anhydrous benzene was prepared. Na was added to the benzene in round bottom flask and the reaction was carried out at 70 °C in argon gas environment. Evaporated water is condensed and collected in a separate flask and this process was repeated several times.

For the reaction, potassium permanganate ($KMnO_4$) was dissolved in 10 mL of acetic acid (CH_3COOH) and 90 mL of anhydrous benzene was added. Reaction was performed in a round bottom flask and placed in a heating mantle. After reflux started the mixture was filtered and the residual $KMnO_4$ was removed from the medium. The medium turned to a purple colour due to the formation of manganese(III)acetate. Then the flask was placed into the heating mantle again and total amount of 1260 mg of enone was added slowly at a molar ratio of 1:4 Enone: $KMnO_4$. Since the separation of undissolved $KMnO_4$ from the medium by filtration was laborious, $Mn(OAc)_3.2H_2O$ was also used instead of $KMnO_4$. For this, $Mn(OAc)_3.2H_2O$ was kept in an oven for 12-24 hours to obtain anhydrous $Mn(OAc)_3$. For the acetylation step 3 g manganese (III) acetate ($Mn(OAc)_3$) in 10 ml acetic acid (CH_3COOH) in a 250 ml flask is used and 90 ml benzene was added to start the reaction. Reaction performed under reflux with Dean-Stark trap and 45 minute later 1000 mg enone was added slowly and the reaction was continued for 2-3 days. TLC controls were performed with 1:1 Hexane: Ethyl acetate system. For extraction, benzene was evaporated, and the product was dissolved in ether and extracted with ethyl acetate. The extraction mixture was neutralized with saturated aqueous $NaHCO_3$ solution. The column was conditioned in 1:1 EtOAc-Hexane and the product collected for separation. Structure analysis of the product was performed by^1 H-NMR.

Enzymatic Hydrolysis of 4-Methoxy-2-Oxocyclohex-3-Enyl Acetate

The starting material 4-methoxy-2-oxacyclohex-3-enylacetate (20 µl) which was prepared in previous steps was dissolved in 200 µl DMSO and added to 10 ml 50 mM phosphate buffer (pH: 7) containing the enzyme Porcine Liver esterase (30 mg) or Lipase (40 mg). The reaction was incubated at 30°C with 100 rpm rotation for 50 hours for racemic product and 22 hours for optically enriched product formation. The reaction progress was monitored by TLC in 1:1 EtOAc:Hexane mobile phase. Product analysis was carried out by HPLC (Chiralpak AD, 90:10 Hexane:Isopropanol, 0.8 ml/min) for determination of the enantiomeric excess (ee) and by H^1 – NMR for structural analysis.

Producing Galactitol Dehydrogenase and Shikimate Dehydrogenase from Recombinant E. coli Cells

Recombinant cells (*E. coli BL21* were grown in sterile LB (Luria Broth) agar containing 100 mg/ml ampicillin and then transferred to 10 mL LB liquid media in 50 mL falcon tube. Ampicillin was added at a concentration of 100 µg/mL. After an overnight incubation (150 rpm 37 °C) cells were transferred to 250 mL volume of the same medium and incubated at the same conditions for approximately 3-4 hours until OD value reaches 0.6-0.7 at 600 nm. At the end of this period, IPTG (Isopropyl-D-thiogalactopyranoside) was added to the culture medium with a final concentration of 1 mM for transcription initiation. IPTG was sterilized before the procedure by passing through a 0.2 µm filter (Millipore®).

After 5-8 hours incubation, cells were separated from the medium by centrifugation (5000 rpm 4 °C, 10 min) and then supernatant was discarded. Cells were resuspended in phosphate buffer (50 mM, pH:7.5) containing 10 mM NaCl, 10 mM imidazole, 10 mL 2 mg lysozyme for extraction. Crude extract was prepared by sonication (2 min, 2 sec intervals) and centrifugation (20000 rpm, 10 min at 4° C). For long-term storage, it was lyophilized for 1-2 days or stored in 20% glycerol at -20° C.

When we stored lyophilized cells instead crude extract, cells were suspended in 50 mM phosphate buffer (pH: 6.5), lysozyme was added and incubated in an ice bath for 5 min. The cells were lysed with the help of a sonicator for a total of 2 min, 2 sec intervals and then the mixture was centrifuged at 9000 rpm for 10 min. The crude extract was used directly in enzymatic reactions or kept in 20% glycerol at -20 °C for long term storage.

Bradford Protein Detection Method

Standard Preparation: Protein was determined according to the Bradford method. 500 µl Bovine Serum Albumin (Sigma) from 1mg/ml stock solution was diluted to 0.5 mg/ml with 500 µl of water. 500 µl of the 0.5% solution was taken and diluted to 0.25 mg/ml with 500 µl of water. A 0.125 solution was also obtained by the same procedure. A blind sample, i.e. one without protein, was also prepared. 50 µl of each of these solutions was added to spectrophotometer cuvettes containing 1.5 ml of Bradford reagent.

Sample Measurement: The samples to be measured for protein concentration were diluted 1:100 and added to 1.5 ml of Bradford reagent and their optical absorbance at 595 nm was measured and protein values were determined.

Biocatalytic Reactions of 4-Methoxy-2-Oxocyclohex-3-Enylacetate with Dehydrogenase Enzymes

Recombinant enzymes (GatDH and ShDH) prepared as described above were added to 50 mM phosphate buffer. For reactions with GatDH, 1 mM $MgCl_2$ was added to the reaction medium. To this mixture 6 mg of NADH in 300 µL DMSO was added. The reaction mixture was left at 35 °C with a rotation speed 80 rpm for 2 days. Reactions with diaphorase enzyme were carried out with 4 mg of lyophilized enzyme (Sigma, 6-8 U/mg). After 24 h, same amount of enzyme was added to the reaction mixture. Product formations were monitored by TLC (1:1 EtOAc: Hexane). The reaction medium was extracted twice with ethyl acetate, once with concentrated NaCl solution and dried over magnesium sulfate (MgSO4). Product was separated on a silica column (Hexane: Ethyl acetate 1:1). Control experiments were carried out with enzyme-free and substrate-free reaction mixtures.

Biocatalytic Reactions of 6-Hydroxy-3-Methoxycyclohex-2-Enon with Dehydrogenase Enzymes

6-Hydroxy-3-methoxycyclohex-2-enon (10 mg) was dissolved in 300 µL DMSO and added to 50 mM pH 6-8 phosphate buffer containing 6 mg NADH, the enzyme used for this conversion were GatDH (28 U) and SDH (100 mg total protein of crude enzme). It was left at 35°C with a rotation speed 80 rpm for 2 days. Reactions with diaphorase enzyme were carried out with 4 mg of lyophilized enzyme (Sigma, 6-8 U/mg). Reactions with enzyme-free and substrate-free mixtures were also performed as control experiments. Product formation was monitored by TLC (EtOAc: hexane 1:1). The reaction medium was extracted twice with ethyl acetate, once with concentrated NaCl solution, dried with magnesium sulphate and cleaned on a silica column (1:1 Ethyl acetate: Hexane). The resulting product was analysed by ^{1}H-NMR.

RESULTS AND DISCUSSION

Synthesis of 4-Methoxy-2-Oxacyclohex-3-Enyl Acetate

We aimed to synthesize a cyclic acetoxy enone structure (4-methoxy-2-oxocyclohex-3-enlylacetate) to use in enzymatic reduction reactions. The first step to obtain this substance was to synthesize 3-methoxy-cyclohex-2-enone from cyclohexane-1,3-dione to prepare the starting material for the acetylation reaction.

The synthesis of enone structure from cyclohexane-1,3-dione was carried out in acidic medium (1:50 mixture of acetic acid and methanol). The product was monitored by TLC and the RF value of the product was determined as 0.802 (EtOAc:Hexane 2:1) and product formation was observed within the first 6 hours. Starting material was completely consumed after 16 hours. According to the proton MNR analysis, the methoxy group in the molecule appeared at 3.63 ppm, the olephinic proton at 5.29 ppm and the other -CH_2 peaks were 2.36-2.33, 2.29-2.25, 2.29-2.25. (^{1} H NMR ($CDCl_3$, 400 MHz):δ 5.29 (s, 2H), 3.63 (s, 3H), 2.36-2.33 (m, 2H), 2.29-2.25 (m, 2H), 1.95-1.88 (m,2H)). Based on this, the results showed that the substance obtained is the structure of the expected enone (Figure 4.2).

4-methoxy-2-oxacyclohex-3-enyl acetate was synthesized as a starting material for two different enzymatic reactions (enzymatic reduction and deacetylation reactions). α, β α' acetoxylation products of unsaturated ketones are important chiral ligands. Many methods have been reported related to synthesis of these compounds. The method had been used in this work is found to be advantageous because there is no arylated by-product is formed. The reaction mechanism as it is shown in the figure 2 proceeds radically.

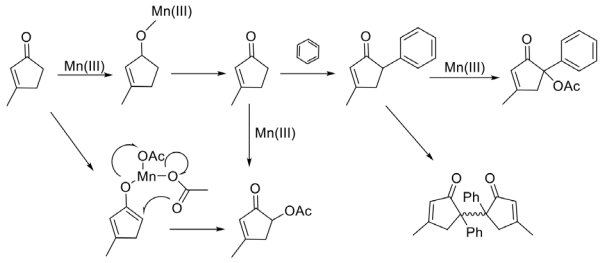

Figure 2. Mechanism of formation of α' acetoxylation products of α, β, unsaturated ketones

3-Methoxy-cyclohex-2-enone acetylated at the 3 enyl position by adding 3 equivalents of manganese acetate in acetic acid: benzene medium (Figure 3). The oxidation product was obtained with 40-60% conversion rate in 48-54 hours. Since the reaction is not proceeded enantioselective the product obtained as racemic mixture (Demir, 1991).

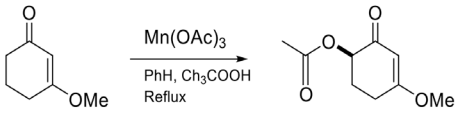

Figure 3. Synthesis of 4-methoxy-2-oxacyclohex-3-enyl acetate

After product formation observed by TLC (Rf: 0.320; 2:1 EtOAc: Hexane) at the end of 20 hrs, anhydrous manganese acetate was added to the reaction medium. To our observation using anhydrous manganese acetate was an important factor affecting the efficiency of the reaction. The reaction was terminated after 54 hours when there is no change was observed. After isolation and purification of the product, it was analysed by NMR (Figure 4). Structure of the product was easily determined by the proton NMR spectrum where the methyl peak of the acetyl group was observed at 2.18 ppm and the alpha proton was observed as dd at about 5.30 ppm. (^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.41 (d, *J=1*.5 Hz, 1H), 5.30 (dd, , *J=5*.3 and 12.6 Hz, 1H), 3.71 (s, 3H), 2.67 (dddd, *J=1*.6, 5.2, 12.0 and 17.4Hz, 1H), 2.52 (ddd, *J=2*.9, 5.3 and 17.8 Hz, 1H), 2.27-2.20 (m,1H), 3.71 (s, 3H), 2.15-2.06 (m, 1H)).

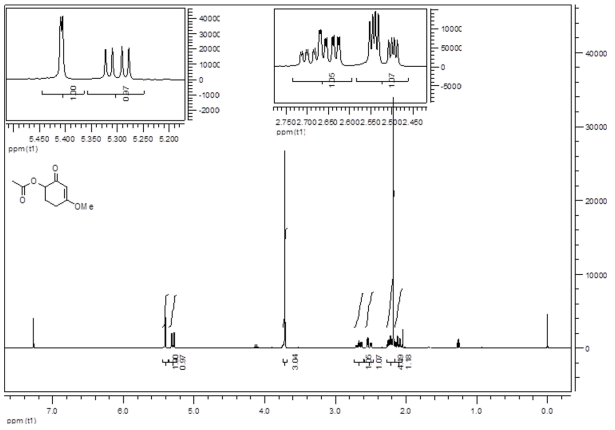

Figure 4. NMR spectrum of 4-methoxy-2-oxacyclohex-3-enyl acetate

Asymmetric Hydrolysis of 4-Methoxy-2-Oxacyclohex-3-Enyl Acetate Catalysed by the Enzymes Lyase and Esterase: Synthesis of 6-Hydroxy-3-Methoxycyclohex-2-Enone

The alpha hydroxy enone structure was formed by deacetylation of the acetoxy enone structure. Enzymes Porcine Liver Esterase (EC. 3.1.1.1.1) (PLE) and Amanolipase (EC. 3.1.1.3.3) were chosen because these enzymes carry out acetyl group transfers (e.g. in amino acid synthesis) in the cell and these reactions are enantioselective reactions (Figure 5). Due to these properties, they are used in biotransformation studies in acetyl group transfers, especially in deacetylation reactions (Tanyeli, 2002). In this way, enantiospecific synthesis of some valuable alcohols of pharmaceutical importance is carried out (Zelinski, 1994).

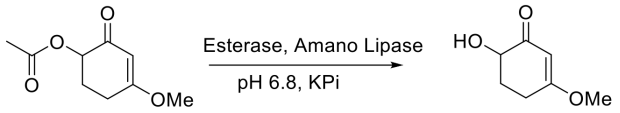

Figure 5. Synthesis of 6-hydroxy-3-methoxycyclohex-2-enone.

The GC-MS analysis of the product we synthesized shows that the molecular weight of product is 142.1 and this can be seen in the mass analysis, in addition, the mass of the molecule formed because of a proton break (m/z=141.1) can also be observed. In the mass analysis of alcohol-containing compounds, the observation of the M-1 peak accompanied by the molecular ion (M^+) peak and the presence of water exit (M^{-18}) peaks are quite distinctive. The M^{-18} peak of the compound was observed at 124.1. The alpha hydroxyketone, which is expected to be formed because of deacetylation by esterase reaction, was analysed by proton NMR as well (Figure 6). The methyl group of the acetyl group of this substance is not observed in the spectrum and the alpha proton peaked at a lower ppm (4.06 ppm) as expected. While the peak should be split into three, it was split into two because the substance is chiral. Apart from these, the proton of the hydroxy group is seen as a doublet at 3.83 ppm, which proves that the substance is the desired product hydroxy enone structure. H NMR ($CDCl_3$, 400 MHz): δ 5.42 (d, *J=1*.6 Hz, 1H), 4.06 (ddd, *J=1*.2, 5.5 and 13.1 Hz, 1H), 3.83 (d, *J=1*.2 Hz, 1H), 3.73 (s, 3H), 2.63 (dddd, *J=1*.7, 5.0, 12.6 and 17.7 Hz, 1H), 2.49 (ddd, *J=2*.2, 5.2 and 17.9 Hz, 1H), 2.37 (dtd, *J=2*.3, 5.2 and 12.7 Hz, 1H), 1.84 (dq, *J=5*.3 and 12.7 Hz, 1H).

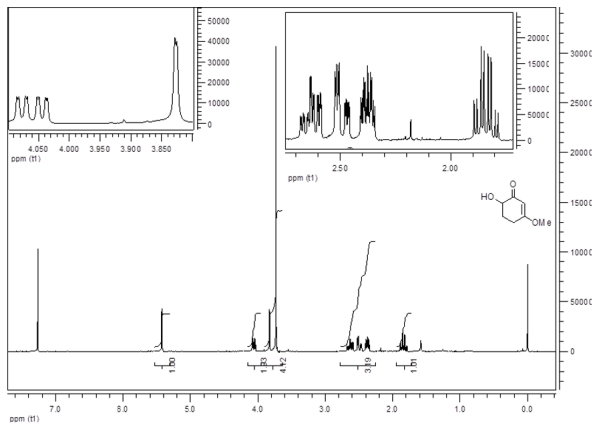

Figure 6. NMR spectrum of 6-hydroxy-3-methoxycyclohex-2-enon

Production of Galactitol Dehydrogenase and Shikimate Dehydrogenase Enzymes and Determination of Enzyme Activity

During an enzyme production, it is necessary to know the time when the enzyme is expressed at the highest level and therefore the time point for enzyme extraction. In this study, *E. coli* strains BL21 and DH5α carrying genes encoding galactitol dehydrogenase and shikimate dehydrogenase enzymes (D GatDH pET and CglSDH-L correspondently) were used for enzyme production. After induction with IPTG, production was terminated after 5-8 hours, and cells were collected by centrifugation. After obtaining the crude extract, enzyme-catalysed reactions were carried out with this crude extract. The enzyme activity in the crude extract was measured via the reduction of GatDH 1,2-hexanedione and the specific activity in the crude extract was calculated as 0.8-1 U/mg. For the enzyme shikimate dehydrogenase, the activity could not be calculated since the natural substrate of the enzyme could not be obtained. However, experiments were carried out with a crude extract mixture with a determined total protein amount (100 mg) to able to corelate the amount of the biocatalyst.

Biotransformation of 4-Methoxy-2-Oxacyclohex-3-Enyl Acetate 2 And 6-Hydroxy-3- Methoxycyclohex-2-Enone with 3 Dehydrogenase Enzymes

Galactitol dehydrogenase, shikimate dehydrogenase and diaphorase are the enzymes selected for biocatalytic conversion of the acetoxy enone 4-methoxy-2-oxacyclohex-3-enyl acetate and the cyclic alpha hydroxy enone 6-hydroxy-3-methoxycyclohex-2-enone. Bioconversion reactions were carried out under optimum reaction conditions for each enzyme according to the reduction reaction in which the co-enzyme (NADH) is oxidized. For dehydrogenase enzymes, the reduction reaction takes place mostly at relatively acidic pH levels compared to oxidation reactions. For this reason, enzymatic reduction reactions were tested at different pH levels in the range of pH 6-8. The reactions were carried out completely in buffer medium, i.e. water. In our study, DMSO (1%) was added to the reaction medium due to the poor solubility of the substrates we synthesized for the reduction reactions in water.

First, acetoxyenone structure was used for reduction transformations (Figure 7). All three enzymes mentioned above were used to test the biocatalytic reduction of this structure. TLC checks at regular intervals from the first 30 minutes of the reactions and a product was observed in all enzymatic transformations during the reactions. However subsequent NMR analysis showed that this compound was a α-hydroxy ketone structure formed by the hydrolysis of the acetyl group. It is thought that this hydrolysis product is not formed because of an enzymatic conversion under the specified conditions (pH: 6-8, 25-30 °C). However, these hydrolysis products, which occurred at different rates and conversion yields for each enzyme, were analyzed by HPLC to determine the enantiomeric excess. The results showed that the products had ee values in the range of 15-22% in experiments with GatDH and SDH. Experiments were also carried out with a control group in buffer medium only and in buffer medium containing NADH. In these control experiments, the deacetylation product was also formed, it was observed that it occurred much slower and in lower yields than the enzymatic reactions. This result was difficult to interpret in terms of biocatalytic conversions. It is well known phenomena that the enzymes are promiscuous for their natural substrate and sometimes also for their natural reaction type. But the evidences here are not clear enough to conclude that this reaction occurred due to this type of a promiscuous mode of action of the correspondent enzymes. Therefore, we need to have more evidence of this kind of deacetylation reactions in aqueous media at different pH to be certain about if this this conversion observed as a result of an enzymatic reaction.

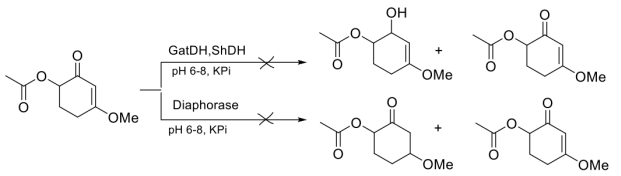

Figure 7. Expected reactions of 4-methoxy-2-oxacyclohex-3-enyl acetate with GatDH, SDH and diaphorase enzymes.

Another starting material 6-hydroxy-3-methoxycyclohex-2-enone was synthesized for bio-reduction reactions. Bioconversion conditions that have been used in previous bio-reduction reactions were again relatively alkaline and aqueous at 25-30 °C. However, no product formation was observed under any of the conditions (Figure 8).

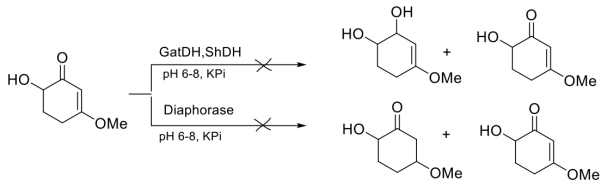

Figure 8. Expected reactions of 6-Hydroxy-3-methoxycyclohex-2-enone with GatDH, SDH and diaphorase enzymes.

For our biodegradation experiments, the enzyme diaphorase was also used since to examine the bio-reduction of double bond of the enone structure. However, according to the NMR and GC- MS analyses no such product was observed as a result of the enzymatic bioconversions with this enzyme for both of the substrates 4-Methoxy-2-oxacyclohex-3-enyl acetate and 6-hydroxy-3-methoxycyclohex-2-enone.

CONCLUSION

This study covers the optimisation of chemoenzymtic synthesis of pharmaceutically important chiral cyclic enol structures. The first step was the synthesis of starting material 3-methoxy-cyclohex-2-enone starting from the cyclic diketone. This was followed by the synthesis of 4-methoxy-2-oxacyclohex-3-enyl acetate by alpha acetylation reaction and the enzymatic synthesis. The formation of the products of these reactions was confirmed by structure determination using proton NMR analysis. The synthesized product was used for biocatalytic digestion of dehydrogenase reactions as well as acetylation with lipase and esterase enzymes to obtain a second substrate for dehydrogenase conversions. The formation of this substrate was also confirmed by both proton NMR and GC-MS analysis and the enantiomeric excess of this chiral structure was calculated by HPLC. It was found necessary to use the racemic starting materials for the enzymatic reactions which was planned to be carried out with GatDH, SDH and diaphorase enzymes. Depending on success of these trials then we could proceed with the optically pure substrates. For this reason, the reaction was first performed until all the product was completed which results in racemic mixture of deacetylation product. For the subsequent experiments, reactions terminated at the end of the 20-24 hour therefore we could obtain 50% pure chiral deacetylation product due to kinetic resolution of the starting material. Following the synthesis of starting materials (4-Methoxy-2-oxacyclohex-3-enyl acetate and 6-hydroxy-3-methoxycyclohex-2-enone) GatDH, SDH enzymes were produced using recombinant *E. coli* cells carrying plasmids containing the genes encoding the enzymes for use in biocatalytic reduction reactions and prepared as crude extracts for biotransformation reactions.

Biocatalytic reduction reactions of substrate in acetoxy enone structure in aqueous medium with DMSO as co-solvent did not produce the expected reduction products. NMR analysis of the product showed that the reduction product of the carbonyl group was not formed but the acetyl group was cleaved. It would be stated as this phenomenon was occurred due to the activity of the other enzymes available in the medium for the bio-reduction reaction performed with crude enzyme SDH. But the same observation was obtained with the pure dehydrogenase GatDH which was used for this reaction. The reason for this may be that the acetyl group is not formed because of an enzymatic reaction but because of spontaneous deacetylation in aqueous medium. HPLC analysis of the substance showed that product has formed with a low enantiomeric excess (ee: 16%) and this also suggests that both of the hypotheses may suggest a explanation.

The fact that the expected product was not formed should not be attributed to the deacetylation of the substrate, but to the fact that the enzyme did not convert this substance into a product under the specified conditions. Because the alpha hydroxy ketone formed as a result of deacetylation was also not converted into any reduction product Moreover deacetylation reaction took place quite slow (up to 16 hours), therefore it is not expected to be competitive with the reduction reaction.

As it is stated before each substrate was also reacted with another enzyme diaphorase for a possible reduction reaction in double bond structure. Deacetylation product that has been formed with GatDH and SDH was not observed with this enzyme under the specified conditions and within the specified time. In biotransformation studies, dehydrogenase reactions are carried out in buffer solution under acidic or basic pH conditions, depending on the direction of the reaction. Therefore, this type of biotransformation method was used in our study. The reactions took place at temperatures where the enzymes showed optimum activity and the results obtained at different pH values did not differ and the expected reduction product was not obtained. Under these conditions, it can be concluded that the dehydrogenase enzymes we used do not prefer the substances 4-methoxy-2-oxacyclohex-3-enyl acetate and 6-Hydroxy-3-methoxycyclohex-2-enone as substrates for bio reduction reactions. These pharmaceutically important compounds can be tested with a different dehydrogenases for optimisation of enantioselective reduction reactions.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest

The authors declared that for this research article, they have no actual, potential or perceived conflict of interest.

Author contribution

The contribution of the authors to the present study is equal. All the authors read and approved the final manuscript. All the authors verify that the Text, Figures, and Tables are original and

that they have not been published before.

Ethical approval

Ethics committee approval is not required.

Funding

This study was supported by Kırşehir Ahi Evran University Scientific Research Projects Coordination Unit (BAP) entitled "Biocatalytic Conversions of Chiral Precursor Molecules Enone and Acetoxyenone Structures by Dehydrogenase Enzymes"

Data availability

Not applicable.

Consent for publication

Not applicable.

Acknowledgements

Authors are thankful to Dr. Gert-Wieland Kohring and Dr. Niefind Karsten for supplying the plasmid with GatDH and SHD encoding gene.

REFERENCES

Ari M. P. (2022). Koskinen Asymmetric Synthesis of Natural Products, John Wiley, USA, ISBN: 978-0-471-93848-4

Bradford, M. M., (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72, 248-251. https://doi.org/10.1016/0003-2697(76)90527-3

Carius, Y., Christian, H., Faust, A., Zander, U., Klink, B.U., Kornberger, P., Kohring, G-W., Giffhorn, F., Scheidig, A. J. (2010). Structural Insight into Substrate Differentiation of the Sugar-metabolizing Enzyme Galactitol Dehydrogenase from *Rhodobacter sphaeroides* D. J. Biol. Chem. 26, 20006-20007. https://doi.org/10.1074/jbc.M110.113738

Csuk, R. Glanzer, B. I. (1990). Baker's yeast mediated transformations in organic chemistry, Chem. Rev. 91, 49-97. https://doi.org/10.1021/cr00001a004

Çelik, İ. N., Arslan, F. K., Ramazan, T. U. N. Ç., and Yıldız, İ. (2021). Artificial Intelligence in Drug Discovery and Development. Journal of Faculty of Pharmacy of Ankara University, 45(2), 400-427. https://doi.org/10.1002/med.21764

Demir, A. S., Emrullahoglu, M., (2007). Manganese(III) acetate: A versatile reagent in organic chemistry, Curr. Org. Synth. Bentham Science Publishers B.V. 4, 321-325. https://doi.org/10.2174/157017907781369289

Demir, A.S. Fındık, H. Köse, E., (2004). A new and efficient chemoenzymatic route to both enantiomers of alpha '-acetoxy-alpha-methyl and gamma-hydroxy-alpha-methyl cyclic enones, Tetrahedron: Asymm., 15, 777- 781. https://doi.org/10.1016/j.tetasy.2003.12.006

Demir, A. S. Reis, Ö. İğdir, Ç. (2004). Reinvestigation of the synthetic and mechanistic aspects of Mn(III) acetate mediated oxidation of enones, Tetrahedron, , 60, 3427-3432. https://doi.org/10.1016/j.tet.2004.02.039

Demir, A. S., Jeganathan, A. A., (1992). Selective Oxidation Of Alpha,Beta-Unsaturated Ketones At The Alpha'-Position, Synthesis, Elsevier Science, ISBN 978-0-08-052349-1, 235-246.

Faber, K., (1996). Introduction and Background Information, Biotransformations in Organic Chemistry Springer, New York. pp 1–30

Fronza,G., Fuganti,C., Mendozza, M., Rigoni,R., Servia, S., and Zucchi,G., (1996). Stereochemical aspects of flavour biogeneration through baker's yeast mediated reduction of carbonyl-activated double bonds. Pure & Appl. Chem., 68, 11, 2065-2071, https://doi.org/10.1351/pac199668112065

Heiba E. I., Dessau R., and Rodewald M., (1974). Oxidation by Metal Salts. X. One-step synthesis of Gamma.-lactones from olefins, J. Am. Chem. Soc., 96, 7977-7981. https://doi.org/10.1021/ja00833a024

Heiba, E. I., Dessau, R. M. (1974) Oxidation by metal salts XI. The Formation of Dihydrofuranes, J. Org.Chem, 39, 3456-3457. ISSSN 0022-3263

Huang Y., and Hayashi T., (2022). Chiral Diene Ligands in Asymmetric Catalysis, Chem. Rev., 122, 18, 14346-14404, https://doi.org/10.1021/acs.chemrev.2c00218

Kataoka, M., Kita, K., Wada, M., Yasohara, Y., Hasagawa, and J. Shimizu. A school-based mental health program for traumatized Latino immigrant children, S. App. Microb. Biotech. (2003), 62, 437-445. https://doi.org/10.1097/00004583-200303000-00011

Kongkona S, Abiram K., R., Ponnusamy S.K. , Sunita V., Mohideen N. , Rashmi L., Jenet G., Vinoth K.V., (2021), Recent advances in biotransformation of 5-Hydroxymethylfurfural: challenges and future aspects. J. Chem. Technol. Biotechnol. 97: 409-419, https://doi.org/10.1002/jctb.6670

Mane, S., (2016). Analytical Methods Racemic Drug Resolution: A Comprehensive Guide, Chemistry, Doi:10.1039/C6AY02015

Mindt M., Kashkooli A. B. , Suarez-Diez M. , Ferrer L. , Jilg T. , Bosch D. , Martins dos Santos V. , Wendisch F. V. and Cankar K., (2022). Production of indole by *Corynebacterium glutamicum* Microbial Cell Factories for Flavor and Fragrance Applications. Microbial Cell Factories Volume 21, Article number: 45. https://doi.org/10.1186/s12934-022-01771-y

Noyori, R., Suzuki, M. (2006). Angewandte, The Journal of Physical Chemistry B. 96, 854-882. https://doi.org/10.1021/ja064500p

Schoepe, J., Niefind, K., Chatterjee, S. and Schomburg, D. (2006) Cloning, expression, purification and preliminary crystallographic characterization of a shikimate dehydrogenase from *Corynebacterium glutamicum* Acta Cyristallographica 62, 635- 637. https://doi.org/10.1107/S1744309106017805

Sachin M., (2016). Racemic drug resolution: a comprehensive guide, Analytical Methods, Royal Society of Chemistry, Issue 42. https://doi.org/10.1039/C6AY02015A

Tanyeli, C., Özdemirhan D., Ezen, B. (2002) ROMP-Polymers in Asymmetric Catalysis: The Role of the Polymer Backbone, Tetrahedron Asymm. Mathematical & Physical Sciences, Scholarly Journals, Elsevier, 58, 9983-9988. https://doi.org/10.1002/1615-4169

Yinhua H., and Tamio H., (2022). Chiral Diene Ligands in Asymmetric Catalysis, Chem. Rev. 2022, 122, 18, 14346-14404 Publication Date: August 16, https://doi.org/10.1021/acs.chemrev.2c00218

Zelinski, T.; Kula, M. R. (1994). A kinetic study and application of a novel carbonyl reductase isolated from *Rhodococcus erythropolis*, Bioorg. Med. Chem. Bioorganic & Medicinal Chemistry Letters, 2, 421-428. https://doi.org/10.1016/0968-0896(94)80010-3

Zhuozhuo He, Lingzi Peng & Chang Guo, (2022). Catalytic stereodivergent total synthesis of amathaspiramide D, Nature Synthesis volume 1, pages 393-400. https://doi.org/10.1038/s44160-022-00063-y