APPLICATION OF Saccharomyces carlsbergensis OLD YELLOW ENZYME IN SYNTHESIS OF CHIRAL KETONES AND BUILDING BLOCKS FOR β-AMINO ACIDS

By

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To my loving parents, Helenie and Janowi Swiderskim.
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APPLICATION OF *Saccharomyces carlsbergensis* OLD YELLOW ENZYME IN SYNTHESIS OF CHIRAL KETONES AND BUILDING BLOCKS FOR β-AMINO ACIDS

By

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This dissertation describes the application of *S. carlsbergensis* Old Yellow Enzyme (an NADPH dependent yeast oxidoreductase) in the biotransformation of different types of activated alkenes. Two classes of compounds, substituted cyclohexenones and β-nitroacrylates, were synthesized and tested as potential substrates for OYE.

Both 2- and 3-substituted 2-cyclohexenones (5a-b and 9a-e) were shown to be reactive with the mentioned protein. Chemo- and stereoselective alkene reductions were observed and no alcohol products were detectable. In most of the cases, biotransformations proceeded with high optical purities, with the exception 2-exo-methylene cyclohexanones (13a-b), which were obtained as racemic mixtures. The enantioselectivities of the reactions were determined based on the chiral GC separation of the derivatized biotransformation products.

Enzymatic reductions of 2-substituted-β-nitroacrylates (20c-f) occurred with 87-96% of enantiomeric excess (e.e.), with larger substrates providing greater stereoselectivities. The products of the biotransformations were further chemically reduced to amino acid esters (22c-f) and derivatized with TFAA (trifluoroacetic anhydride) in order to assess the enantiomeric excess values. The acid hydrolysis of esters gave optically active β2-amino acids (23c-f), important drug
intermediates and subjects of biological studies. In the case of 3-substituted-\(\beta\)-nitroacrylates (20a-b), only racemic products were observed.
CHAPTER 1
HISTORICAL BACKGROUND OF OLD YELLOW ENZYME

Discovery and Structure of an Old Flavoprotein

*Saccharomyces carlsbergensis* old yellow enzyme (OYE) is known as the first discovered and characterized flavoprotein. It was isolated from brewers’ bottom yeast by Warburg and Christian in 1932 during their studies on the oxidation of glucose-6-phosphate by methylene blue. They discovered that the reaction takes place only when oxygen is reduced to hydrogen peroxide in the presence of additional elements called at that time “Zwischenferment”-identified later as glucose-6-phosphate dehydrogenase, “Gelbe Ferment”-yellow protein, and a heat-stable “Coferment”-NADP⁺ (1-1).

![Reaction system of Warburg and Christian.](image)

A few years after this discovery, another yeast flavoenzyme (D-amino acid oxidase) was isolated and named “das neue gelbe Ferment”. This led to the final name given to the Warburg’s protein: old yellow enzyme. In 1935, Hugo Theorell purified OYE and showed that it consists of two components: a colorless apoprotein and a yellow dye, both essential for the enzyme activity. In 1955, he identified the yellow element as flavin mononucleotide (FMN), which was later found to bind non-covalently to the enzyme active site (Figure 1-2).
Despite the fact that OYE had been known since 1932, its first crystallization was not achieved for nearly another 20 years by Hugo Theorell in 1955. Unfortunately, the quality of the crystals was not sufficient for X-ray studies, which were later explained as a probable result of the heterogeneity of natural OYE arising from the two genes present in S. carlsbergensis. Since then, several X-ray data were published mainly by Fox and Karplus, who solved the crystal structure at a resolution of 2.0 Å for the oxidized and reduced forms of recombinant intact Oye1 and for its complex with p-hydroxybenzaldehyde.

Based on Fox’s results, the enzyme was found to exist as an α/β barrel in the form of a dimer, with a monomer of ~45 kDa. Each single domain binds non-covalently the molecule of flavin mononucleotide (FMN) that interacts by hydrogen bonds with surrounding it amino acids.

Fox and Karplus used also spectroscopic techniques to obtain some additional information on solvent accessibility to the active site of the OYE. $^{13}$C and $^{15}$N NMR together with the X-ray
experiments revealed that it is the *si* face of the FMN that is exposed to solvent, when the *re* face is buried by interactions with the protein.

Since that time, OYE has been characterized in great detail, especially by Vincent Massey’s laboratory, and it has served as a model for studying other flavoproteins.\(^1,6\) Unfortunately, despite the extensive knowledge on the structure and reactivity of the old flavoprotein, the physiological role of OYE has remained unknown.

**Occurrence and Physiological Importance of OYE Family Members**

Several proteins with amino acid sequence homologous to OYE have been found in yeasts, plants and bacteria and their postulated physiological function gives some idea on the importance of old yellow enzyme in Nature.\(^7\) Based on those reports a number of possible functions for *Saccharomyces carlsbergensis* OYE were suggested, from assembly of the yeast cytoskeleton\(^6\) to oxidative stress response in yeast.\(^8\)

In *Saccharomyces cerevisiae*, two enzymes have been discovered, OYE2 and OYE3, with sequence closely related to OYE1. The most recent studies on acrolein toxicity in this strain of yeast (*Saccharomyces cerevisiae*) point to the role of OYE2 as a main agent mediating resistance to small \(\alpha,\beta\)-unsaturated carbonyl compounds, such as acrolein.\(^9\) However, based on the previous reports on the role of OYE in the control of the redox state of the actin cytoskeleton, the authors suggested that this group of enzymes is likely to have physiological functions beyond the simple detoxification of harmful metabolites.

There have been found some other strains of yeasts containing enzymes with genes related to OYE, such as

- **Candida albicans**: contains estrogen binding protein (*EBP1*), which possesses desaturase activity and reduces 19-nor-testosterone (OYE1 can only aromatize it).
• **Hansenula polymorpha**: contains hansenula yellow enzymes (*HYE1*, *HYE2* and *HYE3*), which have been found to increase the resistance towards high concentrations of allyl alcohol in the presence of alcohol oxidase (AO).

• **Kluyveromyces lactis**: contains kluyveromyces yellow enzyme (*KYE1*), whose physiological role is unknown.

• **Yarrowia lipolytica**: contains *N*-ethylmaleimide reductase.

• **Candida macedoniensis**: contains old yellow enzyme (*oye*).

A number of enzymes related to OYE have been identified in bacteria in the following strains

• **Gluconobacter suboxydans**: contains old yellow enzyme, physiological role unknown.

• **Pseudomonas putida M10**: contains morphinone reductase (*morB*), which reduces double bonds of both morphinone and codeinone, producing important pharmaceutical drugs like hydromorphone and hydrocodone, respectively.

• **Pseudomonas putida II-B and Pseudomonas fluorescenc I-C**: contain nitroester reductases (*XenA* and *XenB*, respectively), responsible for nitrate ester degradation.

• **Agrobacterium radiobacter**: contains glycerol trinitrate reductase (*Ner*), responsible for nitrate ester degradation.

• **Enterobacter cloacae Pb2**: contains pentaerythritol tetranitrate reductase (*Orn*), which is known for degradation of explosives, such as nitroglycerine (GTN) and 2,4,6-trinitrotoluene (TNT).

• **Escherichia coli JM109**: contains *N*-ethylmaleimide reductase (*NemA*)

• **Bacillus subtilis**: contains YqjM (*B14911_08447*), involved in detoxification.

• **Shewanella oneidensis**: contains SYE1, SYE3 and SYE3 (*AAN55488.1*, *AAN57126.1*, *AAN56390.1*, respectively), physiological role unknown.

Plant homologues of OYE were first identified during studies on elucidation of octadecanoid biosynthesis by Vick and Zimmerman, who isolated 12-oxophytodienoic acid reductase (OPR) from *Zea mays*, strain found in corn. The enzyme was found to catalyze one of the steps in jasmonic acid biosynthesis, the reduction of the double bond of 12-oxo-10,15(Z)-hytodienoic acid (OPDA). After this discovery several other plant strains, which contain proteins related to OYE, were identified, such as
- **Corydalis sempervirens**: contains 12-oxophytodienoic acid reductase (OPRI), which reduces \((9R,13R)\)-cis and \((9S,13R)\)-trans diastereoisomers of OPDA.

- **Arabidopsis thaliana**: contains 12-oxophytodienoate reductase 1 (OPR1), which reduces \((9R,13R)\)-cis and \((9S,13S)\)-cis diastereoisomers of OPDA.

- **Arabidopsis thaliana**: contains 12-oxophytodienoate reductase 2 (OPR2), which reduces \((9R,13R)\)-cis diastereoisomer of OPDA.

- **Arabidopsis thaliana**: contains 12-oxophytodienoate reductase 3 (OPR3), which reduces \((9R,13R)\)-cis, \((9S,13S)\)-cis, \((9R,13S)\)-trans and \((9S,13R)\)-trans diastereoisomer of OPDA.

- **Oryza sativa L. (rice)**: contains 12-oxophytodecanoic acid reductase (opda).

### Compounds Bound by OYE

In the process of searching for the physiological role of old yellow enzyme, researchers identified a number of compounds capable of inhibiting this protein.\(^{12-14}\) The OYE ligands bind with milimolar to micromolar affinities to the oxidized form of the enzyme.

Aromatic compounds with an ionizable hydroxyl substituent are one of the most studied group of inhibitors of OYE (Figure 1-3). Upon binding, the phenolic ligand OYE forms deeply colored long wavelength charge-transfer complexes, which can be detected by their green color.\(^15\)

Two other classes of binding ligands are simple monovalent anions (e.g., acetate, chloride or azide) and pyridine nucleotide derivatives such as the acid hydrolysis products of reduced form of nicotinamide adenine dinucleotide phosphate (NADPH). All three types of inhibitors, as well as substrates, share a common active site on the \(si\)-face of the flavin.

### Catalytic Properties of an Old Flavoprotein

The first reports on the reactivity of old yellow enzyme can be found in the same studies that helped Warburg discover this protein.\(^1\) During the attempts to elucidate the nature of glucose6-phosphate oxidation process, he identified OYE as the component oxidizing NADPH and reducing oxygen to hydrogen peroxide. Since then, several experiments proved that NADPH
Figure 1-3. Examples of typical ligands of OYE could be the physiological reductant for the old flavoprotein. There are some other compounds capable of reducing OYE, like reduced form of nicotinamide adenine dinucleotide (NADH) or sodium dithionite, but the efficiencies of the reactions are significantly lowered compared to NADPH.16,17

Substrates capable of oxidizing OYE include methylene blue, Fe^{3+}, quinones, cytochrome c and ferricyanide.18 The reoxidation of the enzyme can also be effected by molecular oxygen, producing H_{2}O_{2}.19 Recently, it has been found that a number of α,β-unsaturated aldehydes, ketones,20,21 and nitro compounds22 could serve as more efficient substrates for this enzyme (Figure 1-4).

These enzymatic reactions are chemoselective with exclusive reduction of the double bond of the olefin, but not the carbonyl or nitro groups (Figure 1-5). The presence of a stoichiometric amount of NADPH or another reductant for OYE is required during the reaction. It has been found by Massey and co-workers that in the absence of an agent capable of reducing OYE, the oxidative aromatization of the cyclic enones occurs.21 In this dismutation reaction, the substrate
Figure 1-4. Examples of α,β-unsaturated aldehydes, ketones and nitro compounds that serve as substrates for OYE

is first dehydrogenated, and subsequently the olefinic bond of a second substrate molecule is reduced (Figure 1-6).

Another interesting class of reactions catalyzed by old yellow enzyme is reduction of nitrate esters. The mechanism of the process is still not completely known but two pathways were proposed:

- **Pathway a:** the reaction involves a hydride transfer from the reduced flavin to the nitrogen of the nitrate residue (Figure 1-7). This step could be followed by or concerted with electron rearrangement, resulting in liberation of nitrite and formation of the alcohol product.

- **Pathway b:** the reduction involves sequential electron and proton transfers as described in Figure 1-8.
Figure 1-5. Example of reduction reaction catalyzed by OYE

Figure 1-6. Dismutation reaction catalyzed by OYE
Two of the compounds were particularly studied: glycerin trinitrate (GTN) and propylene dinitrate. The reactions resulted in product mixtures as the rate of reduction for primary and secondary nitrate was different (Figure 1-9).

**Mechanism of Old Yellow Enzyme**

There are several residues that are assumed to have a major role in the catalytic function of OYE. At least one of them, Thr-37, was found to strongly affect the reactivity of the enzyme by direct interaction with the flavin molecule.\(^{19}\) It was suggested that this residue plays an important
role in controlling the redox potential of the enzyme by stabilizing the negative charge of the reduced flavin by hydrogen bonding with the C-4 oxygen of the FMN (Figure 1-10).

![Chemical structures](image)

Figure 1-8. Nitrate reduction by OYE1: Pathway b

\[
\text{glycerine trinitrate (GTN)} \xrightarrow{\text{OYE, NADPH}} \text{HO} - \text{ON} - \text{ONO}_2 + \text{O}_2\text{NO} - \text{ON} - \text{ONO}_2
\]

Figure 1-9. Denitration reaction

\[
\text{propylene dinitrate} \xrightarrow{\text{OYE, NADPH}} \text{HO} - \text{ON} - \text{ONO}_2 + \text{O}_2\text{NO} - \text{OH}
\]
The determination of the crystal structure\textsuperscript{4} of the complex between Oye1 and $p$-hydroxybenzaldehyde was critical in identifying two important residues of the enzyme: histidine 191 and asparagine 194, which direct the positioning of the reactants (substrate or inhibitor) in the active site of OYE by formation of hydrogen bonds (Figure 1-11). These interactions are particularly important in the catalytic process, since they stabilize the anionic form of the reactant, which acts as an electron acceptor and is involved in charge-transfer interaction with the FMN group.

![Figure 1-10. Interaction between Thr-37 and FMN. In this projection the view is on the $\textit{si}$-face of the flavin.](image)

Further studies on the mechanistic role of the amino acid residues from the active site of OYE provided some important information about the catalytic cycle of this enzyme.\textsuperscript{24} The investigations were especially focused on tyrosine 196, which was suggested to be a proton donor in the substrate double bond reduction. The experiments were performed with different enones, including 2-cyclohexenone and 1-nitrocyclohexene. The results confirmed the importance of Tyr-196 only in the case of the ketones, where single mutation of the tyrosine residue to phenylalanine (Y196F) inhibited the process of reduction. Completely different
situation was observed for the nitro compound, where the same mutation (Y196F) almost did not affect the reaction and it was possible to obtain a saturated product without a visible inhibition.

\[ \text{Asn 194}^{\delta+} \]
\[ \text{His 191}^{\delta+} \]

Figure 1-11. Interaction between the substrate and Asn 194 and His 191 from OYE1 active site.

These results suggest different mechanisms for those two types of compounds

- **Mechanism a**: utilized by \(\alpha,\beta\)-unsaturated ketones and aldehydes. This reaction may be best described as a concerted mechanism in which the transfer of a hydride is possible by the presence of Tyr-196 primed for protonation (Figure 1-12).

- **Mechanism b**: utilized by 1-nitrocyclohexenones, may involve the formation of an \(aci\)-nitro intermediate by transfer of hydride to the substrate followed by its protonation either by Tyr-196 or water. In this case, the process does not depend on the presence of the tyrosine residue (Figure 1-13).

\[ \text{Asn 194}^{\delta+} \]
\[ \text{His 191}^{\delta+} \]

Figure 1-12. Reduction mechanism for ketones and aldehydes catalyzed by OYE

In order to complete the model of the catalytic cycle for old yellow enzyme, some additional studies were performed to establish the mechanism of the hydride transfer to the flavin from NADPH, which is assumed to be the physiological reductant for OYE.\(^2\) According to the
results of this investigation, the pro-$R$-hydrogen of NADPH is transferred as a hydride to the flavin $N_5$, then this is followed by the transfer of the same hydride to the $\beta$-carbon of the substrate (Figure 1-14). Base on the studies on the interaction between $p$-hydroxybenzaldehyde ligand and the active site of OYE, the flavin should be always positioned in the $re$ face of the bound substrate that means that the hydride uptake is possible only from this direction, which would make the reduction highly stereospecific (Figure 1-15).

![Reduction mechanism for nitrocyclohexene catalyzed by OYE](image)

Figure 1-13. Reduction mechanism for nitrocyclohexene catalyzed by OYE

It was suggested that NADPH and the substrate bind to the same site of the enzyme, requiring the protein to act by a ping-pong mechanism, which is consistent with the steady state kinetics of all forms of the enzyme studied (Figure 1-16).\textsuperscript{15,19,24,25}

**Purification of Old Yellow Enzyme**

Old yellow enzyme was first isolated in the form of a homogeneous, crystalline protein by Theorell and Åkerson in 1956 as a result of long studies started by Warburg. However, the original purification method was time-consuming with a very low efficiency. Since then, several attempts were made in order to improve this process. During the development of the procedure, it was discovered that the oxidized enzyme forms green colored charge-transfer complex with a
Figure 1-14. Catalytic cycle for old yellow enzyme

low molecular weight compound.\textsuperscript{12,13} Later, the ligand was identified as any aromatic molecule with an ionizable hydroxyl substituent, e.g. naturally occurring \( p \)-hydroxybenzaldehyde.\textsuperscript{14}

Additionally, it was found that the green complex dissociates upon reduction of the enzyme. This characteristic interaction between phenolic compounds and OYE helped Abramovitz and Massey to develop a simple purification procedure based on affinity chromatography.\textsuperscript{17} They used 4-hydroxy-\( N \)-\( n \)-butylbenzamide as an affinity matrix (Figure 1-17). In this method, protein isolation process consists of 3 steps. First, the solution of crude extract of brewer’s bottom yeast is loaded on the column and only OYE binds to the agarose containing phenolic ligand. The formation of the complex can be easily noticed as the gel changes color from white to green. In order to remove all the unbound proteins the column is washed several times with a Tris-HCl buffer (pH 8.0) that contains \((NH_4)_2SO_4\) and PMSF. The
presence of the last reagent methylsulfonyl fluoride proved to increase the efficiency of the process by inhibiting proteolysis.

Figure 1-15. Active site of OYE in complex with p-hydroxybenzaldehyde.

Figure 1-16. Kinetic mechanism of OYE

N-(4-hydroxybenzoyl)aminoheptyl agarose

Figure 1-17. Affinity matrix for OYE purification
The desired protein is eluted from the column by using the same washing buffer but with addition of 3 mM sodium dithionite, which acts as a reducing agent for the enzyme and releases it from the complex with the ligand from the matrix. Upon elution from the column, the protein becomes oxidized and returns to its yellow color. This procedure delivers the enzyme in nearly homogeneous form with high efficiency. Additionally, this way of purification is simple and does not require too many steps in comparison to the previous methods.
CHAPTER 2
CHIRAL CYCLOHEXANONES

Introduction

Optically active cyclic ketones, especially those bearing a stereogenic center α or β to the carbonyl group, are important reaction intermediates (synthons) for asymmetric synthesis. Among this group, chiral cyclohexanones are one of the most interesting synthons due to their broad applications in the production of biologically active substances. The synthesis of (-)-agarospirol, (-)-α-acorenol or (+)-β-acorenol, a family of sesquiterpenes used as ethereal oils in perfumery, can serve as an interesting example (Figure 2-1).²⁵,²⁶

Another class of compounds that find their origin in chiral α- or β-substituted cyclohexanones are lactones (Figure 2-2), which are present in various forms in numerous naturally occurring substances like antibiotics and essential oils (Figure 2-2).²⁸,²⁹

Figure 2-1. Application of 3-substituted chiral cyclohexanones.

Figure 2-2. Application of 3-substituted chiral cyclohexanones.
Figure 2-2. Application of 3- and 2-substituted chiral cyclohexanones.

Chemical and Enzymatic Methods toward Chiral Cyclohexanones

Considering the variety of applications for optically active substituted cyclohexanones, many research groups concentrate their work on developing methodologies to obtain these important chiral intermediates. To date, several procedures, both chemical and biochemical, have been developed. The two most widely used strategies are: i) the conjugate addition of organometallic reagents to $\alpha,\beta$-unsaturated compounds (Figure 2-3), and ii) the asymmetric conjugate reduction of cyclic enones (Figure 2-4). Unfortunately, these methods are limited only to formation of $\beta$-alkylated ketones.

Another procedure that is commonly applied in the synthesis of optically enriched $\alpha$- or $\beta$-substituted ketones is enantioselective reduction of the C-C double bond of the corresponding enone, which can be carried out using both chemical (Figure 2-5) and biochemical (Figure 2-6) catalysts.
Figure 2-3. Enantioselective conjugate addition of R$_2$Zn compounds to cyclic enones catalyzed by copper phosphoramidite.$^{28-30}$

Figure 2-4. Enantioselective conjugate reduction of β-substituted cyclic enones.$^{31,32}$
Figure 2-5. Chemical reduction of double bond of β-substituted cyclohexenones.

Figure 2-6. Enzymatic hydrogenation of C-C double bond of enones.

Most of the enzymatic reductions of α,β-unsaturated cyclic ketones yield not only saturated cyclohexanones but also the corresponding alcohols\(^{37-39}\) (Figure 2-7). One of the newest methods
leading to chiral $\alpha$-substituted cyclohexanones is enantioselective decarboxylative protonation recently reported by Stoltz and co-workers$^{40}$ (Figure 2-8).

Enantioselective alkylation of ketones via chiral enamines, first reported by Horeau,$^{41}$ subsequently modified by others, such as Meyers et al.,$^{42}$ delivers a highly enantioselective and efficient way of obtaining chiral $\alpha$-substituted cyclohexanones (Figure 2-9).

$$\text{Reductase} \rightleftharpoons \text{Synechococcus sp. PCC 7942}$$

Figure 2-7. Enzymatic hydrogenation of $\alpha,\beta$-unsaturated ketones.

$$\text{Pd}_{2}(\text{dba})_{3} + \text{L} \xrightarrow{\text{Et}_{2}\text{O, rt, 10 h}} \text{HCO}_{2}\text{H}$$

Figure 2-8. Catalytic enantioselective decarboxylative protonation.

Among other interesting approaches to $\alpha$-alkylated cyclohexanones is enzymatic hydrolysis of prochiral $\alpha$-substituted enol esters catalyzed by a number of esterases. The intermediates of the reaction, $\alpha$-substituted enols, undergo enantioselective rearrangement in the active site of the enzyme to yield optically active ketones.$^{28,43}$
Figure 2-9. Enantioselective alkylation of ketones via chiral enamines.

One of the most recent works characterizing this type of reaction describes esterases I and II isolated from cultured plant cells of *Marchantia polymorpha* (Figure 2-10). Another route to optically active substituted cyclohexanones is enantioselective protonation of prochiral enolates using chiral imides. An interesting example of this type of reaction was reported by Yamamoto et al. (Figure 2-11).

Figure 2-10. Enantioselective hydrolysis of enol esters.

Despite the variety of existing methods that lead to optically active cyclohexanones, there is constant demand for easier, environmentally friendly and universal for both alkyl substitutions
(α and β) synthetic routes. This fact forces chemists to explore new chemical and biochemical procedures.

![Chemical structure](image)

R = Me, n-Bu, Ph, Bz  4-67% e.e.

Figure 2-11. Enantioselective protonation of prochiral enolates using chiral imides.

**Old Yellow Enzyme Family Approach to Formation of Chiral Cyclohexanones**

Asymmetric hydrogenation by chiral rhodium or ruthenium phosphines has resulted in an impressive number of enantioselective alkene reductions during the last 20 years.\(^46,47\) Despite the tremendous progress in this area, high stereoselectivities nearly always depend on olefin proximity to highly polar groups such as amides, acids and alcohols.\(^48\) Attempts to generalize these procedures to aprotic oxygen functionalities such as aldehydes, ketones, esters or nitro groups have been much less successful; although some exceptions were reported (those examples were mentioned in the previous paragraph of this chapter). Moreover, the fact that organometallic approach requires preparation of complex chiral reducing agents and extreme reaction conditions makes this methodology unattractive, especially with respect to its environmental issues. Enzymatic alkene reductions might be one useful solution to problem.
While several isolated enzymes have been reported to reduce α,β-unsaturated cyclic ketones producing optically active saturated ketones,\textsuperscript{49,50} those of the old yellow enzyme (OYE) family have been characterized most thoroughly. The Stott et al. study reported several enones as good substrates for NADPH-mediated reduction by OYE1, among those many α,β-unsaturated cyclic ketones were found as quite reactive species (Figure 2-12).\textsuperscript{21}

Based on preliminary results on the reactivity of OYE1, many research groups extended their studies to the search for the other OYEs from different organisms. OYE2 and OYE3 were isolated from \textit{S. cerevisiae} and both of them showed activity towards α,β-unsaturated cyclic ketones.\textsuperscript{51,52} Similar results were obtained with old yellow enzyme from \textit{Candida macedoniensis}. The protein was discovered during the screening of different fungal species for the ability to reduce stereoselectively C-C double bond of ketoisophorone (KIP) to produce (6R)-levodione, a biologically important chiral synthon (Table 2-1).\textsuperscript{53} Reduction of unsaturated cyclic ketones was also reported among some of the bacterial relatives of old yellow enzyme.

![Chemical structures](image)

**Figure 2-12.** Substituted cyclohexenones as substrates for OYE1 ordered from the least to the most reactive enone.
In 1994, Bruce’s laboratory isolated morphinone reductase from *Pseudomonas putida* M10, enzyme responsible for reduction of olefin double bonds of morphinone and codeinone.\(^{54,55}\) The same group demonstrated the ability of this enzyme to reduce 2-cyclohexenone in an NADH-dependent manner.

OYEs have been also detected in other *Pseudomonas* species\(^ {54}\) and other bacteria, like: *Enterobacter cloacae*;\(^ {56}\) *Escherichia coli*;\(^ {57}\) *Bacillus subtilis*;\(^ {58}\) or *Shewanella oneidensis*.\(^ {59}\) All of them showed some reactivity towards 2-cyclohexenone (Table 2-2). Several OYEs were also isolated from plants. Among those, some demonstrated ability to reduce double bond of 2-cyclohexenone (Table 2-3).\(^ {60-63}\)

Despite the extensive studies on the crystal structure and substrate specificity of proteins from the old yellow enzyme family, the stereoselectivities of reductions catalyzed by those enzymes were never determined. There are only two reported examples that employed homologs of the *S. carlsbergensis* old yellow enzyme, both of which described production of \((6R)\)-levodione used in the synthesis of \((4R,6R)\)-actinol (Figure 2-13).\(^ {52,64}\) The above results suggested a new view on old yellow enzyme as a stereoselective catalyst that may be used in multi step reaction.

![Figure 2-13. Two-step conversion of ketoisophorone to \((4R,6R)\)-actinol using old yellow enzyme homologs and LVR.](image)

Combining all these results and properties, the protein appears to be an effective and inexpensive catalyst. In this work, we examined the substrate specificity and stereoselectivity of
the *S. carlsbergensis* old yellow enzyme and point to ways in which it can be employed in chiral building block production. Our approach was based on the chemical synthesis of the starting materials for the OYE1, in this case we concentrated on $\alpha,\beta$-unsaturated cyclohexenones, followed by their biohydrogenation using isolated enzyme or whole cells overexpressing OYE1.

Table 2-1. List of OYEs from yeasts and corresponding substrate specificity.

<table>
<thead>
<tr>
<th>Organism and Protein</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td></td>
</tr>
<tr>
<td>Old Yellow Enzyme 2</td>
<td>2-cyclohexenone</td>
</tr>
<tr>
<td>Old Yellow Enzyme 3</td>
<td>menadione</td>
</tr>
<tr>
<td><em>Candida macedoniensis</em></td>
<td></td>
</tr>
<tr>
<td>Old Yellow Enzyme</td>
<td>duroquinone</td>
</tr>
<tr>
<td></td>
<td>4-oxo-isophorone</td>
</tr>
</tbody>
</table>

Table 2-2. List of OYEs from bacterias and corresponding substrate specificity.

<table>
<thead>
<tr>
<th>Organism and Protein</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas putida II-B</em></td>
<td></td>
</tr>
<tr>
<td>Nitroester reductase</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens I-C</em></td>
<td></td>
</tr>
<tr>
<td>Nitroester reductase</td>
<td></td>
</tr>
<tr>
<td><em>Enterobacter cloacae</em></td>
<td></td>
</tr>
<tr>
<td>Pentaerythritol reductase</td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
</tr>
<tr>
<td>N-ethylmaleimide reductase</td>
<td></td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td></td>
</tr>
<tr>
<td>YqJIM</td>
<td></td>
</tr>
<tr>
<td><em>Shewanella oneidensis</em></td>
<td></td>
</tr>
<tr>
<td>SYE1, SYE3, SYE4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2-cyclohexenone</td>
</tr>
<tr>
<td></td>
<td>$R = \text{H, morphinone}$</td>
</tr>
<tr>
<td></td>
<td>$R = \text{Me, codeinone}$</td>
</tr>
</tbody>
</table>
Table 2-3. List of OYE s from plants and corresponding substrate specificity.

<table>
<thead>
<tr>
<th>Organism and Protein</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Corydalis sempervirens</em></td>
<td><img src="" alt="2-cyclohexenone" /></td>
</tr>
<tr>
<td>OPRI</td>
<td></td>
</tr>
<tr>
<td><em>Arabidopsis thaliana</em></td>
<td>12-oxophytodienoate reductase 2 and 3</td>
</tr>
<tr>
<td><em>Lecopersicon esculentum</em></td>
<td>12-oxophytodienoate reductase 1, LeOPR1</td>
</tr>
<tr>
<td><em>Pisum sativum</em></td>
<td>PsOPR1-6</td>
</tr>
</tbody>
</table>
CHAPTER 3
APPLICATION OF *Saccharomyces carlsbergensis* OLD YELLOW ENZYME IN SYNTHESIS OF CHIRAL CYCLOHEXANONES

**Synthesis of α,β-Unsaturated Cyclic Enones**

A number of compounds were synthesized in order to serve as starting materials for the enzymatic reductions with *S. carlsbergensis* old yellow enzyme. Early experiments on ligand binding to OYE suggested that the enzyme should be reactive with some α,β-unsaturated cyclohexenones.\(^\text{21,25}\) Moreover, the studies on the mechanism of ligand binding and interaction with the active site of OYE\(^1\) suggested that the process may be highly stereoselective. Based on already published data, we proposed three groups of substituted cyclohexenones that were examined as substrates for OYE, as described in Table 3-1.

<table>
<thead>
<tr>
<th>Cyclohexenones</th>
<th>Substituents</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-alkyl-2-cyclohexen-1-ones</td>
<td>(R = \text{Me, Et})</td>
</tr>
<tr>
<td>3-alkyl-2-cyclohexen-1-ones</td>
<td>(R = \text{Me, Et, } n-\text{Pr, } i-\text{Pr, } n-\text{Bu})</td>
</tr>
<tr>
<td>2-alkyldienecyclohexan-1-ones</td>
<td>(R = \text{Me, Et})</td>
</tr>
</tbody>
</table>

**General Procedure for Preparation of 2-Alkyl-2-Cyclohexen-1-ones**

The first effort to obtain this class of compounds was concentrated on the procedure reported by Ohta and coworkers,\(^\text{65}\) which consisted of two steps: a) bromination of 2-alkyl-cyclohexan-1-one with *N*-bromosuccinimide and b) subsequent dehydrobromination with aniline to give the corresponding 2-alkyl-2-cyclohexen-1-one (Figure 3-1). Unfortunately, after several attempts no positive result was obtained.
We turned our attention to another procedure, which consisted of three-steps resulting in the desired product. Although the first step of this method proceeded with very low yield, all the reagents were readily available and inexpensive. The synthesis of 2-alkyl-2-cyclohexen-1-ones began with a Friedel-Crafts acylation of the appropriate carboxylic acid with glutaryl chloride in the presence of aluminum chloride to yield the 2-alkylcyclohexane-1,3-diones and , which were converted to the corresponding 2-alkyl-3-isobutoxy-2-cyclohexenones and by reaction with and . The resulting vinylogous esters were reduced by lithium aluminum hydride (LAH) to afford the desired 2-alkyl-2-cyclohexen-1-ones (Figure 3-2).

**General Procedure for Preparation of 3-Alkyl-2-Cyclohexen-1-ones**

Our approach to 3-alkyl-2-cyclohexen-1-ones was based on a widely applied Grignard reaction. The reason why we chose this route was practical. First of all, the reagents used were readily available; second, the intermediates for all the 3-alkyl-2-cyclohexen-1-ones were the same (cyclohexane-1,2-dione and 3-isobutoxy-2-cyclohexenone ). The synthesis began by reacting 6 with 1-isobutanol to yield 7. This reaction was followed by addition of the appropriate Grignard reagent 8a-e to 7, and then the crude product was hydrolyzed in aqueous acid. The final enones 9a-e were purified by silica gel column chromatography (Figure 3-3).
General Procedure for Preparation of 2-Alkylidenecyclohexan-1-ones

The synthesis was based on procedure developed by Huang et al.\textsuperscript{80} which gave acceptable chemical yields. Although the authors reported that the method results in a mixture of cis- and trans-isomers, always one of them is obtained in high excess. The procedure began with aldol condensation of cyclohexanone with readily available aldehydes and gave expected aldols which were dehydrated by mesylation, followed by treatment with DBU.\textsuperscript{81,82} Enones were obtained as a mixture of isomers (80\% of \textit{E}; 20\% of \textit{Z}) (Figure 3-4).\textsuperscript{83,84} Unfortunately, attempts to separate the isomers did not give positive results.
Biotransformation of α,β-Unsaturated Cyclic Enones Using Old Yellow Enzyme

There are two major approaches in biotechnology when it comes to the physical state of biocatalyst, which can be applied either as isolated enzyme or in the form of whole microorganism. The final decision as to which of them should be used depends on many factors, such as: (i) the type of reaction, (ii) if there are cofactors to be recycled and (iii) the scale in which the biotransformation has to be performed.  

\[
\begin{array}{c}
\text{10} \\
\text{11 \ a \ R = Me} \\
\text{b \ R = n-Pr}
\end{array}
\]

Figure 3-4. General synthesis of 2-alkylidene cyclohexane-1-ones 13a and 13b.

In our studies, we first needed to examine the reactivity and stereoselectivity of the enzyme. The most secure way to accomplish this task was by using purified protein. In this way, we ensured that OYE is the source of chirality in the bioreduction products. The next step was to scale up (up to 1 gram) the characterized reactions by using whole cells and determine their efficiency.

Biotransformation Using Isolated OYE

The old yellow enzyme plasmid (pOYE-pET3b) (the plasmid was a gift from Professor Massey’s Laboratory) was transformed into BL21 (DE3) cells. The overexpressed protein was purified based on the procedure developed by Massey and coworkers.

Each of the ketones was tested as a substrate for the OYE using NADPH, which was supplied by a cofactor regeneration system. This was based on the conversion of NADP\(^+\) and
glucose-6-phosphate into NADPH and 6-phosphoglucono-δ-lactone, respectively, catalyzed by glucose-6-phosphate dehydrogenase (Figure 3-5). The presence of NADPH was required in order to avoid the dismutation reaction, catalyzed by OYE in the absence of this cofactor, which results in formation of phenol compounds.²¹

![Diagram of biotransformation of cyclic enones using isolated OYE and NADPH regeneration system.](image)

Figure 3-5. Biotransformation of cyclic enones using isolated OYE and NADPH regeneration system.

The first step in the biotransformation of enones was to test them as potential substrates for the enzyme. This procedure was performed on small scales, with substrate concentration of 3 mM, and the ketones were introduced to the reaction in the form of stock solutions with ethanol, which increased the solubility of organic compounds in aqueous buffer. The reductions were carried out at room temperature. The progress of the biotransformations was monitored by taking 30 µL samples and extracting them with 30 µL of EtOAc. The organic phase was analyzed by non-chiral-phase gas chromatography and mass spectrometry. Each of the reactions went to completion, except 9e and 13b, which were not reduced.

The next step was to define the stereoselectivity of the enzyme. This was achieved by scaling up the reactions to 20 mg of substrate, an amount sufficient for further analysis. A stoichiometric quantity of β-cyclodextrin was added to promote the substrate solubility in the
reaction solution. The enantiomeric excess of the products obtained was assessed by chiral-phase
gas chromatography. The complete separation of the chiral ketones was possible only after their
derivatization with \((2R,3R)-(-)-2,3\)-butanediol\(^{86}\). The same procedure was applied to the
corresponding racemic cyclohexanones to demonstrate baseline resolution of enantiomers
(Figure 3-6). Product absolute configurations were revealed by comparison with authentic
standards available from earlier studies.\(^{87}\)

![Figure 3-6. Derivatization of cyclic enones.](image)

According to the results of the scaled up biotransformation with isolated enzyme, after 24
hours, only the least substituted substrates (5a and 9a) were completely reduced. Larger ketones
were not fully reduced, even after 24 h and the conversion did not proceed further after this time
(Table 3-2). Based on GC analysis, the conversion decreased with the length of the substituent,
with two examples of no conversion (9e and 13b).

The enzyme displayed high enantioselectivity towards 2- and 3-substituted cyclohexenones
(series of 5 and 9) with enantiomeric excess values raging from 90 to 97% (Table 3-2).
Moreover, the results of the absolute configuration assessment support the OYE reduction
mechanism proposed by Massey and coworkers. According to this model, reduction of
3-substituted cyclohexenones should deliver the S enantiomers, if we assume that the double
bond is at the right hand side as illustrated in Figure 3-7. This is a result of hydride donation by
reduced FMN from the re-face. With simultaneous α-face protonation by the phenol of Tyr 196,
which can be seen as formation of 2-substituted cyclohexanone with excess of its $R$ enantiomer (Figure 3-7). Attempted reductions of 2-alkylidenecyclohexanones resulted in very low extents of conversions and almost racemic mixtures of products (Figure 3-8).

**Biotransformation Using Whole Cells of *E. coli* BL21(DE3)(pOYE-pET3b)**

The application of isolated enzymes in the biotransformation of organic compounds has its pros and cons. One of the major disadvantages of this approach is the requirement for cofactor recycling. In the case of old yellow enzyme, the regeneration of NADPH becomes very costly, especially when applied to bigger scale reactions. One useful solution to this problem may be biocatalysis mediated by whole cells.

Our strategy was to increase the scale of the enone reductions using whole cells of *E. coli* BL21(DE3)(pOYE-pET3b) that overproduced the old yellow enzyme from *S. carlsbergensis*. Flavoprotein expression was induced by adding isopropylthio-β-D-galactoside (IPTG) when the cultures reached the early logarithmic phase of growth. After 30 min, the appropriate ketone and stoichiometric amount of β-cyclodextrin (to increase substrate solubility) were added. The reactions were allowed to proceed at room temperature until the bioconversions ceased.

![Figure 3-7. Schematic diagram of the *S. carlsbergensis* old yellow enzyme active site.](image-url)
Figure 3-8. Biotransformation of substituted cyclic enones using isolated old yellow enzyme.

Table 3-2. Reduction of substituted cyclic enones by isolated old yellow enzyme.

<table>
<thead>
<tr>
<th>Ketone</th>
<th>R</th>
<th>Conversion (%)</th>
<th>ee (%)</th>
<th>Configuration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5a</td>
<td>Me</td>
<td>100</td>
<td>97</td>
<td>R</td>
</tr>
<tr>
<td>5b</td>
<td>Et</td>
<td>40</td>
<td>92</td>
<td>R</td>
</tr>
<tr>
<td>9a</td>
<td>Me</td>
<td>100</td>
<td>96</td>
<td>S</td>
</tr>
<tr>
<td>9b</td>
<td>Et</td>
<td>81</td>
<td>95</td>
<td>S</td>
</tr>
<tr>
<td>9c</td>
<td>n-Pr</td>
<td>30</td>
<td>90</td>
<td>S</td>
</tr>
<tr>
<td>9d</td>
<td>i-Pr</td>
<td>23</td>
<td>92</td>
<td>S</td>
</tr>
<tr>
<td>9e</td>
<td>n-Bu</td>
<td>NR(^a)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13a</td>
<td>Me</td>
<td>40</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13b</td>
<td>n-Pr</td>
<td>NR(^a)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\) No reaction

Experiments suggested that the enones were ultimately toxic to one or more of the reaction components after extended periods. The biotransformation with whole cells delivered similar results to those obtained with isolated enzyme (Table 3-3). The decrease in substrate conversion was observed as the size of the substituents increased. 2-Exo-methylene cyclohexanones gave either very low or no conversion. This was also the case for pure enzyme reactions. Additionally,
the enantioselectivity of the biotransformations mediated by whole cells was just slightly lower than with isolated protein. One of the reasons for this may be the presence of other reductases in the cells *E. coli* that could to small extent affect the reduction by OYE (Figure 3-9), (Table 3-3).

![Chemical structures](image)

**Figure 3-9.** Biotransformation of substituted cyclic enones using whole cells.

<table>
<thead>
<tr>
<th>Ketone</th>
<th>R</th>
<th>Conversion (%)</th>
<th>ee (%)</th>
<th>Configuration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5a</td>
<td>Me</td>
<td>100</td>
<td>96</td>
<td>R</td>
</tr>
<tr>
<td>5b</td>
<td>Et</td>
<td>16</td>
<td>90</td>
<td>R</td>
</tr>
<tr>
<td>9a</td>
<td>Me</td>
<td>100</td>
<td>94</td>
<td>S</td>
</tr>
<tr>
<td>9b</td>
<td>Et</td>
<td>76</td>
<td>95</td>
<td>S</td>
</tr>
<tr>
<td>9c</td>
<td>n-Pr</td>
<td>25</td>
<td>89</td>
<td>S</td>
</tr>
<tr>
<td>9d</td>
<td>i-Pr</td>
<td>18</td>
<td>90</td>
<td>S</td>
</tr>
<tr>
<td>9e</td>
<td>n-Bu</td>
<td>NR(^a)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13a</td>
<td>Me</td>
<td>40</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13b</td>
<td>n-Pr</td>
<td>NR(^a)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\) No reaction

**Biotransformation Using Sodium Dithionite as Reducing Agent for Old Yellow Enzyme**

The concept of using sodium dithionite as a reductant for the enzyme, instead of NADPH, was based on the fact that the hydride that reduces the substrate does not come directly from
nicotinamide cofactor but from the flavin and so any chemical reagent (in this case Na$_2$S$_2$O$_4$) that can reduce the FMN can substitute for NADPH (Figure 3-10).

![Figure 3-10. Biotransformation of substituted cyclic enones using sodium dithionite as reducing agent for old yellow enzyme.]

Since reactions with sodium dithionite require basic conditions and exclusion of oxygen, which causes autooxidation of Na$_2$S$_2$O$_4$, the buffer used for the biotransformations was degassed and its pH increased from 7.0 to 8.0. Substrates (5a, 5b and 9a-e) and β-cyclodextrin were added in a 1:1 ratio in the presence of the excess Na$_2$S$_2$O$_4$. After 24 hours, although product formation was detected, none of the reactions proceeded to completion (Table 3-4). Two major issues that might have contributed to the decreased conversion are:

- Oxidation of sodium dithionite by oxygen from the air that was not completely eliminated.
- Reaction between the substrates and sodium dithionite.

It has been reported in the literature that Na$_2$S$_2$O$_4$ can serve as a strong reducing agent for cyclohexenones (Figure 3-11). In this case, dithionite may reduce both enzyme and starting material. The GC/MS spectra suggested that this may be the reason for the loss of substrate without product conversion. On the other hand in the reaction analysis data there was no sign of
phenol compounds formation which could be the result of the dismutation reaction between OYE and the cyclohexenones.

\[
S_2O_4^{2-} + H_2O \rightarrow HSO_2^- + HSO_3^- \\
HSO_2^- + S_2O_4^{2-} \rightarrow HSO_3^- + S_2O_3^{2-}
\]

Figure 3-11. Reduction of α,β-unsaturated ketones by Na₂S₂O₄.

Table 3-4. Reduction of substituted cyclic enones using sodium dithionite as reducing agent for Old Yellow Enzyme.

<table>
<thead>
<tr>
<th>Ketone</th>
<th>R</th>
<th>Conversion (%)</th>
<th>ee (%)</th>
<th>Configuration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5a</td>
<td>Me</td>
<td>60</td>
<td>97</td>
<td>R</td>
</tr>
<tr>
<td>9a</td>
<td>Me</td>
<td>60</td>
<td>96</td>
<td>S</td>
</tr>
<tr>
<td>9b</td>
<td>Et</td>
<td>20</td>
<td>95</td>
<td>S</td>
</tr>
<tr>
<td>9c</td>
<td>n-Pr</td>
<td>5</td>
<td>90</td>
<td>S</td>
</tr>
<tr>
<td>9d</td>
<td>i-Pr</td>
<td>NRᵃ</td>
<td>92</td>
<td>S</td>
</tr>
</tbody>
</table>

ᵃ No reaction

Conclusions

Sketching the rough outlines of old yellow enzyme’s substrate- and stereoselecivities was the goal of this study, which employed a homologous series of simple alkyl-substituted enones.⁴⁸ The enzyme displayed gratifying enantioselectivity. Moreover, the absolute configurations of the products could be predicted reliably from a simple model derived from X-ray crystallography.
data (Figure 3-7). Because of hydrogen bonding with the carbonyl oxygen and the requirement that the β-carbon lie above \( N_5 \) of the flavin, reduced FMN must deliver hydride to the \( re \) face of the bound cyclohexenones while the protonation must occur from the \( si \) face.

Increasing the extents of conversion, particularly for larger substrates, is a key challenge the must be overcome before the enzyme can be considered synthetically useful. Nonetheless, the present results underscore the high potential of \( S. \) \textit{carlsbergensis} \( \) old yellow enzyme and probably related proteins in stereoselective organic synthesis.
CHAPTER 4
CHIRAL β-AMINO ACIDS

Introduction

β-Amino acids occur in nature both in free and bound form, and even though they are less abundant than their α-analogues, they have become one of the most investigated subjects in chemistry and biology. Especially interesting for scientists are the properties of oligomers composed exclusively of β-amino acids (so called β-peptides), which are stable to metabolism, exhibit slow microbial degradation and are stable to proteases and peptidases.89

There are three general types of open-chain chiral β-amino acids, depending on whether the substitution takes place at the carbon bearing the carboxyl group (α-position), the carbon bearing the amino group (β-position), or at both positions (α,β-disubstitution) (Figure 4-1).90 Recently, Seebach and co-workers91,92 proposed the terms β2- and β3-amino acid, where the numbers indicate the position of the side chains, in order to distinguish positional isomers (Figure 4-2).

![Figure 4-1. Linear β-amino acids](image)

![Figure 4-2. Nomenclature proposed by Seebach and co-workers.](image)
Enantiopure $\beta^2$-phenylalanine and $\beta^2$-homovaline were recently synthesized by Gellman and co-workers\textsuperscript{93} to provide access to new $\beta$-peptides with specific conformations and particular functions (Figure 4-3). Besides their importance for peptidomimetic studies, $\beta$-amino acids are also gaining attention as potential precursors for natural products and pharmaceuticals. Several examples of this kind of amino acid structure can be found as an essential component of biologically active compounds.\textsuperscript{94} One of the simplest $\beta$-amino acids, (R)-2-methyl-3-aminopropionic acid, is a residue in cryptophycin, a potent antitumor depsipeptide (Figure 4-4).\textsuperscript{95}

![Figure 4-3. Enantiopure $\beta^2$-phenylalanine and $\beta^2$-homovaline.](image)

![Figure 4-4. Cryptophycin and its precursor.](image)

Carbocyclic $\beta$-amino acids, like cispentacin, and their heterocyclic analogues, such as methylphenidate, are useful intermediates for the enantioselective synthesis of antifungal antibiotics\textsuperscript{96} and mental disorder medications, respectively (Figure 4-5).\textsuperscript{97}
(R)-(+-)-3-Amino-3-phenyl-2,2-dimethylpropionyl derivative NSL-95301 is a novel trisubstituted \( \beta \)-amino acid exhibiting potent inhibition of platelet aggregation, which makes it promising antithrombotic agent (Figure 4-6).\(^{98}\)

\[
\text{cispentacin} \quad \text{methylphenidate}
\]

Figure 4-5. Carbocyclic and heterocyclic \( \beta \)-amino acids.

\[
\text{(R)-(+-)3-amino-3-phenyl-2,2-dimethylpropanoic acid} \quad \text{(R)-(+-)NSL-95301}
\]

Figure 4-6. (R)-(+-)-3-Amino-3-phenyl-2,2-dimethylpropanoic acid and its derivative.

**Chemical and Enzymatic Routes to \( \beta \)-Amino Acids**

The importance of \( \beta \)-amino acids and their derivatives in the field of pharmacology and in peptide chemistry is well represented by the multitude of reports that have been published in the past decades. Additionally, since the far-reaching discovery that \( \beta \)-peptides form much more stable structures than their \( \alpha \)-peptidic natural counterparts, there has been an ever-growing interest in synthesizing \( \beta \)-amino acids with various substitution patterns. In particular, the preparation of enatiomerically pure \( \beta \)-amino acids has become an important and challenging endeavor for organic chemists.\(^{89}\) There are eight main approaches available till date for
stereoselective synthesis of β-amino acids: homologation of α-amino acids, enzymatic resolution, addition of enolates to imines, Curtius rearrangement, conjugate addition of a nitrogen nucleophile to α,β-unsaturated esters or imides, hydrogenation, amino hydroxylation and β-lactam synthesis.99

A number of homologation reaction examples in the synthesis of β-amino acids have been reported,100-102 since this method is considered to be the best for one carbon chain elongation of carboxylic acid. Seebach and co-workers have utilized the Arndt-Eistert procedure in β-peptide synthesis to produce β-amino acid derivatives in enantiomerically pure form (Figure 4-7).103 Unfortunately, this method is limited to the synthesis of β3-amino acids, with only few exceptions to their α-substituted equivalents. One of the examples of the application of the Arndt-Eistert homologation in the synthesis of β2-amino acids is proposed by Yang and co-workers104 synthesis of α-substituted-β-amino esters (Figure 8).

Figure 4-7. Arndt-Eistert homologation.

Figure 4-8. Arndt-Eistert homologation in β2-amino acids synthesis.

Enzymatic resolution of β-amino acids is a cheap and environmentally friendly approach to their optically active derivatives. This method can be applied in several ways, but two commonly
used strategies are stereoselective acylation of one enantiomer of the racemic β-amino esters\textsuperscript{105} and hydrolysis of \(N\)-phenylacetyl derivatives, both of which are catalyzed by lipases\textsuperscript{106} (Figure 4-9 and 4-10).

\[
\begin{align*}
\text{NH}_2 & \quad \text{O} \\
\text{R} & \quad \text{OEt}
\end{align*}
\]

\[
\begin{align*}
\text{lipase} & \quad \text{PrCOOR}_1 \\
\text{Pr} & \quad \text{NH} \quad \text{O}
\end{align*}
\]

\[
\begin{align*}
\text{R} & = \text{Me, Et, } \text{n-Pr, } \text{i-Pr} \\
\text{R}_1 & = \text{CH}_2\text{CF}_3 \text{ or } \text{Bu}
\end{align*}
\]

**lipase - lipase A from *Candida antarctica***

Figure 4-9. Stereoselective acylation of one enantiomer of the racemic β-amino esters.

\[
\begin{align*}
\text{NH}_2 & \quad \text{O} \\
\text{R} & \quad \text{OH}
\end{align*}
\]

\[
\begin{align*}
\text{Et}_3\text{N} & \quad \text{Ph} & \quad \text{O} \\
\text{Cl} & \quad \text{O}
\end{align*}
\]

\[
\begin{align*}
\text{acylase} & \quad \text{H}_2\text{O} \\
\text{acetylase} & \quad \text{H}_2\text{O}
\end{align*}
\]

\[
\begin{align*}
\text{R} & = \text{C}_6\text{H}_5, \text{4-F-C}_6\text{H}_4, \\
& \text{2-F-C}_6\text{H}_4, \text{4-MeO-C}_6\text{H}_4
\end{align*}
\]

**acylase - penicillin acylase from *E. coli* ATCC 9637**

Figure 4-10. Hydrolysis of \(N\)-phenylacetyl derivatives of β-amino esters.

Similar chemical procedures may be achieved by formation of diastereomeric salts via complexation with a chiral base, for example (-)-ephedrine (Figure 4-11).\textsuperscript{107} The diastereomeric salts a and b can be separated by fractional crystallization due to their difference in solubility in
a suitable solvent (Figure 4-11). However, the process of multistep recrystallizations is long and tedious.

Enzymatic resolution was also applied as one of the steps in the synthesis of α-hydroxy β-amino acids, which are important class of compounds that possess interesting bioactivities. Cardillo and Gentilucci reported a two-step approach to the production of syn-α-hydroxy β-amino acids (Figure 4-12). The two key steps in this process were PGA catalyzed kinetic resolution of a racemic amino acid ester, followed by the highly diastereoselective formation of trans-oxazoline. Escalante and Juaristi have demonstrated the utilization of pyrimidinones in the synthesis of α-hydroxy β-amino acids (Figure 4-13).

![Figure 4-11. Chemical resolution of β-amino acids.](image)

![Figure 4-12. Synthesis of α-hydroxy β-amino acids.](image)
chiral β-amino acids is transformation of the carboxy into an amino group by means of a Curtius rearrangement (Figure 4-14). Sibi and Deshpande used this methodology in stereoselective preparation of iturinic acid and 2-methyl-3-aminopropanoic acid, components of biologically important peptides iturin and cryptophycin.\textsuperscript{110}

Among various strategies available to date, conjugate addition of an amine nucleophile to α,β-unsaturated carboxylic acid derivatives represents one of the most attractive methods for the stereoselective synthesis of β-amino acids.\textsuperscript{94,111} There are basically three ways to achieve asymmetric induction using this methodology

Figure 4-13. Asymmetric synthesis of β-amino acids derivatives.

Figure 4-14. Stereoselective preaparation of iturinic acid and 2-methyl-3-aminopropanoic acid using Curtius rearrangement. \* 1) Et\textsubscript{3}N, ClCO\textsubscript{2}Et, acetone, 0°C, 1h; 2) NaN\textsubscript{3}, H\textsubscript{2}O, acetone, 0°C, 1h; 3) toluene, heat, 1h; 4) t-BuOH, heat, 12-24h
• **Method 1:** addition of a “chiral ammonia” equivalent to an acceptor (Figure 4-15).

• **Method 2:** addition of a nitrogen nucleophile to a chiral acceptor (Figure 4-16).

• **Method 3:** asymmetric catalysis (Figure 4-17).\(^9^9\)

The first case is well represented by the protocol developed by Davies and co-workers,\(^{11^2}\) who used lithium amides as synthetic equivalents of ammonia (Figure 4-18). The second case can be described by the addition of diphenylmethanamine to chiral crotonates, reported by Chiaroni and co-workers (Figure 4-19).\(^{11^3}\) The synthesis of β-aryl-β-amino acid derivatives using catalytic amounts of a chiral Lewis acid can serve as an example for the third case of conjugate addition (Figure 4-20).\(^{11^4}\)

![Figure 4-15. Addition of a “chiral ammonia” equivalent to an acceptor.](image)

\[ Z = \text{achiral template} \]

![Figure 4-16. Addition of a nitrogen nucleophile to a chiral acceptor.](image)

\[ X_c = \text{chiral auxiliary} \]

![Figure 4-17. Conjugate addition of amine nucleophile by asymmetric catalysis.](image)
\[ \text{Figure 4-18. Asymmetric synthesis of } \beta\text{-amino acids via conjugate addition of chiral metallated amines.} \]

\[ \text{Figure 4-19. Addition of a nitrogen nucleophile to a chiral acceptor.} \]

\[ \text{Figure 4-20. Asymmetric catalysis in conjugate addition.} \]

Reductions of \( \alpha,\beta \)-unsaturated esters or nitriles can serve as another interesting approach to enantiopure \( \beta \)-amino acids, which can be achieved in two ways: 1) catalytic reduction and 2) reductive amination. These two strategies are exemplified by
• **Synthesis of β-amino esters and synthesis of β\(^2\)-amino acids:** proceed through rhodium catalyzed hydrogenation of 3-aminoacylates (Figure 4-21)\(^{115}\) and rhodium-catalyzed hydrogenation of β-phthalimide acrylates (Figure 4-22).\(^{116}\)

• **Synthesis of β-aryl-β-amino esters and synthesis of β-amino esters:** proceed through hydrogenation of β-enamino esters catalyzed by Pearlman’s catalyst (Figure 4-23)\(^{117}\) and direct reductive amination of β-keto esters with NH\(_4\)OAc and H\(_2\) in the presence of (\(R\))-L-Ru catalyst (Figure 4-24).\(^{118}\)

One of the most important derivatives of β-amino acids are β-lactams with a broad application in the production of biologically active substances. Despite their significance, there are only few protocols for their construction. The development of the efficient methods for their asymmetric synthesis is still a very active area of research.\(^{119}\) The Staudinger reaction, which involves [2+2] ketene-imine cycloaddition, is one of the most reliable methods available for the construction of β-lactam rings.\(^{120}\) Lecka et al. developed a modified Staudinger reaction, where nucleophilic ketene was generated in the presence of 10 mol% of benzoylquinine (BQ) (Figure 4-25).\(^{121}\)

Each of mentioned above approaches has advantages along with limitations.

The most important advantages of available approaches are:

• Arndt-Eistert homologation uses ready available, inexpensive and high enantiomerically pure α-amino acids as starting materials.

• Asymmetric addition of enolates or silyl enolates to imines gives very high e.e., around 98%.

• Catalytic hydrogenation process mostly uses the systems that tolerate an \(E/Z\) mixture of the substrates (derivatives of acrylic acid or nitrile), which simplifies the starting material preparation process.

The major disadvantages of mentioned methods are:

• Arndt-Eistert homologation is not suitable for large scale synthesis due to the high cost of the silver catalyst and danger of working with the hazardous reagent CH\(_2\)N\(_2\).
Figure 4-21. Rhodium catalyzed hydrogenation of 3-aminoacrylates.

Figure 4-22. Rhodium-catalyzed hydrogenation.
Figure 4-23. Hydrogenation of β-enamino esters catalyzed by Pearlman’s catalyst.

Figure 4-24. Direct reductive amination of β-keto esters with (R)-L-Ru catalyst
• Classical resolution of β-amino acids requires multistep fractional recrystallization, and therefore the sequence is long and tedious.

• The enzymatic resolution reactions need to be stopped at 50% of the conversion and so yield is usually low; another problem is a narrow tolerance of the enzymes toward racemic β-amino acids.

• Oppolzer’s sultam-chiral auxiliary is unstable at temperatures greater than -45°C.

• Evans’ chiral auxiliary is too expensive for the large-scale synthesis.

• Catalytic hydrogenation requires the use of expensive rhodium catalysts.

• All the mentioned methods (besides enzymatic resolution) are environmentally unfriendly chemical processes.

Based on above information on the synthesis of chiral β-amino acids, the development of an efficient, easy to operate, inexpensive and suitable for large-scale synthesis process, still remains a significant issue.

Old Yellow Enzyme Approach to Chiral β-Amino Acids

Old yellow enzyme is well known for its reduction of the olefinic bond of α,β-unsaturated carbonyl compounds by using NADPH as a cofactor. Moreover, we proved that these reactions are highly stereospecific.\textsuperscript{122}

Another class of similar compounds is that of unsaturated nitro compounds. Like a carbonyl group, a nitro group exerts a strong electron attracting influence within the molecule, enhancing the acidity of the hydrogen atoms attached to the carbon α to the substituent group. Nitro compounds also exhibit a tautomerism analogous to keto-enol tautomerism. Massey and co-workers found that old yellow enzyme is capable of reducing double bond conjugated with a nitro group (Figure 4-26).\textsuperscript{18}
Similar reactions can be catalyzed by other members of old yellow enzyme family, such as old yellow enzyme from *Candida macedoniensis*, N-ethylmaleimide reductase from *Escherichia coli* or YqjM from *Bacillus subtilis*.

It was discovered that the reduction catalyzed by OYE1 proceeds in a stepwise manner, with formation of a nitronate intermediate which is the result of hydride transfer to the β-carbon of the olefin. This process is followed by protonation of the nitronate at α-carbon to form the saturated nitroalkane. Both steps are catalyzed by the enzyme.

Meah and Massey suggested, based on results from theoretical model of interaction between active site of the protein and nitrocyclohexenone, that reduction of nitro-olefins by old yellow enzyme may proceed via a *trans*-addition across the double bond. If that is the case, it seemed reasonable to conclude that the reaction may be stereoselective, like it was showed for unsaturated cyclohexenones.

Based on this information, we extended our studies on the stereoselectivity and substrate specificity of old yellow enzyme to the substituted nitro acrylates. The reduction products served as intermediates for β-amino acids synthesis.

![Synthesis of β-lactams via modified Staudinger reaction](image)

Figure 4-25. Synthesis of β-lactams via modified Staudinger reaction.
Figure 4-26. Reduction of nitro-olefins by *S. carlsbergensis* old yellow enzyme.
CHAPTER 5
APPLICATION OF *Saccharomyces carlsbergensis* OLD YELLOW ENZYME IN SYNTHESIS OF CHIRAL β-AMINO ACIDS

**Synthesis of Mono-Substituted β-Nitroacrylates**

The preparation of β-nitro acrylic esters has been reported by several groups. Shechter et al. proposed their method based on nitration of corresponding acrylic esters with dinitrogen tetroxide (Figure 5-1). The reaction usually yielded a mixture of products in the form of methyl 3-nitroacrylate, methyl 2-hydroxy-3-nitropropionate, oxalic acid dihydrate and nitrogen-containing polymers of methyl acrylate.

![Figure 5-1. Synthesis of methyl 3-nitroacrylate using N₂O₄](image)

Besides this, nitryl chloride (Figure 5-2), nitrosyl chloride (Figure 5-2) and NaNO₂ in aqueous solution of CH₃CO₂H (Figure 5-3) have also been employed to synthesize
β-nitroacrylates from the corresponding acrylic esters. Unfortunately, the low boiling point of some of these reagents makes them inconvenient, especially if the reactions are carried out on small scales.

![Synthesis of β-nitroacrylates using NO2Cl or NOCl.](image)

**Figure 5-2.** Synthesis of β-nitroacrylates using NO2Cl or NOCl.

![Synthesis of β-nitroacrylates using nitrous acid.](image)

**Figure 5-3.** Synthesis of β-nitroacrylates using nitrous acid.

Recently, Vankar et al. reported their approach using NaNO2-ceric ammonium nitrate (CAN) in CH3CN in order to convert acrylic esters into β-nitro alcohols that were dehydrated via their mesylates by following modified McMurry’s method (Figure 5-4). The reaction is believed to proceed via radical intermediates, which may be the reason for formation of side
products and low yield of reaction (13-25%) (Figure 5-5). Despite our attempts to improve the efficiency (10-13% average yield) of the Vankar’s procedure, we were not able to obtain sufficient amount of products for further application.

![Chemical Reaction](image)

Figure 5-4. NaNO2-Ceric ammonium nitrate mediated conversion of acrylic esters into β-nitroacrylates.

![Chemical Reaction](image)

Figure 5-5. Proposed radical mechanism of 2-hydroxy-3-nitroacrylates formation.

After considering several synthetic methods to β-nitro acrylic esters, we decided to follow the protocol developed by Palmieri. The first step of this approach was a nitroaldol (Henry) reaction carried out under heterogeneous catalysis using a solid-phase base (Amberlyst A-21) along with the appropriate α-keto-ester (either commercially available or prepared by the method of Macritchie et al.) and nitroalkane. The NMR spectral data for nitroaldol adducts matched those reported previously. These were converted to the corresponding
nitroacrylates via mesylate derivatives (Figure 5-6). Both 3-alkyl-substituted nitroacrylates 19a and b were obtained predominantly in the \((E)\)-form (~90%)\(^{127}\) whereas the \((Z)\)-isomers predominated for 2-substituted alkenes (~80%) 19c-g, possibly as a result of E\(_1\)cB reaction on the corresponding mesylates. Because olefin geometry may directly impact the stereoselectivity of enzymatic reactions, the major alkene isomers were chromatographically enriched (>95% geometric purity).

Figure 5-6. Amberlyst A-21 mediated conversion of acrylic esters into \(\beta\)-nitroacrylates.

**Biotransformation of \(\beta\)-Nitroacrylates and Synthesis of \(\beta\)-Amino Acids**

We studied two approaches towards the bioreduction of \(\beta\)-nitroacrylic esters: i) biotransformation using isolated enzyme and ii) in the form of enzyme extract. The first method served for determination of the stereo- and enantioselectivity of the protein towards nitroacrylates. The reactions with extract were used to scale up the process.

**Biotransformation Using Isolated Enzyme**

Biocatalytic reductions utilized old yellow enzyme that had been purified by affinity chromatography. NADPH was supplied by a cofactor regeneration system (glucose-6-phosphate / bakers’ yeast glucose-6-phosphate dehydrogenase). Preliminary studies had revealed that olefin isomerization was more rapid under alkaline conditions, and pH 6.93 was selected to minimize this side-reaction while maintaining acceptable enzyme efficiency. A two-fold molar excess of
β-cyclodextrin (relative to the nitroacrylate) was also included to enhance substrate solubility under aqueous conditions. Unfortunately, the solubility of the β-cyclodextrin / 20g complex was still too low for efficient reduction, and the observed conversion was too low for further analysis of the product. Both the substrates (20a-f and glucose-6-phosphate) and the two enzymes were added portionwise to enhance the longevity of the processes, which were monitored by GC/MS and complete substrate consumption was observed after ca. 8 hr in all cases except for (E)-20b. The NMR and GC analysis of the crude products verified that only the double bond of the olefins had been reduced and the nitro groups remained intact. No significant levels of side products were observed and yields after purification ranged from 74-98%. Because it was not possible to determine the optical purities of the obtained products by chiral-phase chromatography, the crude materials were hydrogenated in the presence of Raney-Ni to the corresponding amines (23a-g) (75-85% yield) (Figure 5-7). Enantiomer separations were then possible by chiral-phase GC following derivatization with trifluoroacetic anhydride.

The racemic standards, required for enantioselectivity assignment, were obtained by hydrogenation of corresponding β-nitroacrylates catalyzed by Raney-Ni. Good optical purities were obtained from 2-alkyl-substituted nitroalkenes 20c-f; by contrast, 3-alkyl-substituted products were obtained in essentially racemic form.105,132-135 The absolute configurations were assigned by the direction of optical rotations of the free β2-amino acids as their hydrochloride salts (obtained by acid hydrolysis in 88-95% yields). Overall yields of β2-amino acids from β-nitroalkenes ranged from 57-73% (Table 5-1).

The results of our previous studies of alkyl-substituted-2-cyclohexenone reductions by old yellow enzyme122 were consistent with the net trans-hydrogenation mechanism elucidated by Massey and Karplus.4,15,24 Hydride β-addition (from reduced FMN) occurs from the re-face
while \( \alpha \)-protonation likely involves the phenol side-chain of Tyr-196. Carbonyl activation is achieved via hydrogen bonding by the side-chains of His-191 and Asn-194. For the acyclic \( \beta \)-nitroacrylates investigated here, analogous binding could occur in which one nitro-oxygen occupies the same location as the carbonyl oxygen and the alkene is positioned similarly (Figure 5-8).

![Diagram](image)

Figure 5-7. Biotransformation of nitroacrylates by OYE towards \( \beta \)-amino acids.

Table 5-1. Reduction of substituted nitroacrylates by isolated old yellow enzyme and production of \( \beta \)-amino acids.

<table>
<thead>
<tr>
<th>Nitroolefin</th>
<th>Conversion (%)</th>
<th>ee (%)</th>
<th>([\alpha]_{D})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(E)-20a</td>
<td>&gt;98</td>
<td>8((R))</td>
<td>-1.1(^\circ) c 1.0 (-39.5(^\circ) c 0.56)(^{136})</td>
</tr>
<tr>
<td>(E)-20b</td>
<td>50</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>(Z)-20c</td>
<td>&gt;98</td>
<td>87((R))</td>
<td>-13.0(^\circ) c 0.94 (-12.6(^\circ) c 1)(^{137})</td>
</tr>
<tr>
<td>(Z)-20d</td>
<td>&gt;98</td>
<td>91((R))</td>
<td>-2.7(^\circ) c 1.0 (-2.9(^\circ) c 1)(^{137})</td>
</tr>
<tr>
<td>(Z)-20e</td>
<td>&gt;98</td>
<td>94((R))</td>
<td>+1.2(^\circ) c 1.0 (3.5(^\circ) c 1)(^{137})</td>
</tr>
<tr>
<td>(Z)-20f</td>
<td>&gt;98</td>
<td>96((R))</td>
<td>-1.6(^\circ) c 1.0 (-14.4(^\circ) c 1)(^{138})</td>
</tr>
</tbody>
</table>

\(^{a}\) Measured in aqueous solution at the indicated concentrations from hydrochloride salts at room temperature.

This substrate binding orientation was verified by carrying out the enzymatic reductions of (E)-20b and (Z)-20c in D\(_2\)O (Figure 4-9) All MS and NMR data were consistent with deuterium incorporation only on nitro-bearing carbon in both cases, although they did not eliminate the possibility of the hydrogen exchange with water after the biotransformation, which could explain the racemization at the \( \beta \)-carbon.
In order to determine whether the mentioned hydrogen incorporation occurs we examined the deuterium exchange between the enzymatic reduction product $21a$ and deuterated phosphate buffer. After only five hours the deuterium incorporation could be detected in high levels in MS chromatogram by measuring the ratio of peak 117 to 116 as observed in Figures 5-10 and Figure 5-11.

Based on the above results, the formation of the racemic center at the $\beta$-position was attributed to the process of the epimerization related to the presence of the acidic hydrogen at the asymmetric $\beta$-carbon (Figure 5-12). Unfortunately, this data was not sufficient to confirm proposed mechanism of the biotransformation and additional tests using deuterated NADPH were required.

![Chemical structures](image)

Figure 5-8. Proposed mechanism of reduction of substituted-2-cyclohexenones and nitroacrylates by old yellow enzyme.
Figure 5-9. Biotransformation of nitroacrylates in D$_2$O.

Figure 5-10. Incorporation of deuterium into 21a.
There are two different approaches to the reactions with isotope labeled nicotinamide: either using isolated NADPD or applying NADPD regeneration system directly to the analyzed reaction. The first method seems to be rather unattractive considering the high cost of the reagents and the time consuming purification process. Instead, we turned our attention to the second method by using *Thermoanaerobium brockii* alcohol dehydrogenase (TBADH) and isopropanol-d8 in OYE reduction of (Z)-20c (Figure 5-13).\(^{139}\)

The MS and NMR data supported proposed net trans-addition of hydrogen to 20c-f that leads to the observed (R)-products. Additionally, in a preliminary experiment, (E)-20d was reduced by the OYE with largely (S)-stereoselectivity, as would be expected from the model
described in Figure 5-14. Within 7 hours, initially pure (Z)-20d incubated in buffer alone afforded three by-products in a combined conversion of ca. 50%. They were identified by GC/MS as (Z)-20d, the alternate acrylate alkene regioisomer of 20d and the water addition product from 20d (in racemic form) (Figure 5-15). These observations underscore the need to reduce β-nitroacrylates rapidly and minimize their exposure to the aqueous medium conditions.

Figure 5-13. Reaction of (E)-20c with NADPD.

Figure 5-14. Biotransformation of (E)-20d by isolated OYE1.

Figure 5-15. Incubation of 20d in KPi buffer.

**Biotransformation Using Cell-Free Extract**

Old yellow enzyme requires NADPH as cofactor which - in purified form - is very expensive in big amounts. The need to scale up the bioreductions forced us to look for
regeneration of NADPH by other means. One solution to this problem is application of the coupled-enzyme regeneration protocol.\textsuperscript{140} The procedure is based on combining cell extracts of glucose dehydrogenase from \textit{B. subtilis} - responsible for cofactor regeneration - and old yellow enzyme \textit{S. carlsbergensis}, both over expressed in \textit{E. coli}. Cells over expressing those two proteins were grown separately in 1 liter of LB medium and harvested just before they reached the stationary phase. Next, they were lysed and centrifuged in order to remove cell debris. The extracts were then mixed together in 100 ml KPi buffer (100 mM) and the biotransformation performed by portionwise addition of the appropriate nitroacrylate (20\textit{a}, \textit{c}, \textit{e} at final concentrations of 13 mM), a two-fold molar excess of β-cyclodextrin (relative to the nitroacrylate) and glucose (at a final concentration of 10 mM). The pH of the solution was constantly controlled and kept at 6.93. The reactions were allowed to proceed in room temperature with gentle stirring until the bioconversions ceased after 11 hours.

Although the chemical yields of the bioreductions were satisfactory (72-85\%), the enantioselectivity of the transformations of 20\textit{c} and 20\textit{e} was lower than in the case of isolated enzyme reactions (Table 5-2). The reason for this could be the presence of small amounts of other reductases in the extract (e.g. old yellow enzyme from \textit{E. coli}: NemA) that to some extent affected the final optical purity of the product.

Another problem may be insufficient amount of intercellular NADP\textsuperscript{+} and decreased production of the NADPH required by OYE1 as a cofactor. Additionally, the MS and NMR analysis identified by-products: (\textit{E})-20\textit{a}, 20\textit{c}, 20\textit{e} and the water addition product from 20\textit{a}, 20\textit{c} and 20\textit{e} (in racemic form) (Figure 5-16).

**Conclusions**

Old yellow enzyme-mediated reductions of 3-alkylsubstituted β-nitroacrylates 20\textit{a} and 20\textit{b} yielded essentially racemic products.\textsuperscript{141} Given the highly stereoselective α-protonation observed
in 2-cyclohexenone reductions, this result was surprising. The isotope wash-in following reduction analysis revealed chiral instability of the β-carbon of the biotransformation product, which undergoes spontaneous epimerization in aqueous media. The above explanation is further supported by Ohta and co-workers who reported racemization of similar α-substituted nitro compounds occurring both in basic and acidic water solutions. Further studies with deuterium labeled NADPH confirmed proposed net trans-addition mechanism by incorporation of deuterium at the α-carbon.

\[ \text{O}_2\text{N} \begin{array}{c} \text{CO}_2\text{Et} \\ \text{R}_1 \end{array} \begin{array}{c} \text{R}_2 \\ \text{Z-20c,e} \end{array} \quad \text{1. } \text{E. coli cell extract with over expressed OYE and GDH} \]

\[ \text{E-20a} \]

\[ \text{H}_2\text{N} \begin{array}{c} \text{CO}_2\text{H} \\ \text{R}_1 \end{array} \begin{array}{c} \text{R}_2 \\ 23\text{a,c,e} \end{array} \quad \text{2. } \text{H}_2, \text{Ra-Ni} \quad \text{3. } \text{HCl, } \Delta \]

Figure 5-16. Biotransformation of nitroacrylates using E. coli cell-free extract with overexpressed OYE and GDH.

<table>
<thead>
<tr>
<th>Nitroolefin</th>
<th>Conversion (%)</th>
<th>e.e. (%)</th>
<th>Configuration</th>
</tr>
</thead>
<tbody>
<tr>
<td>(E)-20a</td>
<td>96</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>(Z)-20c</td>
<td>98</td>
<td>73</td>
<td>R</td>
</tr>
<tr>
<td>(Z)-20e</td>
<td>97</td>
<td>89</td>
<td>R</td>
</tr>
</tbody>
</table>

\(^a\) Determined by chiral-phase GC following nitro group reduction and derivatization with trifluoroacetic anhydride.

In conclusion, our results have uncovered a new application for S. carlsbergensis old yellow enzyme in synthesizing optically active β²-amino acids. The synthetic route is concise and utilizes inexpensive starting materials. The major difficulties lie in suppressing alkene isomerization prior to reduction and ensuring active site protonation of the nitronate intermediate.
CHAPTER 6
EXPERIMENTAL SECTION

General Methods and Instrumentation

Standard media and techniques for growth and maintenance of E. coli were applied. Luria-Bertani (LB) medium used for bacterial cultivation contained 1% Bacto-Tryptone, 0.5% Bacto-Yeast Extract and 1% NaCl. Synthetic reactions were carried out under argon atmosphere, with the exception of water containing reactions. Dichloromethane, diethyl ether and tetrahydrofuran were dried on an MBRAUN solvent purification system using a double 4.8 L activated alumina columns type A2. Triethylamine was dried by distillation in the atmosphere of argon and stored over molecular sieves in -20°C. An ion-exchange resin Amberlyst A-21 was used in the water-moist free base form in nitroaldol reaction procedures. Reactions were monitored by thin-layer chromatography (TLC), using precoated silica gel plates (EMD Chemicals), or by gas chromatography using DB-17 column (0.25 mm x 25 m x 0.25 µm thickness) with a flame ionization detector. Products were purified by flash chromatography on Purasil silica gel 230-400 mesh (Whatman). For chiral separation, gas chromatography was applied which utilized a Chirasil-Dex CB column (0.25 mm x 25 m x 0.25 µm thickness) with mass spectrometric detection. NMR spectra were measured in CDCl₃, CDOD or D₂O solutions and recorded at room temperature on a Varian Mercury 300 spectrometer operating at 300 MHz for ¹H and 75 MHz for ¹³C, respectively, with chemical shifts (δ, ppm) reported relative to tetramethylsilane (¹H NMR) or residual solvent (¹³C NMR). IR spectra were obtained as neat films on NaCl plates using a Perkin-Elmer Spectrum One FT-IR spectrophotometer. Racemic β-amino acid esters were prepared from the corresponding nitroacrylates by hydrogenation at 500 psi in the presence of Raney nickel. A solution of 6M HCl was used in hydrolysis procedures of β-amino acid esters.
Purification of *S. carlsbergensis* Old Yellow Enzyme

All enzyme purification steps were carried out in 4°C. The plasmid encoding *S. carlsbergensis* old yellow enzyme (pOYE-pET3b) was a gift from Professor V. Massey’s laboratory. Routine *E. coli* strain BL21(DE3) (Novagen) transformations with pOYE-pET3b were performed by electroporation. The affinity matrix (N-(4-hydroxybenzoyl)aminohexyl agarose) was synthesized based on reported procedure.\(^{14}\)

**Cell Growth and Extract Preparation for Protein Isolation**

An overnight culture of *E. coli* (BL21(DE3)(pOYE-pET3b)) grown in LB medium containing 100 µg/ml ampicillin was diluted 1:100 into 4 L of the same medium in a New Brunswick M19 fermenter. The culture was stirred at 700 rpm with aeration at 4 L/min in 37°C until the optical density at 600 nm reached 0.8, then the enzyme overproduction was induced with isopropylthio-β-D-galactoside (IPTG) at a final concentration of 400 µM and the culture was stirred for an additional 2.5 hours at room temperature. The cells were harvested by centrifugation (5,000 rpm for 15 min at 4°C), washed twice with cold sterile water and then resuspended in 15 mL of buffer (40 mM Tris-Cl, 10 mM MgCl\(_2\), 10 mM DTT, 200 mM KCl, 1 mM PMSF and 10% glycerol, pH 8.0). The cells were lysed using a French Press and debris was removed by centrifugation at 20,000 rpm for 60 min at 4°C. The pH of supernatant was adjusted to 8.5 by adding concentrated ammonium hydroxide and the solution was made to 78% saturation with ammonium sulfate added portionwise over an hour. Then it was left for another 30 min stirring and the mixture was centrifuged at 20,000 rpm for 60 min at 4°C. The pellet was resuspended in 20 mL of buffer (0.1 M Tris-Cl, 0.1 M ammonium sulfate, 10 µM PMSF, 10 µM sodium dithionite, pH 8.0). The resulting solution was dialyzed against Tris-buffer (0.1 M Tris-Cl, 0.1 M ammonium sulfate, 10 µM PMSF, 10 µM sodium dithionite, pH 8.0) which was
changed three times over 20 hours, the second and third time without sodium dithionite. After 20 hours of dialysis the crude material was centrifuged at 20,000 rpm for 10 min at 4ºC.

**Isolation of Old Yellow Enzyme**

A 10 mL affinity column was washed with 600 mL of the starting buffer (0.1 M Tris-Cl, 0.1 M ammonium sulfate, 10 µM PMSF at pH 8.0) at a flow rate of 0.5 mL/min. The crude extract was applied and the column was washed with 1 L of the same buffer until the absorbance at 280 nm was less than 0.2. The enzyme was eluted with washing buffer (0.1 M Tris-Cl, 0.1 M ammonium sulfate, 10 µM PMSF, pH 8.0) which had been degassed and flashed with oxygen-free argon in a 500 ml suction flask and then supplemented with 3 mM sodium dithionite. The enzyme was collected in three or four 4 mL fractions in test tubes, concentrated by ultrafiltration and stored at -20ºC.

**Regeneration of Affinity Matrix**

The $N$-(4-hydroxybenzoyl)aminohexyl agarose was regenerated by washing with 0.2 M sodium acetate buffer, pH 5.0, containing 6 M guanidine HCl. Storage of the gel in Tris buffer, pH 8.0, with 1 mM sodium azide prevented microbial damage.

**Enzyme Activity Assay**

Old yellow enzyme activity was assayed by measuring the rate of NADPH oxidation at 340 nm at 25ºC. The standard assay system contained 0.2 mM NADPH (10 µL of 20 mM stock solution prepared immediately before use in 0.1 M KP$_i$ buffer, pH 7.0), 2.5 mM 2-cyclohexenone (100 µL of 25 mM stock solution in EtOH) and 1 µL of enzyme in a total volume of 1 mL. Glucose Dehydrogenase activity was assayed by measuring the rate of NADP$^+$ reduction at 340 nm at 25ºC. The standard assay system contained 0.5 mM of glucose (0.5 µL of 1 M stock solution in water), 0.1 mM NADP$^+$ (5 µL of 20 mM stock solution in KP$_i$ buffer) and 1 µL of GDH. The slope was calculated and used to find the specific activity. The background NADPH
oxidation was measured using an identical to above mentioned solution in which the amount of substrate was replaced by phosphate buffer. A unit of enzyme activity was defined as the quantity sufficient to oxidize 1 µmol of NADPH per minute in the mixture described above. Unit per ml of enzyme preparation were calculated based on the following equation:

\[
\text{Units / mL} = \frac{[(dA / dt \times 1000) / (\varepsilon_{340} \times l)] \times (V_{\text{assay}} / V_{\text{enzyme}}) \times \text{Dilution}}{\text{Dilution}, \text{where } dA / dt \text{ is the slope in AU / min, } \varepsilon_{340} = 6270 \text{ L / mol cm, } l = 1 \text{ cm, } V_{\text{assay}} = 1 \text{ mL, } V_{\text{enzyme}} = \text{volume of enzyme added in mL, Dilution = dilution factor.}}
\]

**Synthesis of 2-Alkyl-cyclohexane-1,3-diones**

**General procedure:** Glutaryl chloride (6.7 g, 40 mmol) and 80 mmol of the appropriate acid were added to a suspension of 13.58 g (103 mM) of AlCl₃ in 13 mL nitromethane with cooling in the atmosphere of argon. The mixture was heated for 3 hours at 80°C, then cooled to 10°C and poured onto 20 g of ice. After cooling to about 0°C, the crude product that had separated was filtered off, washed with 5 ml of cold water, and recrystallized from water (with charcoal). The aqueous phase of the filtrate was boiled with charcoal, filtered and extracted with ether. A second fraction crystallized after concentration of the ether extract.

**2-Methylcyclohexane-1,3-dione (3a).** White solid (1.0 g, 20%). \(^1\)H NMR (CDCl₃): \(\delta\) 0.75 (s, 3H), 1.80 (m, 2H), 2.29 (t, 2H), 3.29 (t, 1H) ppm. \(^13\)C NMR (CDCl₃): 7.7, 21.0, 33.0, 110.0 ppm (C2 signal not observed).

**2-Ethylcyclohexane-1,3-dione (3b).** Beige solid (0.84 g, 15%). \(^1\)H NMR (CDCl₃): \(\delta\) 0.75 (s, 3H), 1.76 (m, 2H), 2.08 (q, 2H), 2.24 (t, 2H), 3.18 (t, 1H) ppm. \(^13\)C NMR (CDCl₃): 12, 17.5, 21.5, 32.5, 108.0 ppm (C2 signal not observed).

**Synthesis of 3-Isobutoxy-2-alkylcyclohex-2-enone**

**3-Isobutoxy-2-methylcyclohex-2-enone (4a).** To a stirred solution of 2-methyl-1,3-cyclohexanedione (3a) (1.08 g, 8.57 mmol) and p-toluenesulfonic acid (108 mg) in
9 ml of benzene was added i-BuOH (2.6 mL). The mixture was heated at reflux under a Dean-Stark trap for 3 hours. The reaction mixture was cooled down to room temperature and poured into 6.5 mL of saturated aqueous NaHCO₃ solution and extracted with ether (3 x 5 mL). The combined extracts were washed with 5 mL of brine and dried over MgSO₄. The solvent was removed under reduced pressure, solution purified by column chromatography (silica gel, hexane/ethyl acetate = 5/1) to give 3-isobutoxy-2-methylcyclohex-2-enone as yellow oil (1.48 g, 95%). ¹H NMR (CDCl₃): δ 0.97 (d, 6H,), 1.71 (s, 3H), 1.93 (m, 1H), 2.31 (t, 2H), 2.51 (t, 2H), 3.39 (t, 2H), 3.73 (d, 2H) ppm. ¹³C NMR (CDCl₃): 7.0, 18.7, 21.3, 27.3, 32.2, 72.3, 105.8, 167.4, 185.9 ppm.

3-Isobutoxy-2-ethylcyclohex-2-enone (4b). To a stirred solution of 2-methyl-1,3-cyclohexanedione (3a) (0.90 g, 6.45 mmol) and p-toluenesulfonic acid (80 mg) in 7 mL of benzene was added i-BuOH (1.95 mL). The mixture was heated at reflux under a Dean-Stark trap for 3 hours. The reaction mixture was cooled down to room temperature and poured into 5 mL of saturated aqueous NaHCO₃ solution and extracted with 3 x 5 mL of ether. The combined extracts were washed with 4 mL of brine and dried over MgSO₄. The solvent was removed under reduced pressure and the residue was purified by column chromatography (silica gel, hexane/ethyl acetate = 5/1) to give 3-isobutoxy-2-ethylcyclohex-2-enone as yellow oil (1.01 g, 80% yield. ¹H NMR (CDCl₃): δ 0.96 (d, 6H,), 1.1 (t, 3H), 1.67 (m, 3H), 2.25 (t, 2H), 2.40 (m, 2H), 3.20 (t, 2H), 3.74 (d, 2H) ppm. ¹³C NMR (CDCl₃): 7.0, 18.7, 21.3, 27.3, 32.2, 72.3, 105.8, 167.4, 185.9 ppm.

Synthesis of 2-Alkylcyclohex-2-enone

**General procedure using NBS:** To a solution of 2-alkylidenecyclohexan-1-one (10 mmol) dissolved in CCl₄ (50 mL) was added N-bromosuccinimide (10 mmol) and the resulting suspension was heated in a water bath at 90ºC for 3 h. The suspension was then cooled to room
temperature, the precipitates filtered off and aniline (10 mmol), was added to the filtrate with cooling (ice-water). The solution was stirred at room temperature for 15 h, washed with 5% HCl (3 x 30 mL) and then with 5% NaHCO₃ (2 x 30 mL). After drying with MgSO₄, the solvent was evaporated to give brownish oil, which was purified by distillation under reduced pressure. NMR analysis did not confirm that desired product was obtained.

**2-Methylcyclohex-2-enone (5a).** A solution of 3-isobutoxy-2-methylcyclohex-2-enone 4a (1.48 g, 8.14 mmol) and dry ether (6 mL) was added dropwise to LAH (115 mg) in dry ether (6 mL) at such a rate that steady reflux was maintained. The mixture was stirred for a further 1 hour, cooled and 10% H₂SO₄ (6 mL) added. The ether layer was removed and the aqueous layer extracted with ether (5x20 mL). The combined ether solutions were dried over MgSO₄ and distilled to obtain 2-methylcyclohex-2-enone (0.80 g, 90%) as yellow oil. \(^1\)H NMR (CDCl₃): δ 1.86 (s, 3H), 1.97 (m, 2H), 2.43 (q, 2H), 2.52 (t, 1H), 5.93 (t, 1H) ppm. \(^13\)C NMR (CDCl₃): 23, 24.5, 39, 45, 116.5, 151.5, 194.4 ppm.

**2-Ethylcyclohex-2-enone (5b).** A solution of 3-isobutoxy-2-ethylcyclohex-2-enone 4a (1.0 g, 5.16 mmol) and dry ether (4 mL) was added dropwise to LAH (73 mg) in dry ether (4 mL) at such a rate that steady refluxing was maintained. The mixture was stirred for a further 1 hour, cooled and a 10% H₂SO₄ (4 mL) added. The ether layer was removed and the aqueous layer extracted with ether (5x10 mL). The combined ether solutions were dried over MgSO₄ and distilled to get 2-ethylcyclohex-2-enone (0.53 g, 84%) as yellow oil. \(^1\)H NMR (CDCl₃): δ 1.1 (t, 3H), 1.96 (m, 2H), 2.0 (m, 2H), 2.44 (q, 2H), 3.1 (t, 2H), 6.2 (t, 1H) ppm. \(^13\)C NMR (CDCl₃): 15.7, 23.2, 24.5, 33.0, 36.0, 134.9, 144.0, 198.0 ppm.

**Synthesis of 1,3-Cyclohexanedione**

**3-Isobutoxy-2-cyclohexeneone (7).** To a stirred solution of 1,3-cyclohexanedione 6 (10 g, 89 mmol) and p-toluensulfonic acid (1.44 g) in 314 ml of benzene was added \(i\)-BuOH (25 mL).
The mixture was heated at reflux under a Dean-Stark trap for 3 hours. The reaction mixture was cooled to room temperature and poured into 56 mL of saturated aqueous NaHCO₃ solution and extracted with ether (3x50 mL). The combined extracts were washed with 45 mL of brine and dried over MgSO₄. The solvent was removed under reduced pressure, and the residue was purified by column chromatography (silica gel, hexane/ethyl acetate = 5/1) to give 3-isobutoxy-2-cyclohexenone as yellow oil (12.71 g, 85%). ¹H NMR (CDCl₃): δ 0.83 (d, 6H), 1.85 (m, 3H), 2.21 (t, 2H), 2.27 (t, 2H), 3.45 (d, 2H), 5.18 (s, 1H) ppm. ¹³C NMR (CDCl₃): 18.7, 20.9, 27.3, 28.6, 36.4, 74.2, 102.3, 177.7, 199.2 ppm.

3-Alkyl-2-Cyclohexen-1-ones

**General procedure:** To the solution of the appropriate Grignard reagent (26.7 mmol) and 20 mL of dry ether, a solution of 3-isobutoxy-2-cyclohexenone (2 g, 17.8 mmol) in 10 mL of dry ether was added and the mixture was stirred for 2 hours. After that time, the Grignard complex was decomposed with diluted sulfuric acid and the solution was extracted with ether. The organic phase was washed with diluted NaHCO₃, water and dried over MgSO₄. After evaporation of solvent the crude product was purified by silica gel column chromatography (hexanes/Et₂O = 3/1) to give the desired 3-alkyl-2-cyclohexen-1-one.

**3-Methyl-2-cyclohexen-1-one (9a).** Yellow oil was obtained (1.62 g, 83%). ¹H NMR (CDCl₃): δ 1.96 (m, 5H), 2.27 (m, 4H), 5.88 (s, 1H) ppm. ¹³C NMR (CDCl₃): 22.3, 24.1, 30.6, 36.7, 126.3, 162.4, 199.2 ppm.

**3-Ethyl-2-cyclohexen-1-one (9b).** Yellow oil was obtained (1.72 g, 78%). ¹H NMR (CDCl₃): δ 1.06 (t, 3H), 1.96 (q, 2H), 2.1 (m, 6H), 5.79 (s, 1H) ppm. ¹³C NMR (CDCl₃): 11.2, 22.7, 29.7, 30.8, 37.4, 124.5, 168.0, 200.1 ppm.
3-\textit{n}-Propyl-2-cyclohexen-1-one (9c). Yellow oil was obtained (1.84 g, 75%). $^1$H NMR (CDCl$_3$): $\delta$ 0.95 (t, 3H), 1.1 (m, 10H), 5.85 (s, 1H) ppm. $^{13}$C NMR (CDCl$_3$): 13.5, 19.9, 22.5, 29.4, 37.7, 39.8, 125.4, 166.4, 199.8 ppm.

3-\textit{i}-Propyl-2-cyclohexen-1-one (9d). Yellow oil was obtained (1.72 g, 70%). $^1$H NMR (CDCl$_3$): $\delta$ 1.05 (d, 6H), 1.97 (m, 2H), 2.33 (m, 5H), 5.83 (s, 1H) ppm. $^{13}$C NMR (CDCl$_3$): 21.1, 23.5, 28.2, 36.2, 38.1, 124.0, 172.4, 200.7 ppm.

3-\textit{n}-Butyl-2-cyclohexen-1-one (9e). Yellow oil was obtained (2.16 g, 80%). $^1$H NMR (CDCl$_3$): $\delta$ 0.94 (t, 3H), 1.30 (m, 2H), 1.52 (m, 2H), 1.98 (m, 2H), 2.24 (t, 2H), 2.32 (t, 2H), 2.40 (m, 2H), 5.88 (t, 1H) ppm. $^{13}$C NMR (CDCl$_3$): 13.9, 22.6, 23.0, 29.3, 29.9, 37.4, 37.9, 125.5, 167.0, 199.8 ppm.

2-(1-Hydroxyalkyl)cyclohexanes

**General procedure:** A solution of cyclohexanone (4.2 mL, 40 mmol) in THF (67 mL) was added dropwise to a stirred and cooled (-78ºC) solution of LDA [generated by dropwise addition of $n$-BuLi (2.5 M, in hexane, 44 mmol, 17.6 mL) to $i$-Pr$_2$NH (42 mmol, 5.88 mL) in THF (200 mL)] at 0ºC, followed, after 15 minutes, by cooling to -78ºC. After 1 hour, the appropriate aldehyde (40 mmol) in THF (100 mL) was added quickly. Stirring was continued for 50 min at -78ºC, and the reaction was quenched with saturated aqueous NH$_4$Cl (80 mL). The cooling bath was removed and stirring was continued until the mixture had reached room temperature. The solution was extracted with ether, washed with water and brine, dried over MgSO$_4$ and the solvent was evaporated. Flash chromatography (EtOAc/hexane = 1/6) of the residue gave final products.

2-(1-Hydroxyethyl)cyclohexane (12a). Pale yellow oil was obtained (4.20 g, 75%). $^1$H NMR (CDCl$_3$): $\delta$ 1.2 (d, 3H), 1.42 (m, 1H), 1.50 (m, 2H), 1.62 (m, 8H), 3.65 (s, 1H), 3.91 (m, 1H) ppm. $^{13}$C NMR (CDCl$_3$): 20.43, 25.55, 28.32, 31.22, 43.34, 58.24, 68.38, 216.31 ppm.
2-(1-Hydroxybuthyl)cyclohexane (12b). Pale yellow oil was obtained (40.0 g, 60%). $^1$H NMR (CDCl$_3$): $\delta$ 0.85 (t, 3H), 1.20 (m, 13H), 3.63 (m, 2H), 3.95 (m, 2H) ppm. $^{13}$C NMR (CDCl$_3$): 13.1, 18.0, 24.7, 27.6, 30.8, 38.1, 42.5, 55.3, 69.9, 215.8 ppm.

2-Alkylidencyclohexanones

2-Ethylidenecyclohexanone (13a). Methanesulfonyl chloride (7.33 mL, 93 mmol) was added dropwise to a stirred and cooled (0°C) solution of alcohol 12a (4.3 g, 30 mmol) and Et$_3$N (21.2 mL, 151 mmol) in CH$_2$Cl$_2$ (160 mL). The cooling bath was left in place, but was not recharged. Stirring was continued for 6 hours, the solution was quenched with saturated aqueous NaHCO$_3$ (37 mL), diluted with Et$_2$O, washed with water and brine, dried over MgSO$_4$. Evaporation of the solvent gave crude mesylate (5.82 g, in 80% yield), which was used immediately for next step. To a stirred solution of the above mesylate in THF (195 mL), DBU (7.5 mL, 50 mmol) was added dropwise. After 1 hour, the mixture was diluted with Et$_2$O, washed with water, 5% hydrochloric acid, and brine, dried over MgSO$_4$ and solvent evaporated. The obtained crude product was purified by flash chromatography (EtOAc/hexane = 1/10) to give 13a (2.64 g, 71%) with $E$ isomer in excess (~ 80%). $^1$H NMR (CDCl$_3$): $\delta$ 6.57 (t, 1H), 2.3 (m, 11H) ppm. $^{13}$C NMR (CDCl$_3$): 21.6, 23.3, 25.7, 29.8, 38.5, 135.2, 138.5, 199.4 ppm.

2-Butylidenecyclohexanone (13b). a) Methanesulfonyl chloride (5.9 mL, 75 mmol) was added dropwise to a stirred and cooled (0°C) solution of alcohol 12a (4.0 g, 24 mmol) and Et$_3$N (16.8 mL, 120 mmol) in CH$_2$Cl$_2$ (128 mL). The cooling bath was left in place, but was not recharged. Stirring was continued for 6 hours, the solution was quenched with saturated aqueous NaHCO$_3$ (34 mL), diluted with Et$_2$O, washed with water and brine, dried over MgSO$_4$. Evaporation of the solvent gave crude mesylate (5.43 g, in 73% yield), which was used immediately for next step; b) to a stirred solution of the above mesylate in THF (178 mL), DBU (6.9 mL, 46 mmol) was added dropwise. After 1 hour, the mixture was diluted with Et$_2$O,
washed with water, 5% hydrochloric acid, and brine, dried over MgSO₄ and solvent evaporated. The obtained crude product was purified by flash chromatography (EtOAc/hexane = 1/10) to give \( 13b \) (7.30 g, 64%) with \( E \) isomer in excess (~ 80%). \(^1\)H NMR (CDCl₃): δ 2.3 (m, 15H), 6.64 (t, 1H) ppm. \(^{13}\)C NMR (CDCl₃): 13.3, 21.6, 23.0, 23.2, 25.9, 29.2, 39.5, 135.7, 138.4, 199.8 ppm.

**Biotransformation of \( \alpha,\beta \)-Unsaturated Cyclic Enones Using Isolated Old Yellow Enzyme**

**General procedure:** Reaction mixtures contained final concentrations of NADP⁺ (10.7 mmol, 8 mg), glucose-6-phosphate (648 mmol, 219.5 mg), glucose-6-phosphate dehydrogenase (256 µg), enone (0.18 mmol), \( \beta \)-cyclodextrin (around 40 mg), purified OYE (20-40 µg) in 100 mM KPi buffer, pH 7.0 in total volume of 25 mL. Conversions were carried out at room temperature. All the reaction components (except the buffer) were added portionwise (10 equal portions during 12 hours) and the mixtures were sampled for GC analysis periodically. After 24 hours the reaction solutions were extracted with Et₂O (3x50 mL). The combined organic extracts were dried over MgSO₄. The final products were purified by filtration through silica gel (EtOAc/hexane = 2/9).

\((\textit{R})\)-2-Methylcyclohexan-1-one (14a). Yellow oil; 97% e.e. (14.0 mg, 69.4%). \(^1\)H NMR (CDCl₃): δ 1.00 (d, 3H), 1.34 (m, 1H), 1.64 (m, 3H), 2.10 (m, 5H) ppm. \(^{13}\)C NMR (CDCl₃): 14.6, 25.3, 28.1, 36.1, 41.7, 45.4, 213.7 ppm.

\((\textit{R})\)-2-Ethylcyclohexan-1-one (14b). Yellow oil; 92% e.e. (5.0 mg, 22%). \(^1\)H NMR (CDCl₃): δ 0.96 (m, 3H), 2.60 (m, 11H) ppm.

2-Ethylcyclohexan-1-one (16a). Yellow oil; racemic mixture (4.0 mg, 17.7%). \(^1\)H NMR (CDCl₃): δ 0.96 (m, 3H), 2.60 (m, 11H) ppm.

\((\textit{S})\)-3-Methylcyclohexan-1-one (15a). Yellow oil; 96% e.e. (16.0 mg, 79.3%). \(^1\)H NMR (CDCl₃): δ 0.92 (m, 3H), 1.31 (m, 1H), 1.64 (m, 1H), 2.00 (m, 4H), 2.32 (m, 3H) ppm. \(^{13}\)C NMR (CDCl₃): 14.6, 25.3, 28.1, 36.1, 41.7, 45.4, 213.7 ppm.
(S)-3-Ethylcyclohexan-1-one (15b). Yellow oil; 95% e.e. (12.3 mg, 54.2%). $^1$H NMR (CDCl$_3$): $\delta$ 0.91 (t, 3H), 1.22 (m, 11H) ppm.

(S)-3-n-Propylcyclohexan-1-one (15c). Yellow oil; 90% e.e. (3.75 mg, 14.88%). $^1$H NMR (CDCl$_3$): $\delta$ 0.89 (t, 3H), 1.34 (m, 5H), 1.95 (m, 3H), 2.00 (m, 1H), 2.05 (m, 1H), 2.25 (m, 1H), 2.34 (m, 1H), 2.42 (m, 1H) ppm.

(S)-3-i-Propylcyclohexan-1-one (15d). Yellow oil; 92% e.e. (3.75 mg, 14.88%). $^1$H NMR (CDCl$_3$): $\delta$ 0.90 (d, 3H), 0.91 (d, 3H), 1.28 (m, 10H) ppm.

Derivatization of Cyclohexanones with (2R,3R)-(-)-2,3-Butanediol

The stereochemical purities of all reduction products were determined by chiral-phase GC after ketalization with optically pure 2,3-butanediol. Derivatizations were carried out by heating a mixture of 1.0 equiv. of crude biotransformation product, 2.0 equiv. of (2R,3R)-(-)-2,3-butanediol and a catalytic amount of p-TsOH in 1.5 mL of CH$_2$Cl$_2$ at reflux for 2 hours. A 1 µL aliquot was directly analysed by GC. Samples of racemic ketones 5a, b, 9a-e and 13a, b were derivatized and analyzed by GC to demonstrate baseline resolution of enantiomers.

Biotransformation of $\alpha,\beta$-Unsaturated Cyclic Enones Using Whole Cells of E. coli BL21(DE3)(pOYE-pET3b)

General procedure: A 500 µL aliquot from an overnight culture of BL21(DE3)(pOYE-pET3b) with an OD$_{600}$ value between 4 and 5 added to 50 mL of LB medium supplemented with 200 μg/mL ampicillin in a 500 mL Erlenmeyer flask. The culture was shaken at 150-200 rpm at 37ºC until it reached an OD$_{600}$ value between 0.4 and 0.5, then isopropylthio-β-D-galactoside (IPTG) was added to final concentration of 0.10 mM. The culture was shaken at 150 rpm at room temperature for an additional 30 minutes, then the ketone and stoichiometric quantity of β-cyclodextrin were added and shaking was continued at room temperature at 150 rpm. Samples for GC analysis were prepared by vortex mixing 50 µL of the reaction mixture with 50 µL of
EtOAc for ca. 30 s. A 1 µL portion of the organic phase was analyzed by GC. At the conclusions of the reactions, the mixture was extracted with EtOAc (3x30 mL), and then the combined organic extracts were dried with MgSO₄ and concentrated by rotary evaporator. The final products were purified by filtration through silica gel (EtOAc/hexane = 2/9).

**(R)-2-Methylcyclohexan-1-one (14a).** Yellow oil; 96% e.e. (12.0 mg, 59.5%). $^1$H NMR (CDCl₃): $\delta$ 1.00 (d, 3H), 1.34 (m, 1H), 1.64 (m, 3H), 2.10 (m, 5H) ppm.

**(R)-2-Ethylcyclohexan-1-one (14b).** Yellow oil; 90% e.e. (3.17 mg, 14%). $^1$H NMR (CDCl₃): $\delta$ 0.96 (m, 3H), 2.60 (m, 11H) ppm.

**2-Ethylcyclohexan-1-one (16a).** Yellow oil; racemic mixture (2.5 mg, 11%). $^1$H NMR (CDCl₃): $\delta$ 0.96 (m, 3H), 2.60 (m, 11H) ppm.

**(S)-3-Methylcyclohexan-1-one (15a).** Yellow oil; 94% e.e. (13.0 mg, 64.4%). $^1$H NMR (CDCl₃): $\delta$ 0.92 (m, 3H), 1.31 (m, 1H), 1.64 (m, 1H), 2.00 (m, 4H), 2.32 (m, 3H) ppm.

**(S)-3-Ethylcyclohexan-1-one (15b).** Yellow oil; 95% e.e. (12.3 mg, 54.2%). $^1$H NMR (CDCl₃): $\delta$ 0.91 (t, 3H), 1.22 (m, 11H) ppm.

**(S)-3-\textit{n}-Propylcyclohexan-1-one (15c).** Yellow oil; 89% e.e. (2.5 mg, 9.9%). $^1$H NMR (CDCl₃): $\delta$ 0.89 (t, 3H), 1.34 (m, 5H), 1.95 (m, 3H), 2.00 (m, 1H), 2.05 (m, 1H), 2.25 (m, 1H), 2.34 (m, 1H), 2.42 (m, 1H) ppm.

**(S)-3-\textit{i}-Propylcyclohexan-1-one (15d).** Yellow oil; 90% e.e. (1.9 mg, 7.4%). $^1$H NMR (CDCl₃): $\delta$ 0.90 (d, 3H), 0.91 (d, 3H), 1.28 (m, 10H) ppm.

**Synthesis of 2-Alkyloxobutanoates**

**General procedure:** The appropriate Grignard reagent (88.6 mmol) was added dropwise to a mixture of diethyl oxalate (10 mL, 73.6 mmol), THF (50 mL) and ether (100 mL) at -78°C and the solution was stirred at this temperature for 4 hours. After quenching with saturated NH₄Cl (100 mL), the mixture was extracted with ethyl acetate (3x100 mL). The organic phases
were combined, dried over MgSO₄ and concentrated in vacuo to give crude products, which were purified by flash chromatography.

**2-Ethyloxobutanoate (17d).** Pale yellow oil (8.13 g, 85%). \(^1\)H NMR (CDCl₃): δ 1.28 (t, J=7.2 Hz, 3H), 1.39 (t, J=7.2, 3H), 2.90 (q, J=7.2, 2H), 4.15 (q, J=7.2, 2H) ppm. \(^{13}\)C NMR (CDCl₃): 6.45, 13.90, 32.69, 62.24, 161.09, 195.04 ppm.

**2-n-Propyloxobutanoate (17e).** Pale yellow oil (7.33 g, 69%). \(^1\)H NMR (CDCl₃): δ 0.7 (m, 8H), 2.81 (t, J=7.1 Hz, 2H), 4.31 (q, J=6.3 Hz, 2H) ppm. \(^{13}\)C NMR (CDCl₃): 14.14, 14.65, 17.15, 41.75, 62.97, 161.86, 195.24 ppm.

**Synthesis of Nitro Alcohols**

**General procedure using CAN:** To a stirred solution of an appropriate acrylic ester (1 mmol) in anhydrous CH₃CN (5 mL) was added CAN (3 mmol) and NaNO₂ (3 mmol) at 0°C under nitrogen. The reaction mixture was vigorously stirred for 24 h at room temperature, diluted with water and extracted sequentially with saturated solution of NaHCO₃, brine and dried over MgSO₄. The residue obtained after evaporation of the solvent was purified by column chromatography.

**General Procedure Using Amberlyst A-21:** A 50 mL two necked flask equipped with a mechanical stirrer was charged with the appropriate nitroalkane (60 mmol) and cooled with ice-water bath. Amberlyst A-21 (5-7 g) was added and the mixture was stirred for 5 minutes before the appropriate 2-oxoacid ethyl ester (60 mmol). After stirring overnight at room temperature the mixture was filtered. The Amberlyst resin was washed with CH₂Cl₂ (4x25 mL), then solvent was evaporated *in vacuo* to yield crude β-nitroalcohols, which were purified by flash chromatography.
**Ethyl 2-hydroxy-3-nitrobutanoate (19a).** Pale yellow oil (9.45 g, 89%). $^1$H NMR (CDCl$_3$): $\delta$ 4.34 (m, 4H), 3.40 (brs, 1H), 1.64 (d, $J$=6.9 Hz, 3H), 1.28 (t, $J$=7.1 Hz, 3H) ppm. $^{13}$C NMR (CDCl$_3$): $\delta$ 171.0, 83.3, 71.8, 62.9, 14.9, 13.9 ppm.

**Ethyl 2-hydroxy-3-nitropentanoate (19b).** Yellow oil (10.2 g, 89%). $^1$H NMR (CDCl$_3$): $\delta$ 4.74 (m, 1H), 4.40 (m, 4H), 2.32 (m, 2H), 1.36 (m, 3H), 1.11 (m, 3H) ppm. $^{13}$C NMR (CDCl$_3$): $\delta$ 170.8, 90.6, 70.6, 62.8, 22.6, 13.8, 10.3 ppm.

**Ethyl 2-hydroxy-2-methyl-3-nitropropanoate (19c).** Pale yellow oil (9.63 g, 91%). $^1$H NMR (CDCl$_3$): $\delta$ 4.87 (dd, $J_1$=13.78 Hz, $J_2$=13.78 Hz, 2H), 4.4 (m, 2H), 1.46 (s, 3H), 1.35 (t, $J$=7.13 Hz, 3H) ppm. $^{13}$C NMR (CDCl$_3$): $\delta$ 173.6, 81.1, 72.6, 63.2, 24.0, 14.1 ppm.

**Ethyl 2-hydroxy-2-(nitromethyl)butanoate (19d).** Yellow oil (11.2 g, 98%). $^1$H NMR (CDCl$_3$): $\delta$ 4.54 (dd, $J_1$=13.59 Hz, $J_2$=13.59 Hz, 2H), 4.33 (m, 2H), 3.79 (s, 1H), 1.69 (m, 2H), 1.31 (t, $J$=7.08 Hz, 3H), 0.90 (t, $J$=7.37 Hz, 3H) ppm. $^{13}$C NMR (CDCl$_3$): $\delta$ 173.0, 80.9, 75.7, 63.2, 29.2, 14.2, 7.2 ppm.

**Ethyl 2-hydroxy-2-(nitromethyl)pentanoate (19e).** Yellow oil (10.80 g, 88%). $^1$H NMR (CDCl$_3$): $\delta$ 4.84 (dd, $J_1$=13.59 Hz, $J_2$=13.59 Hz, 2H), 4.37 (m, 2H), 1.69 (m, 5H), 1.36 (t, $J$=7.07 Hz, 2H), 0.95 (t, $J$=7.36 Hz, 3H) ppm. $^{13}$C NMR (CDCl$_3$): $\delta$ 173.1, 81.1, 75.4, 63.2, 38.8, 16.3, 14.4, 14.1 ppm.

**Ethyl 2-hydroxy-3-methyl-2-(nitromethyl)butanoate (19f).** Yellow oil (12.70 g, 99%). $^1$H NMR (CDCl$_3$): $\delta$ 4.85 (dd, $J_1$=13.54 Hz, $J_2$=13.3 Hz, 2H), 4.39 (m, 2H), 4.39 (q, $J$=7.13 Hz, 2H), 2.00 (m, 1H), 1.36 (t, $J$=7.13 Hz, 3H), 1.00 (dd, $J_1$=6.89 Hz, $J_2$=6.89 Hz, 6H) ppm. $^{13}$C NMR (CDCl$_3$): $\delta$ 173.1, 80.0, 63.6, 34.7, 17.4, 16.8, 14.7 ppm.

**Ethyl 2-hydroxy-3-nitro-2-phenylpropanoate (19g).** Yellow oil (14.97 g, 98%). $^1$H NMR (CDCl$_3$): $\delta$ 7.44 (m, 5H), 5.29 (dd, $J_1$=16.5 Hz, $J_2$=15.0 Hz, 2H), 4.49 (m, 2H), 1.36 (t,
\( J = 7.01 \) Hz, 3H) ppm. \(^{13}\)C NMR (CDCl\(_3\)): \( \delta \) 171.8, 136.6, 129.3, 129.0, 125.4, 80.9, 76.1, 63.8, 14.1 ppm.

**Synthesis of 3- and 2-Alkyl Substituted \( \beta \)-Nitroacrylates**

**General procedure:** The appropriate \( \beta \)-nitroalcohol (17 mmol) was dissolved in 17 mL of CH\(_2\)Cl\(_2\) at -78°C under an argon atmosphere, then 1 equiv. of methanesulfonyl chloride (17 mmol) was added in one portion. After 30 minutes, triethylamine (51 mmol) was added dropwise, and the reaction mixture was stirred for 4 hours at -78°C. The reaction mixture was then transferred to a separatory funnel with the aid of 17 mL of CH\(_2\)Cl\(_2\), then it was washed with water, 5% aqueous HCl, and brine. The final product was purified by flash chromatography.

(**E**)-Ethyl 3-nitrobut-2-enoate (**E-20a**). Yellow oil (1.08 g, 40%). \(^1\)H NMR (CDCl\(_3\)): \( \delta \) 7.00 (s, 1H), 4.20 (q, \( J = 7.2 \) Hz, 2H), 2.50 (s, 3H), 1.20 (t, \( J = 7.2 \) Hz, 3H) ppm. \(^{13}\)C NMR (CDCl\(_3\)): \( \delta \) 164.2, 160.0, 121.5, 61.8, 14.1, 14.0 ppm. IR \( \nu \max \) 1735, 1533, 1355, 1227 cm\(^{-1}\).

(**E**)-Ethyl 3-nitropent-2-enoate (**E-20b**). Yellow oil (1.35 g, 46%). \(^1\)H NMR (CDCl\(_3\)): \( \delta \) 6.97 (s, 1H), 4.32 (q, \( J = 7.08 \) Hz, 2H), 3.11 (m, 2H), 1.37 (t, \( J = 9.91 \) Hz, 3H), 1.23 (t, \( J = 8.47 \) Hz, 3H) ppm. \(^{13}\)C NMR (CDCl\(_3\)): \( \delta \) 187.0, 185.5, 120.8, 61.9, 21.2, 14.4, 12.5 ppm. IR \( \nu \max \) 1735, 1533, 1352, 1227 cm\(^{-1}\).

(**Z**)-Ethyl 2-methyl-3-nitroacrylate (**Z-20c**). Yellow oil (1.46 g, 54%). \(^1\)H NMR (CDCl\(_3\)): \( \delta \) 6.89 (s, 1H), 4.39 (q, \( J = 7.1 \) Hz, 2H), 2.11 (s, 3H), 1.37 (t, \( J = 7.4 \) Hz, 3H) ppm. \(^{13}\)C NMR (CDCl\(_3\)): \( \delta \) 166.7, 141.5, 136.3, 63.0, 18.2, 14.4 ppm. IR \( \nu \max \) 1737, 1533, 1355, 1227 cm\(^{-1}\).

(**Z**)-Ethyl 2-(nitromethylene)butanoate (**Z-20d**). Yellow oil (0.109 g, 37.2%). \(^1\)H NMR (CDCl\(_3\)): \( \delta \) 6.84 (s, 1H), 4.39 (q, \( J = 7.4 \) Hz, 2H), 2.48 (m, 2H), 1.37 (t, \( J = 7.4 \) Hz, 3H), 1.2 (t, \( J = 7.4 \) Hz, 3H) ppm. \(^{13}\)C NMR (CDCl\(_3\)): \( \delta \) 166.1, 146.7, 135.1, 62.5, 25.5, 14.0, 11.2 ppm. IR \( \nu \max \) 1735, 1533, 1353, 1222 cm\(^{-1}\).
(Z)-Ethyl 2-(nitromethylene)pentanoate (Z-20e). Yellow oil (1.11 g, 35%). $^1$H NMR (CDCl$_3$): $\delta$ 6.85 (s, 1H), 4.39 (m, 2H), 2.41 (m, 2H), 1.66 (m, 2H), 1.37 (t, $J$=7.4 Hz, 3H), 1.28 (t, $J$=7.4 Hz, 3H), ppm. $^{13}$C NMR (CDCl$_3$): $\delta$ 166.1, 145.3, 135.6, 62.5, 33.9, 20.2, 14.0, 13.5 ppm. IR $\nu_{\text{max}}$ 1735, 1532, 1353, 1221 cm$^{-1}$.

(Z)-Ethyl 3-methyl-2-(nitromethylene)butanoate (Z-20f). Yellow oil (0.388 g, 12.2%). $^1$H NMR (CDCl$_3$): $\delta$ 6.83 (s, 1H), 4.40 (q, $J$=7.1 Hz, 2H), 2.77 (m, 1H), 1.39 (t, $J$=7.4 Hz, 3H), 1.23 (d, $J$=7.1 Hz, 6H) ppm. $^{13}$C NMR (CDCl$_3$): $\delta$ 165.8, 150.9, 134.9, 62.4, 31.6, 20.5, 14.0 ppm. IR $\nu_{\text{max}}$ 1736, 1533, 1352, 1221 cm$^{-1}$.

(Z)-Ethyl 3-nitro-2-phenylacrylate (Z-20g). Yellow oil (2.25 g, 60%). $^1$H NMR (CDCl$_3$): $\delta$ 7.53 (m, 5H), 7.35 (s, 1H), 4.51 (q, $J$=7.1 Hz, 2H), 1.42 (t, $J$=7.1 Hz, 3H) ppm. $^{13}$C NMR (CDCl$_3$): $\delta$ 164.5, 143.1, 134.2, 129.3, 127.2, 62.6, 13.6 ppm. IR $\nu_{\text{max}}$ 1737, 1533, 1355, 1227 cm$^{-1}$.

**Biotransformation of $\beta$-Nitro Acrylates Using Isolated Old Yellow Enzyme**

**General procedure:** Reaction mixtures contained final concentrations of NADP$^+$ (20 µmol, 15 mg), glucose-6-phosphate (1.27 mmol, 429 mg), glucose-6-phosphate dehydrogenase (500 µg), nitroacrylate (25 mM), and purified OYE (20-40 µg) in 100 mM KP$_i$, pH 6.93 in total volumes of 50 mL. Conversions were carried out at room temperature. Reaction components (except for KP$_i$ buffer) were added in 10 equal portions every 45 minutes and the mixtures were sampled for GC analysis periodically. After nearly all of the substrates had been consumed, the reaction mixture was extracted with Et$_2$O (3 x (5 x reaction volume)). The combined organic extracts were washed with brine (1 volume) and water (1 volume), dried with MgSO$_4$, and concentrated in vacuo.

Ethyl 3-nitrobutanoate (21a). Yellow oil (0.201 g, 98%). $^1$H NMR (CDCl$_3$): $\delta$ 4.98 (m, 1H), 4.21 (q, $J$=7.1 Hz, 2H), 3.18 (dd, $J_1$=8.8 Hz, $J_2$=8.8 Hz, 1H), 2.73 (dd, $J_1$=5.0 Hz, $J_2$=5.0 Hz, 2H), ppm. IR $\nu_{\text{max}}$ 1736, 1533, 1353, 1221 cm$^{-1}$.
Hz, 1H), 1.63 (d, J=6.9 Hz, 3H), 1.29 (t, J=7.1 Hz, 3H) ppm. $^{13}$C NMR (CDCl$_3$): δ 169.3, 78.6, 61.4, 38.6, 19.5, 14.1 ppm.

**Ethyl 3-nitropentanoate (21b).** Yellow oil (0.096 g, 95%). $^1$H NMR (CDCl$_3$): δ 4.89 (m, 1H), 4.20 (q, J=6.5 Hz, 2H), 3.18 (dd, J$_1$=9.4 Hz, J$_2$=9.4 Hz, 1H), 2.71 (dd, J$_1$=4.2 Hz, J$_2$=4.5 Hz, 1H), 2.02 (m, 2H), 1.28 (t, J=7.1 Hz, 3H), 1.02 (t, J=7.4 Hz, 3H) ppm. $^{13}$C NMR (CDCl$_3$): δ 169.4, 84.6, 61.5, 36.9, 27.2, 14.2, 10.0 ppm.

(R)-**Ethyl 2-methyl-3-nitropropanoate (21c).** Yellow oil (0.191 g, 93%). $^1$H NMR (CDCl$_3$): δ 4.76 (dd, J$_1$=8.1 Hz, J$_2$=8.1 Hz, 1H), 4.44 (dd, J$_1$=5.7 Hz, J$_2$=5.7 Hz, 1H), 4.24 (q, J=7.0 Hz, 2H), 3.29 (m, 1H), 1.30 (m, 6H) ppm. $^{13}$C NMR (CDCl$_3$): δ 172.6, 76.6, 61.7, 37.8, 14.5, 14.2 ppm.

(R)-**Ethyl 2-(nitromethyl)butanoate (21d).** Yellow oil (0.151 g, 75%). $^1$H NMR (CDCl$_3$): δ 4.78 (dd, J$_1$=9.1 Hz, J$_2$=9.4 Hz, 1H), 4.46 (dd, J$_1$=5.1 Hz, J$_2$=4.8 Hz, 1H), 4.22 (q, J=7.1 Hz, 2H), 3.18 (m, 1H), 1.76 (m, 2H), 1.30 (t, J=7.1 Hz, 3H), 1.01 (t, J=7.7 Hz, 3H) ppm. $^{13}$C NMR (CDCl$_3$): δ 172.2, 75.1, 61.5, 44.4, 22.7, 14.3, 11.2 ppm.

(R)-**Ethyl 2-(nitromethyl)pentanoate (21e).** Yellow oil (0.187 g, 93%). $^1$H NMR (CDCl$_3$): δ 4.77 (dd, J$_1$=9.3 Hz, J$_2$=9.3 Hz, Hz, 1H), 4.44 (dd, J$_1$=4.8 Hz, J$_2$=4.8 Hz, 2H), 4.20 (q, J=7.4 Hz, 2H), 3.24 (m, 1H), 1.72 (m, 2H), 1.44 (m, 2H), 1.30 (t, J=7.1 Hz, 3H), 0.97 (t, J=7.4 Hz, 3H) ppm. $^{13}$C NMR (CDCl$_3$): δ 172.5, 75.4, 61.5, 42.9, 31.5, 20.1, 13.9, 14.3 ppm.

(R)-**Ethyl 3-methyl-2-(nitromethyl)butanoate (21f).** Yellow oil (0.182 g, 90.5%). $^1$H NMR (CDCl$_3$): δ 4.83 (dd, J$_1$=10.5 Hz, J$_2$=10.5 Hz, 1H), 4.44 (dd, J$_1$=4.0 Hz, J$_2$=4.0 Hz, 1H), 4.25 (q, J=7.1 Hz, 2H), 3.12 (m, 1H), 2.11 (m, 1H), 1.30 (t, J=7.1 Hz, 3H), 1.01 (m, 6H) ppm. $^{13}$C NMR (CDCl$_3$): δ 172.4, 74.3, 61.8, 49.4, 29.4, 20.4, 20.3, 14.7 ppm.
Synthesis of β-Amino Acids

General procedure: Crude biotransformation products (ca. 1.5 mmol) were hydrogenated at 500 psi in the presence of Raney nickel (200 mg) in EtOH (50 mL) at room temperature. After 16 hours, the resulting solution was filtered through Celite and the solvent was evaporated. A portion of the residue (50 mg) was dissolved in 6 M HCl and the solution was held on reflux overnight. The solution was concentrated under reduced pressure to afford yellow oil, which was washed with EtOAc to remove any non-polar impurities. Water was removed by rotary evaporator to yield the β-amino acids as hydrochloride salts.

3-Aminobutanoic acid (23a). Ethyl 3-aminobutanoate (22a) was obtained as pale yellow oil (0.150 g, 74.5%), which was further hydrolyzed to give 23a as a white precipitate (0.105 g, 89%). \(^{1}H\) NMR (D\(_2\)O): \(\delta\) 3.88 (m, 1H), 2.8 (dd, \(J_1=17.5\) Hz, \(J_2=5.8\) Hz, 1H), 2.72 (dd, \(J_1=17.5\) Hz, \(J_2=7.2\) Hz, 1H), 1.36 (d, \(J=6.7\) Hz, 3H) ppm. \(^{13}\)C NMR (D\(_2\)O): \(\delta\) 174.1, 44.4, 37.7, 17.8 ppm.

3-Aminopentanoic acid (23b). Ethyl 3-aminopentanoate (22b) was obtained as pale yellow oil (0.064 g, 81%), which was further hydrolyzed to give 23b as a white precipitate (0.047 g, 91%). \(^{1}H\) NMR (D\(_2\)O): \(\delta\) 3.63 (m, 1H), 2.88 (dd, \(J_1=4.53\) Hz, \(J_2=4.81\) Hz, 1H), 2.74 (dd, \(J_1=8.21\) Hz, \(J_2=8.21\) Hz, 1H), 1.79 (m, 2H), 1.03 (t, \(J=7.64\) Hz, 3H) ppm. \(^{13}\)C NMR (D\(_2\)O: \(\delta\) 174.7, 49.7, 35.8, 25.3, 8.9 ppm.

3-(R)-Aminomethylpropanoic acid (23c). Ethyl 3-amino-2-methylpropanoate (22c) was obtained as pale yellow oil (162.40 g, 85%), which was further hydrolyzed to give 23c as a white precipitate (0.143 g, 88%). \([\alpha]^{25}_{D}=13.0\) (c=0.94; 1M HCl). \(^{1}H\) NMR (D\(_2\)O): \(\delta\) 3.30 (dd, \(J_1=8.77\) Hz, \(J_2=8.5\) Hz, 1H), 3.16 (dd, \(J_1=4.81\) Hz, \(J_2=4.81\) Hz, 1H), 2.97 (m, 1H), 1.30 (d, \(J=7.36\) Hz, 3H) ppm. \(^{13}\)C NMR (D\(_2\)O: \(\delta\) 177.7, 41.3, 37.1, 14.3 ppm.

2-(R)-(Aminomethyl)butanoic acid (23d). Ethyl 2-(aminomethyl)butanoate (22d) was obtained as pale yellow oil (0.106 g, 85%), which was further hydrolyzed to give 23d as a white precipitate (0.065 g, 70%). \([\alpha]^{25}_{D}=6.94\) (c=0.94; 1M HCl). \(^{1}H\) NMR (D\(_2\)O): \(\delta\) 3.64 (m, 1H), 2.78 (dd, \(J_1=16.7\) Hz, \(J_2=14.8\) Hz, 1H), 2.47 (dd, \(J_1=8.21\) Hz, \(J_2=8.21\) Hz, 1H), 1.01 (t, \(J=7.64\) Hz, 3H) ppm. \(^{13}\)C NMR (D\(_2\)O: \(\delta\) 174.7, 49.7, 35.8, 25.3, 8.9 ppm.
precipitate (0.077 g, 90%). \([\alpha]^{25}_D=-2.7 (c=1, 1\text{M HCl})\). \(^1\)H NMR \((\text{D}_2\text{O})\): \(\delta\) 3.33 (dd, \(J_1=9.06\text{ Hz}, J_2=9.06\text{ Hz}, 1\text{H}\)), 3.19 (dd, \(J_1=4.53\text{ Hz}, J_2=4.81\text{ Hz}, 1\text{H}\)), 2.83 (m, 1H), 1.78 (m, 2H), 0.99 (t, \(J=7.36\text{ Hz}, 3\text{H}\)) ppm. \(^{13}\)C NMR \((\text{D}_2\text{O})\): \(\delta\) 177.1, 43.9, 39.6, 22.7, 10.2 ppm.

**2-(R)-(Aminomethyl)pentanoic acid \((23e)\).** Ethyl 2-(aminomethyl)pentanoate \((22e)\) was obtained as pale yellow oil (0.131 g, 85%), which was further hydrolyzed to give \(23e\) as a white precipitate (0.102 g, 95%). \([\alpha]^{25}_D=1.16 (c=1, 1\text{M HCl})\). \(^1\)H NMR \((\text{D}_2\text{O})\): \(\delta\) 3.33 (dd, \(J_1=9.34\text{ Hz}, J_2=9.06\text{ Hz}, 1\text{H}\)), 3.21 (dd, \(J_1=4.25\text{ Hz}, J_2=4.24\text{ Hz}, 1\text{H}\)), 2.93 (m, 1H), 1.75 (m, 2H), 1.41 (m, 2H), 0.93 (t, \(J=7.36\text{ Hz}, 3\text{H}\)) ppm. \(^{13}\)C NMR \((\text{D}_2\text{O})\): \(\delta\) 177.3, 42.4, 39.9, 31.4, 19.3, 13.1 ppm.

**2-(R)-(Aminomethyl)-3-methylbutanoic acid \((23f)\).** Ethyl 2-(aminomethyl)-3-methylbutanoate \((22f)\) was obtained as pale yellow oil (0.124 g, 81%), which was further hydrolyzed to give \(23f\) as a white precipitate (0.095 g, 93%). \([\alpha]^{25}_D=1.6 (c=1.03, \text{H}_2\text{O})\). \(^1\)H NMR \((\text{CD}_3\text{OD})\): \(\delta\) 3.16 (dd, \(J_1=10.47\text{ Hz}, J_2=10.15\text{ Hz}, 1\text{H}\)), 2.96 (dd, \(J_1=3.4\text{ Hz}, J_2=3.68\text{ Hz}, 1\text{H}\)), 2.52 (m, 1H), 2.09 (m, 1H), 0.95 (t, \(J=6.61\text{ Hz}, 6\text{H}\)) ppm. \(^{13}\)C NMR \((\text{CD}_3\text{OD})\): \(\delta\) 175.6, 50.2, 39.3, 30.2, 20.2, 19.7 ppm.

**Derivatization of \(\beta\)-Amino Acid Esters with TFAA.**

\(\beta\)-Amino acid ester (0.03 mmol) was stirred with TFAA (0.9 mmol) in 60°C for 30 minutes. After this time trifluoroacetic acid was evaporated in the stream of argon and the residue was dissolved in EtOAc (1 mL).

**Biotransformation of \(\beta\)-Nitroacrylates Using Cell Extract**

**General procedure:** An overnight cultures of \(E.\ coli\) (BL21(DE3)(pOYE-pET3b)) and \(E.\ coli\) overexpressing glucose dehydrogenase (GDH) from \(B.\ subtilis\), grown separately in LB medium containing 100 µg/mL ampicillin were diluted 1:100 into 1 L of the same medium. The cultures were shaken in 37°C until the optical densities at 600 nm reached 0.8, then the enzymes overproduction was induced with isopropylthio-\(\beta\)-D-galactoside (IPTG) at a final concentration...
of 400 μM and the cultures were stirred for an additional 2.5 hours at room temperature. The cells were harvested by centrifugation (5,000 rpm for 15 min at 4°C), washed twice with cold sterile water and then resuspended in 25 mL of 100 mM KP$_i$ buffer pH 6.93 (with addition of PMSF to final concentration of 10 μM). The cells were lysed using a French Press and debris was removed by centrifugation at 20,000 g for 60 min at 4°C. The pH of supernatant was adjusted to 6.93 by adding diluted HCl. The extracts were mixed together and diluted with the same buffer to the final volume of 100 mL and NADP$^+$ was added (to the final concentration of 0.05 mM). To this mixture were added every 45 minutes portions of: glucose (to the final concentration of 10 mM), β-cyclodextrin 40 mg and 20 mg of the substrate (20a, 20c, 20e) (to the final concentration of 17 mM). The pH of the solution was controlled and kept at 6.93 by addition of 3M NaOH. The concentration of glucose was monitored by using glucose assay. When the starting material was completely consumed (after 11 hours) the protein was precipitated with sodium chloride and the mixture was centrifuged at 15,000 rpm for 15 minutes at 4°C. The supernatant was extracted with Et$_2$O, dried over MgSO$_4$ and solvent was evaporated.

**Ethyl 3-aminobutanoate (22a).** Obtained in 72% yield as a racemic mixture.

**Ethyl 3-amino-2-methylpropanoate (22c).** Obtained in 75% yield with 73% e.e.

**Ethyl 2-(aminomethyl)pentanoate (22e).** Obtained in 71% yield with 89% e.e.

**Glucose Assay**

The glucose concentration in the reaction was determined using a Trinder assay kit commercially available from Diagnostic Chemicals Limited, Canada. The absorbance of the reaction, which consists of 5 μL of the reaction media and 1 mL of the Trinder reagent mixed by inversion and incubated at 37°C for 15 minutes, was measured at 505 nm. The concentration of the glucose in the reaction was measured by comparing it to the standard reference containing 0.4 g/L glucose.
**Incubation of 21a in D2O.**

Compound 21a (20 mg, 0.012 mmol) was stirred in KPi buffer (prepared in D2O) (5 mL) overnight. Samples for GC/MS analysis were taken every hour. The product of the incubation was analyzed by $^1$H NMR spectroscopy: $^1$H NMR (CDCl$_3$): $\delta$ 4.21 (q, $J=7.1$ Hz, 2H), 3.18 (dd, $J_1=8.8$ Hz, $J_2=8.8$ Hz, 1H), 2.73 (dd, $J_1=5.0$ Hz, $J_2=5.0$ Hz, 1H), 1.63 (s, 3H), 1.29 (t, $J=7.1$ Hz, 3H) ppm.

**Biotransformation of (Z)-20c Using NADPD.**

Tris-HCl buffer (250 µL of 1M, pH 8.0) was added to 4.595 mL of KPi buffer, 5 µL of (Z)-20c in 50 µL of isopropanol-d8 was added to that solution added to that solution and stirred for few minutes. After that time 9 mg of NADP$,^+$, 3 mg of TBADH and 100 µL of OYE1 were added to the mixture and stirred. After 6 hours the reaction was completed and the reaction mixture was extracted with ether (3x8 mL), the solvent was dried over MgSO$_4$ and evaporated. The product was analyzed by GC-MS and $^1$H NMR spectroscopy: $^1$H NMR (CDCl$_3$): $\delta$ 4.76 (dd, $J_1=8.1$ Hz, $J_2=8.1$ Hz, 1H), 4.44 (dd, $J_1=5.7$ Hz, $J_2=5.7$ Hz, 1H), 4.24 (q, $J=7.0$ Hz, 2H), 1.30 (m, 6H) ppm.
Figure A-1. GC chromatogram of 14a, 15b and 15c.
Figure A-2. GC chromatogram of 14b, 15a and 15d.
Figure A-3. GC chromatogram of TFA derivatives of 22c and 22d.
Figure A-4. GC chromatogram of TFA derivatives of **22e** and **22f**.
Figure B-1. $^{13}$C NMR of spectrum of 20c.
Figure B-2. $^1$H NMR spectrum of 20c.
Figure B-3. $^{13}$C NMR spectrum of 20d.
Figure B-4. $^1$H NMR spectrum of 20d.
Figure B-5. $^{13}$C NMR spectrum of 20e.
Figure B-6. $^1$H NMR spectrum of 20e.
Figure B-7. $^{13}$C NMR spectrum of 20f.
Figure B-8. $^1$H NMR spectrum of 20f.
Figure B-9. $^{13}$C NMR spectra of 23a and b.
Figure B-10. $^{13}$C NMR spectra of 23c and d.
Figure B-11. $^{13}$C NMR spectra of 23e and f.
Figure B-15. $^1$H NMR spectrum of 21a incubated in D2O.
Figure B-16. $^1$H NMR spectrum of 21a.
Figure B-17. $^1$H NMR spectrum of deuterated 21c.
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BIOGRAPHICAL SKETCH

Magdalena Alicja Swiderska was born in Mrągowo, Poland, on February 1st, 1978. In 2002 Magdalena graduated from the University of Warsaw, Poland with a master's degree in chemistry under the supervision of Prof. Zbigniew Czarnocki. In 2003 Magdalena moved her scientific career to University of Florida, joined the Stewart’s group and began her Ph.D. studies in the field of biocatalysis and bioorganic chemistry. After 5 years of research, she graduated from University of Florida with a Doctor of Philosophy degree in biochemistry.