APPLICATION OF *Saccharomyces carlsbergensis* OLD YELLOW ENZYME IN THE ENANTIOSELECTIVE HENRY REACTION

By

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To my loving parents and family

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Abstract of Thesis Presented to the Graduate School
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APPLICATION OF *Saccharomyces carlsbergensis* OLD YELLOW ENZYME IN THE ENANTIOSELECTIVE HENRY REACTION

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In this thesis, we studied the application of *Saccharomyces carlsbergensis* Old Yellow Enzyme (a kind of flavin dependent enzyme) in the Henry reaction between β-nitroacrylate and acetylaldehyde.

The reaction was monitored every hour until it went to completion after 12 hours, and four different stereoisomers were observed on GC-MS with different ratio. A control reaction between the β-nitroalkane and acetylaldehyde was conducted and showed that the phosphate buffer solution (KP_i, 100mM, pH~7.0/pH~6.0) catalyzed this Henry reaction and that enzyme catalysis was not involved. Further studies should focus on the rate of carbanion protonation and the reaction between the carbanion and acetylaldehyde, modifying the reaction media to inhibit the carbanion protonation, and choosing better substrates.

CHAPTER 1
INTRODUCTION

History of Old Yellow Enzyme

The flavin-dependent old yellow enzyme (OYE) of yeast was the first discovered flavoprotein. It was isolated from Brewer's bottom yeast by Warburg and Christian in 1932 during their study of biological oxidation.[1] In their study, they tried to use methylene blue to oxidize glucose 6-phosphate in the presence of "*Zwischenferment*," which is glucose-6-phosphate dehydrogenase, and a small heat-stable "*Coferment*," which is $NADP^+$. Warburg found that the addition of a yellow enzyme could allow the reaction system to form a respiratory chain when molecular oxygen was introduced (Fig.1-1).[2]

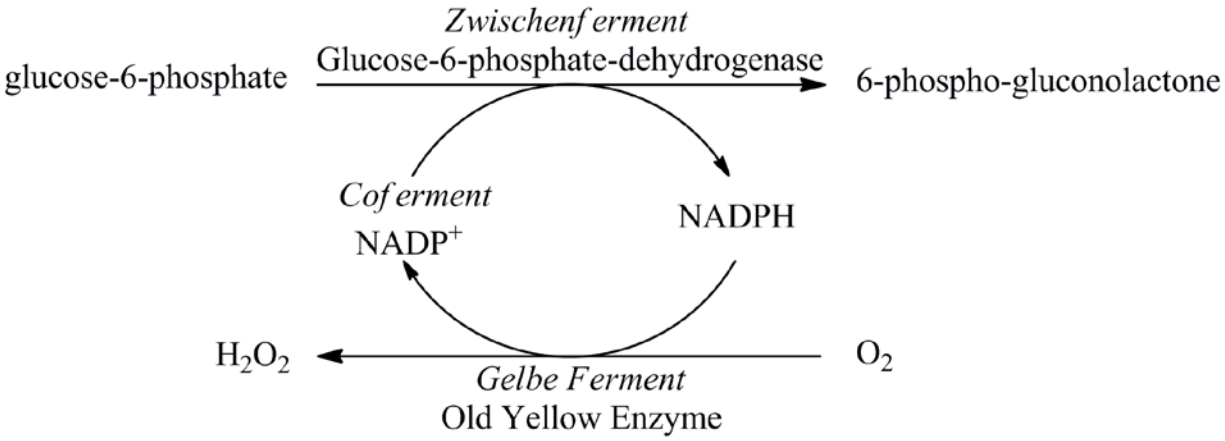


Figure 1-1. Reaction system of Warburg and Christian.

This yellow enzyme was later named old yellow enzyme. The reason for this peculiar name comes from an interesting history: when Warburg first discovered it, he gave it the name "*das gelbe Ferment*." After two years, another yellow enzyme was isolated from yeast by Theorell and named "*das neue gelbe Ferment*." Therefore, Warburg and Christian creatively dubbed the first one "old yellow enzyme," a name that has persisted to today.[3]

Although Warburg and Christian successfully isolated the old yellow enzyme, it was very impure with respect to the high-molecular constituent parts, which consisted mainly of polysaccharides. Theorell studied the yellow pigment in OYE and surprisingly found that this color faded away on reduction and returned on oxidation. Therefore, he came to the conclusion that the enzymatic reaction has something to do with the yellow pigment. After his purification in 1955, Theorell proved that the old yellow enzyme includes two parts: the yellow part is the flavin mononucleotide (FMN), and the other part is the colorless enzyme protein (Fig.1-2).[4]

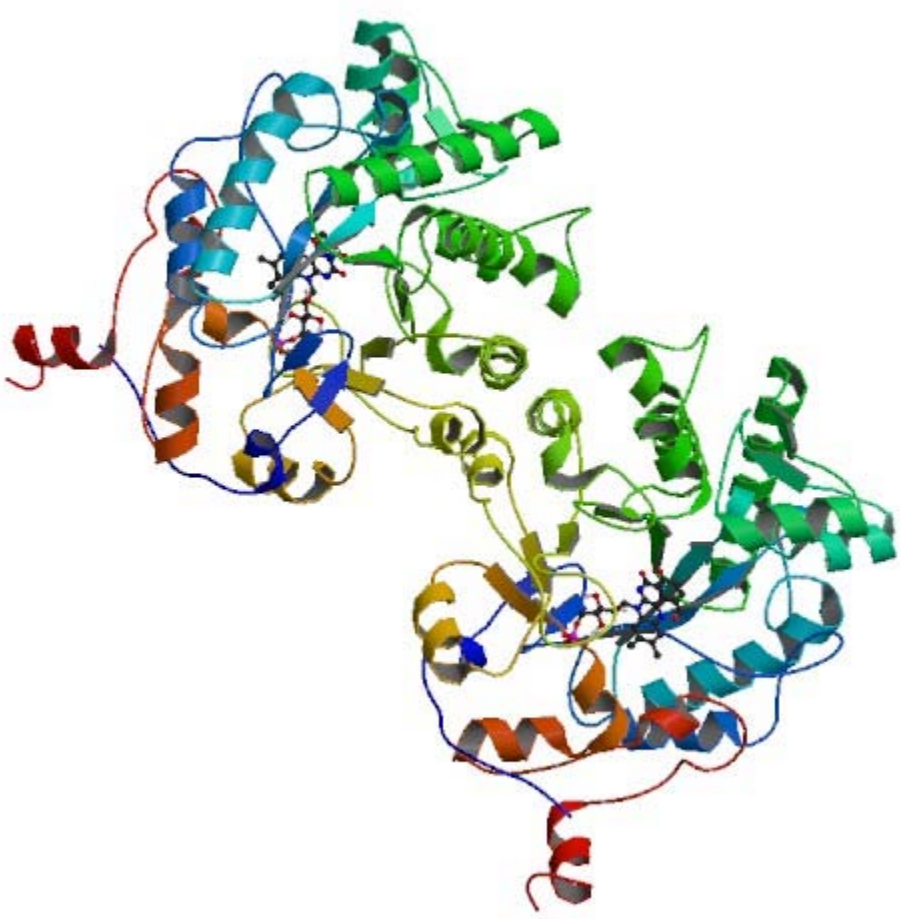


Figure 1-2. A ribbon diagram illustrating the structure of a dimer of OYE.

The Old Yellow Enzyme Family

After the first discovery of OYE, there was a growing interest among scientists in this flavin-dependent enzyme and a number of OYE family members had been found. These homologous proteins have been found in yeasts, plants and bacteria, but not in animals.[5] Through genomic sequencing, many more uncharacterized relatives were predicted.[2]

Yeast Homologues of OYE

In 1991, Saito cloned a gene encoding an isoform of OYE from *Saccharomyces carlsbergensis,* and named it OYE1. After two years, another OYE from *Saccharomyces cerevisiae* was cloned by him and given the name OYE2. Both contain 400 amino acid residues and have very similar molecular weights.[6,7] In 1995, Miranda did a sequence analysis study on a genomic DNA fragment from the yeast *Kluyveromyces lactis* fragment, and found a full length open reading frame (ORF) which encodes for a protein homologous to the OYE. The deduced amino acid sequence of this ORF predicted a protein of 398 residues with 84% similarity in its full length to OYE1from *Saccharomyces carlsbergensis* and OYE2 from *Saccharomyces cerevasiae*.[8] In addition, Buckman and Miller characterized the estrogen-binding protein (EBP1) of *Candida albicans* and showed that it was similar in many ways to OYE in 2000.[9]

Plant Homologues of OYE

Plant homologues of OYE were first identified during studies of octadecanoid biosynthesis by Vick and Zimmerman. They characterized the 12-oxo-phytodienoic acid reductase (OPR), which is a protein related to OYE, from the kernel and seedling of corn in 1985.[10] After their discovery, several more plant strains that contain homologues of OYE were identified. F. Shaller and Weiler purified OPR to homogeneity from

Corydalis sempervirens in 1997,[11] and subsequently cloned the OPR1 from *Arabidopsis thaliana.*[12] Straßner and A. Shaller also isolated and characterized an OYE related enzyme *Le*OPR from tomato in 1999.[13]

Bacterial Homologues of OYE

There are also some reports on bacterial enzymes with homology to OYE. Pentaerythritol tetranitrate (PETN) reductase from *Enterobacter cloacae PB2*[14] can sequentially remove two of the four nitro groups of PETN. *Agrobacterium radiobacter* that contains glycerol trinitrate (GTN) reductase is also responsible for the nitrate ester degradation through catalyzing the reductive scission of GTN to glycerol dinitrates. The difference between the two enzymes is their preference for NADPH and NADH cofactor respectively.[15] Morphinone reductase produced by *Pseudomonas putida M10* can catalyze the NADH-dependent saturation of the carbon-carbon double bond of morphinone and codeinone, and is believed to be involved in the metabolism of morphine and codeine.[16] OYE 1, OYE2, OYE3 and NAD(P)H-dependent-2-cyclohexen-1-one reductase from *Zymomonas mobilis* were expressed in *Escherichia coli* recently and used to reduce the carbon double bond in α,β-unsaturated alkenals and alkenones.[17]

Old Yellow Enzyme Crystal Structure and Mechanism

Theorell first obtained crystals of OYE in 1955; however, the quality was too low for X-ray studies. This was due to heterogeneity of native OYE arising from the two genes present in *S. carlsbergensis*.[2] Fox and Karplus studied the crystal structures of oxidized and reduced form of OYE at 2Å resolution.[18] They came to the conclusion that OYE belonged to the α/β barrel FMN dependent enzyme family, which is closely related with trimethylamine dehydrogenase.[18] The overall structure of OYE is an α/β barrel, with

the flavin binding to the protein at the C-terminal end and the isoalloxazine ring perpendicular to the barrel axis. The crystal structure revealed a long solvent channel through the loops at the C-terminal end, and this exposes the *si* face of the flavin to the solvent. By contrast, the *re* face is completely buried by interactions with protein main chain and side chain groups. The dimethylbenzyl ring of the flavin is solvent-accessible both from the *si* face and along the edge, whereas the other two rings are accessible only from the *si* face.[19] From representation of the active site of OYE in complex with *p*-hydroxybenzaldehyde (Fig 1-3), it can be seen that the phenolic ring undergoes a stacking interaction with the flavin (grey bonds). The phenol oxygen forms two hydrogen bonds to His-191 and Asn-194, displacing a chloride ion bound in the empty oxidized enzyme structure.[2]

After the determination of OYE crystal structure, scientists began to investigate the catalytic properties of OYE. Massey's studies showed that OYE could catalyze the NAD(P)H-dependent reduction of quinines. It was also effective in reducing many α,β-unsaturated carbonyl compounds such as 2-cyclohexenone, in which the olefinic double bond is reduced while the carbonyl functional group remains untouched.[3] These reactions proved to be faster than the NAD(P)H oxidase reaction and are limited in rate by the reduction of enzyme flavin by NAD(P)H.[3]

The NADPH-dependent reduction of OYE was found to proceed through a ping-pong mechanism, in which the product and $NAD(P)^+$ leave the enzyme before the reaction with acceptor (Figure 1-4). In this mechanism, the oxidized flavin-containing OYE first binds with the cofactor NAD(P)H, then hydride transfer happens between NAD(P)H and the flavin in OYE. This forms the EFl_{ox} intermediate and releases

$NAD(P)^+$. After protonation from the solvent, the intermediate will convert to the reduced flavin-containing OYE, and bind with a new substrate. After hydride transfer between the $FMNH_2$ and the substrate, the reduced substrate will be released and the oxidized flavin-containing OYE goes to the next regeneration cycle. In the ping-pong mechanism, only after the first substrate is released can another substrate bind and react with the modified enzyme, regenerating the unmodified enzyme.

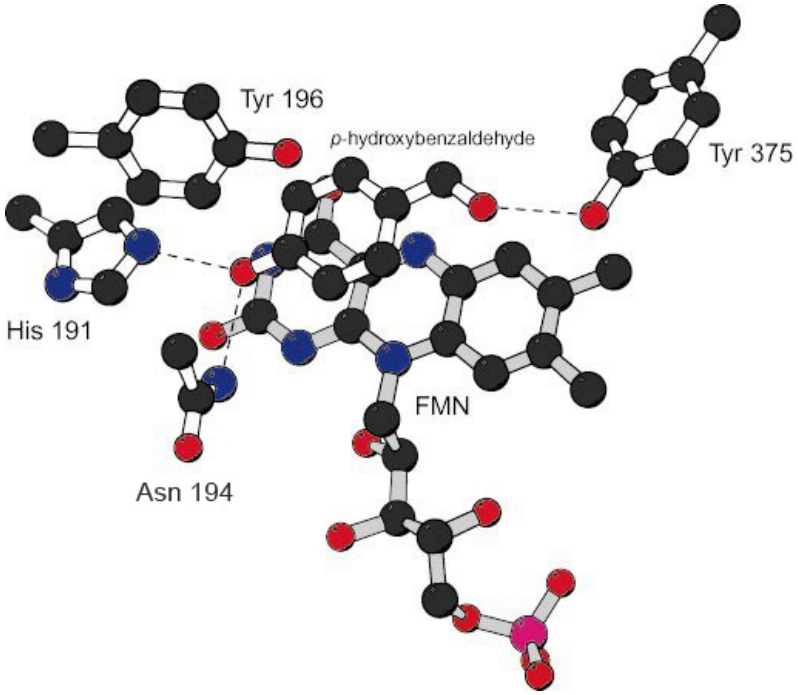


Figure 1-3. Active site of OYE in complex with *p*-hydroxybenzaldehyde.

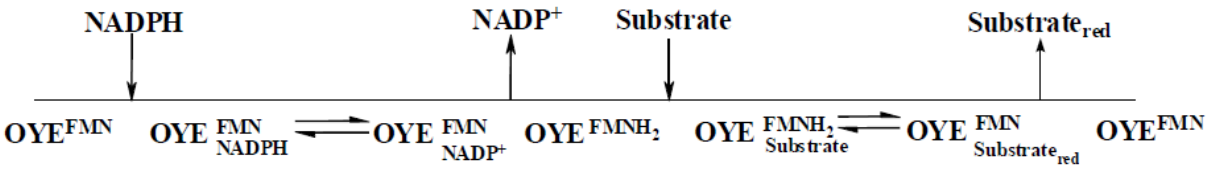


Figure 1-4. Kinetic mechanism of OYE.

Since the nicotinamide cofactor NAD(P)H is expensive, an efficient coenzyme recycling system is desired in order to use the cofactor in catalytic amount. In this case, a number of dehydrogenase and reductase are used to regenerate NAD(P)H cofactors

and the overall mechanism could be summarized as Fig 1-5. An alkene is first activated by an electron withdrawing group attached on it, then the flavin that is contained in OYE delivers a hydride to the β carbon and the carbanion uptakes a solvent derived proton at the α position. The reduced FMN is regenerated in the presence of NAD(P)H cofactor while the cofactor itself is regenerated by adopting a hydride from the dehydrogenase.

Purification of Old Yellow Enzyme

The old yellow enzyme was first isolated as a homogeneous, crystalline protein by Theorell and Åkeson in 1956, however, this method was very time-consuming and the efficiency was low.[20] It was later found that the enzyme activity was associated with a distinctly green fraction and that this fraction was a charge-transfer complex formed by oxidized enzyme and a low molecular weight small molecule. The classical old yellow enzyme could be regenerated from the distinctive green complexes by dialysis of the reduced enzyme and subsequent re-oxidation, the old yellow enzyme was therefore isolated as a complex.[21] Later, Massey developed a simple method of old yellow enzyme purification by binding enzyme to an affinity matrix containing a phenolic substituent followed by release from the matrix by flavin reduction using sodium dithionite. This strategy was based on the finding that many phenolic compounds bound strongly to old yellow enzyme when the flavin was in the oxidized state, but not in the reduced state.[22]

Henry Reaction

The Henry reaction, which is also known as the nitroaldol reaction, is a base-catalyzed C-C bond-forming reaction between nitroalkanes and aldehydes or ketones. The significance of this reaction is that the nitroalcohol formed from this carbonyl

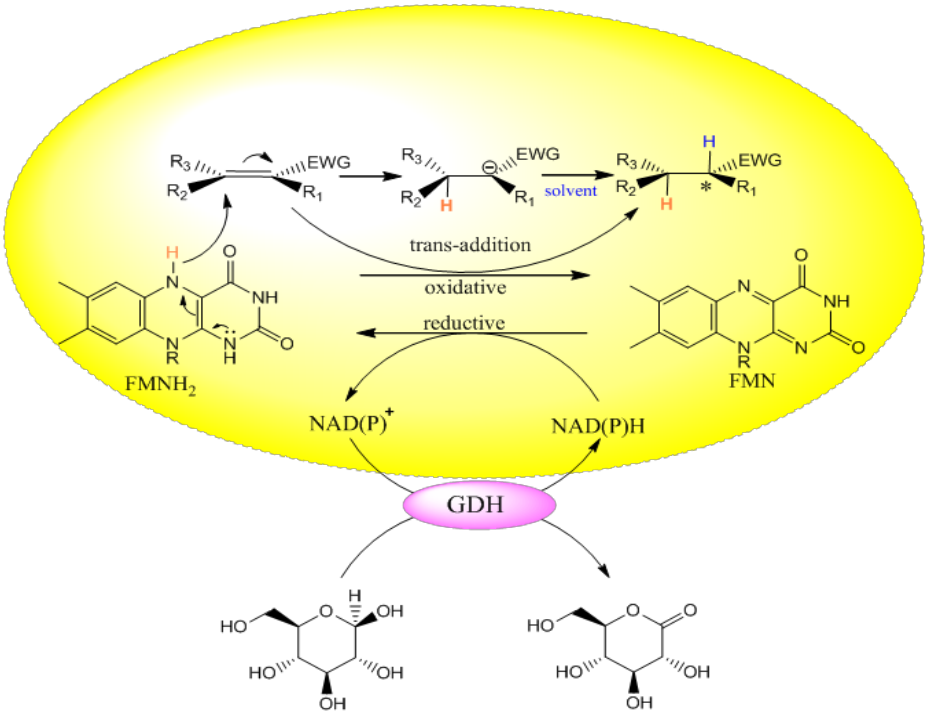


Figure 1-5. Catalytic cycle for old yellow enzyme in the reduction of activated alkene.

addition may be transformed into valuable building blocks[23], which can be very useful in pharmaceutical and natural product synthesis[24] (Fig. 1-6). Recent efforts have therefore focused on the development of catalytic enantioselective reaction variants.

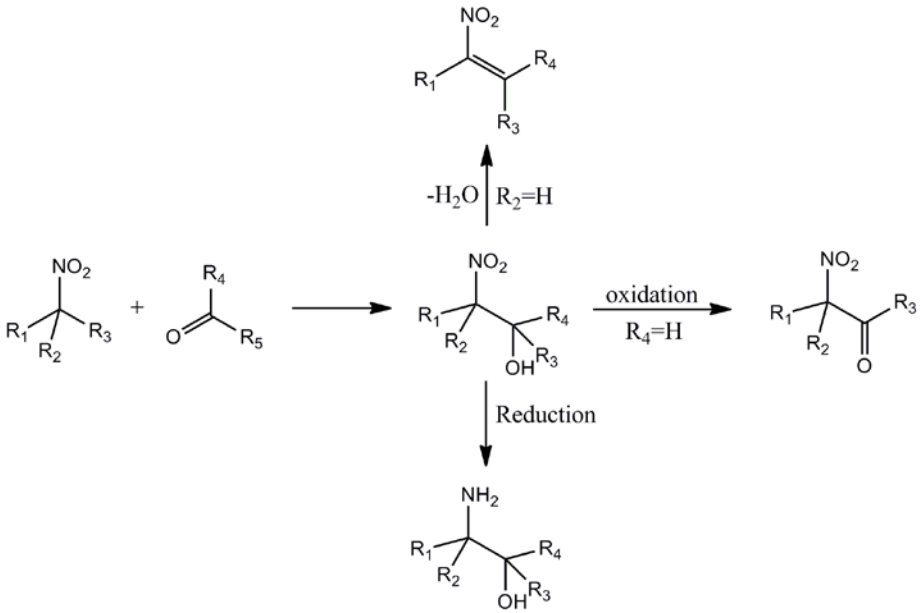


Figure 1-6. Transformation of nitroalcohol to different building blocks.

The first example of a catalytic asymmetric Henry reaction was reported by Shibasaki. In this reaction, (*S*)-(-)-binaphthol in conjunction with a lanthanum alkoxide were used as the reagent system, and an enantiomeric excess of 73%-90% was obtained (Fig. 1-7).[25] After the first successful trial using the chiral binaphthol/rare earth reagent system, a series of enantioselective Henry reactions were developed by Shibasaki using catalysts derived from parent binaphthol/lanthanum alkoxide.[26-29] In 2002, Trost reported a novel type of asymmetric zinc catalyst which involves a dinuclear zinc complex center with a chiral semi-azacrown ligand (Fig. 1-8). This was the first time zinc catalyst had been applied in enantioselective Henry reaction, and this catalyst proved to be successful when applied to enantioselective, direct nitroaldol reactions involving various ketone nucleophiles and aldehyde electrophiles, even when aldehyde substitutes are bulky.[30] (Fig. 1-8).

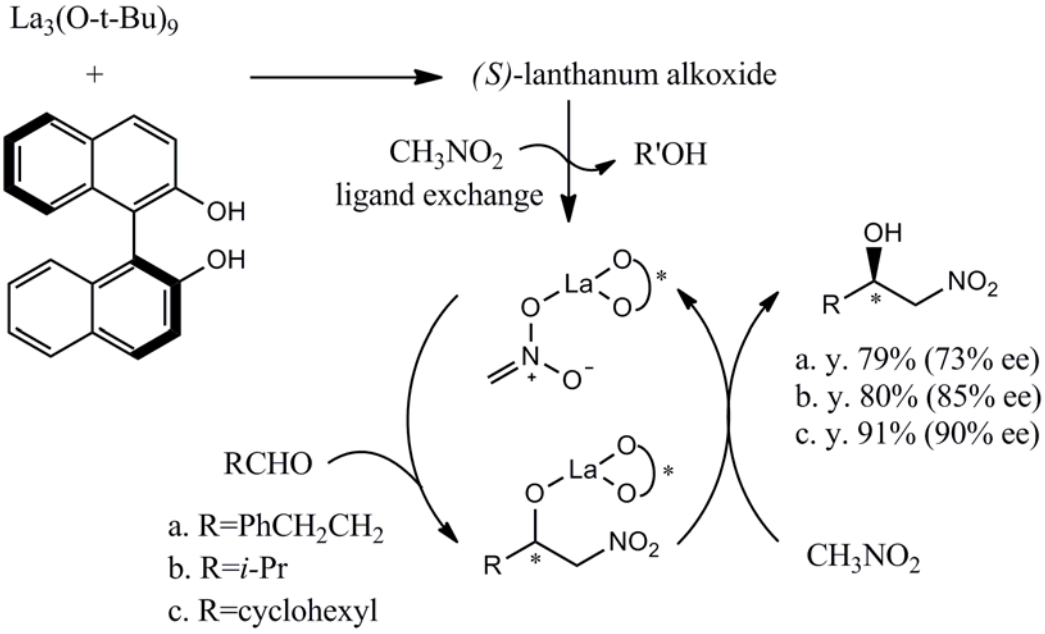


Figure 1-7. Reaction mechanism of the first catalytic asymmetric nitroaldol reaction with the binaphthol/rare earth protocol.

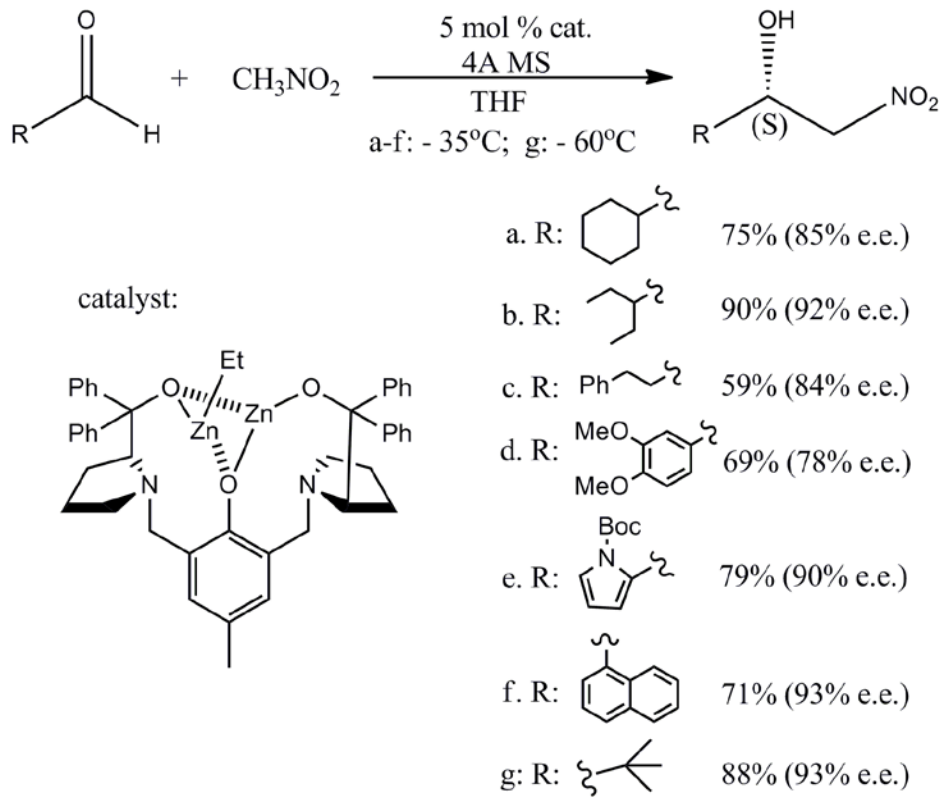


Figure 1-8. Zn catalyst developed by Trost and enantioselective nitroaldol reaction with CH_3NO_2.

Chiral copper complexes have also found wide applications in the catalytic Henry reactions. A notable example was developed by Evans, which involved the copper acetate-bis(oxazoline) catalyzed enantioselective Henry reaction between nitromethane and aldehydes.[23] This method is very general for a range of both aliphatic and aromatic aldehydes and works well under mild reaction conditions (room temperature, ethanol as solvent). A transition state model involving a Jahn–Teller effect on Cu^{II} coordination and positioning of reactants in the most favorable orientations according to steric and electronic considerations has been proposed.[23]

Besides inorganic catalysts, research on small organic molecules in the organocatalytic Henry reactions has also attracted considerable attention. There have been reports on using chiral guanidine species, but the enantiomeric excess obtained

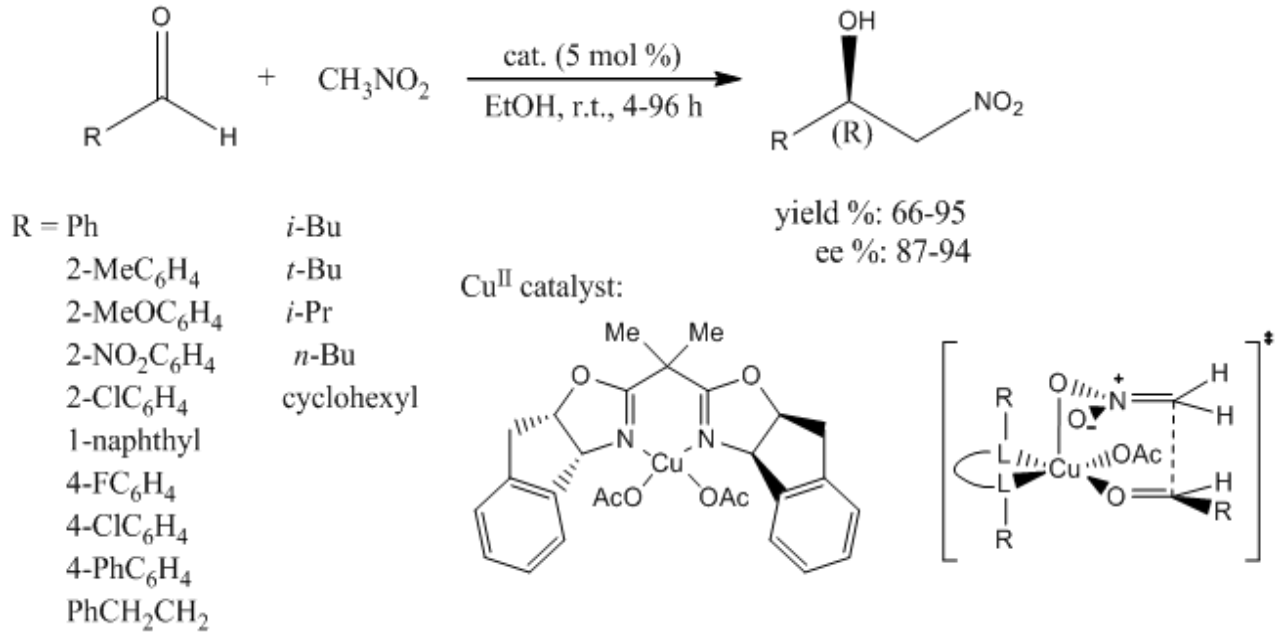


Figure 1-9. Bifunctional $Cu(OAc)_2$–BOX catalyst for broad-scope enantioselective Henry reactions developed by Evans, together with the proposed TS model.

was relatively low (20%-56%).[31-33] The minimum requirement for effective catalytic activity in a nitroaldol reaction was found to involve organic molecules bearing both a thiourea and an amine residue. Hiemstra's catalyst serves as a good example. It catalyzed the nitroaldol reactions between nitromethane and aromatic aldehydes with 50-99% yields and 85-92% ee values (Fig. 1-10).[34]

Although the stereoselective chemical catalyzed Henry reaction is relatively mature, the enzymatic catalyzed Henry reaction is still in the stage of development. The first biocatalytic asymmetric Henry reaction was not reported until 2007, when Griengl used hydroxynitrile lyase from *Hevea brasiliensis* to conduct the Henry reaction between aldehyde and nitromethane (Fig. 1-11, Table 1-1).[35] His idea was based on the fact that the hydroxynitrile lyase can not only catalyze the formation and cleavage of cyanohydrins but also the reaction of nitroalkanes with aldehydes (Henry reaction).

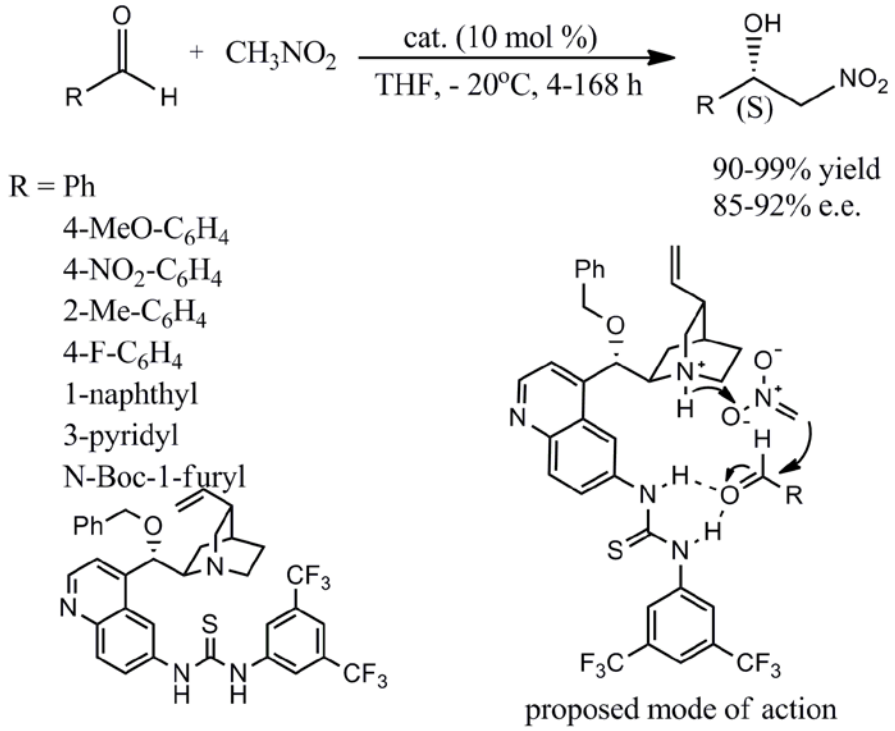


Figure 1-10. A quinine-derived bifunctional amine-thiourea organocatalyst and its performance in Henry reactions.

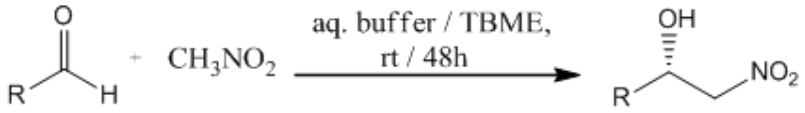


Figure 1-11. *Hb*HNL-catalyzed Henry reactions of aldehydes with nitromethane

Table 1-1. *Hb*HNL-catalyzed Henry reactions of aldehydes with nitromethane

R	pH 7.0 Yield (%)	pH 7.0 ee (%)	pH 5.5 Yield (%)	pH 5.5 ee (%)
a. Ph	63	92	32	97
b. 3-HOC_6H_4	46	18		
c. 4-$NO_2C_6H_4$	77	28	57	64
d. *n*-hexyl	25	89	34	96
e. $Ph(CH_2)_2$	9	66	13	66
f. 2-furyl	57	72	43	88
g. $CH_3(CH_2)_4CHCH_3$	2	88		
h. thienyl			29	98
i. 3-furyl			16	89
j. 2-ClC_6H_4			23	95
k. 3-ClC_6H_4			36	98
l. 4-ClC_6H_4			25	97
m. 4-$MeOC_6H_4$			20	99
n. cyclohexyl			18	99

CHAPTER 2
OLD YELLOW ENZYME IN ASYMMETRIC C=C BOND BIOREDUCTIONS

Asymmetric C=C Bond Reductions

Asymmetric C=C bond reductions offer especially attractive routes to chiral building blocks since up to two adjacent stereocenters could be established by a single reaction. This technique is of great importance due to the increasing demand to produce enantiomerically pure pharmaceuticals, agrochemicals, flavors, and other fine chemicals.[36] The start of the development of catalysts for asymmetric hydrogenation was the concept of replacing the triphenylphosphine ligand of the Wilkinson catalyst with a chiral ligand.[37] Knowles and Horner reported the earliest examples of enantioselective hydrogenation, however the optical purity was very low (Fig 2-1).[38,39]

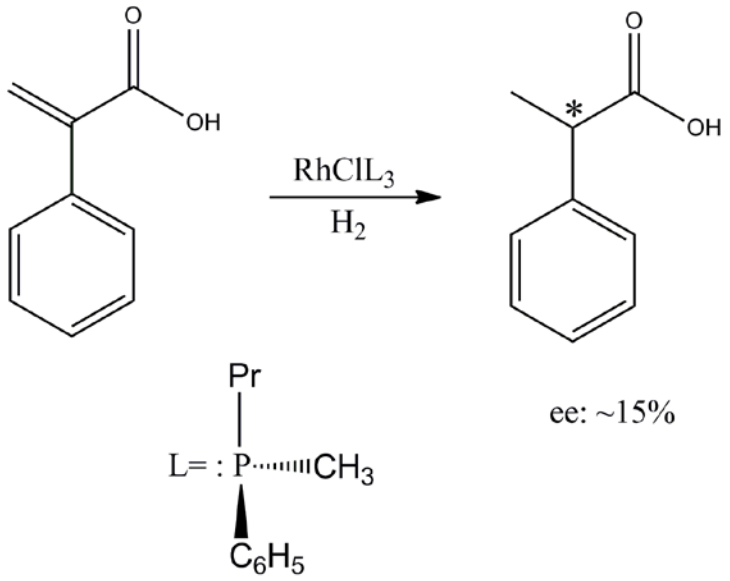


Figure 2-1. First use of a chiral ligand in the hydrogenation of an olefin.

After the initiation of asymmetric hydrogenation, studies on making chiral ligands to control the enantioselectivity began to attract scientists' interest. A notable work was Noyori's research on BINAP-Ru catalysts for asymmetric hydrogenation, which opened up opportunities for efficient hydrogenations of a variety of substrates. The enantiomeric

excess could reach up to 100% in the presence of the BINAP-Ru catalysts (Fig 2-2).[40,41] His distinguished work on chirally catalyzed hydrogenation reactions won him the Nobel Prize in chemistry in 2001.

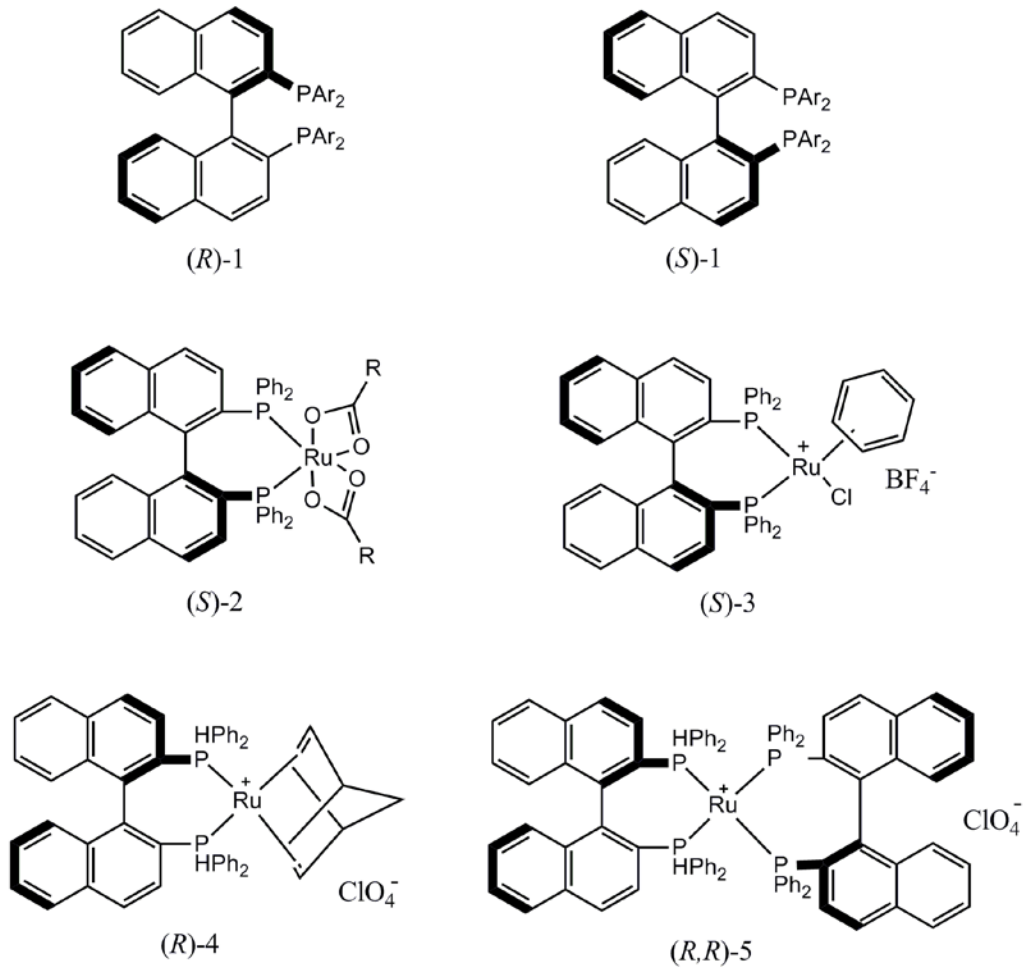


Figure 2-2. BINAP-Ru catalysts that are developed by Noyori.

Despite of the tremendous development in this area, high stereoselectivity nearly always depends on the polarity of the functional group that is attached to the alkene. Highly polar groups such as amides, acids and alcohols can lead to higher stereoselectivity while less polar groups such as ketones, esters or nitro groups usually result in lower enantiomeric excess values. In addition, because complex chiral ligands for chemical reduction are usually required, they are expensive, toxic and difficult to

make. Also, high hydrogen pressures are often required for the optimal results. These drawbacks have prompted chemists to explore biocatalytic alternatives, and enzymatic reduction proved to be a useful solution to this problem, especially the whole cell mediated reductions.[42-45]

Asymmetric α,β-Unsaturated Carbonyl Bioreductions

The use of whole cells for alkene reductions avoids the need for external cofactor recycling. Although the stereoselectivities achieved were often excellent, chemoselectivities of whole cell bioreductions with respect to C=C versus C=O bond reduction are often poor, which is due to the presence of competing alcohol dehydrogenase. As a result, the selective asymmetric bioreduction of conjugated enals or enones to furnish the corresponding saturated aldehydes or ketones is severely impeded by side reactions (Fig 2-3).[46] Finally, the overall reduction yield is low because native cofactor regeneration pathways in whole cells are often inefficient. All of these drawbacks could be avoided by using flavin-containing old yellow enzyme with suitable dehydrogenase in the presence of nicotinamide cofactors.[47]

The asymmetric synthesis of citral is a challenging task in terms of chemo-, regio- and stereoselectivity. Therefore, this reaction was chosen to investigate the potential of the flavin-dependent OYE in chemical synthesis. Citral, a mixture of the geometric isomers geranial (trans-isomer) and neral (cis-isomer), is an important chiral building block. Citral is reduced by the OYE from *Gluconobacter oxydans*,[48] OPR1 and OPR3 from *Lycopersicon esculentum* (tomato), and YqjM from *Bacillus subtilis* into citronellal (Fig 2-4).[47] The reduction was performed with high regio- and chemoselectivity, only the activated C=C bond was reduced while the other isolated C=C bound and the carbonyl moiety stayed untouched. Also, the reaction conversion reached up to 100%, and for

the stereoselectivity of the product, the enzymes produced the (*S*)-citronellal with an excellent ee of >99%.

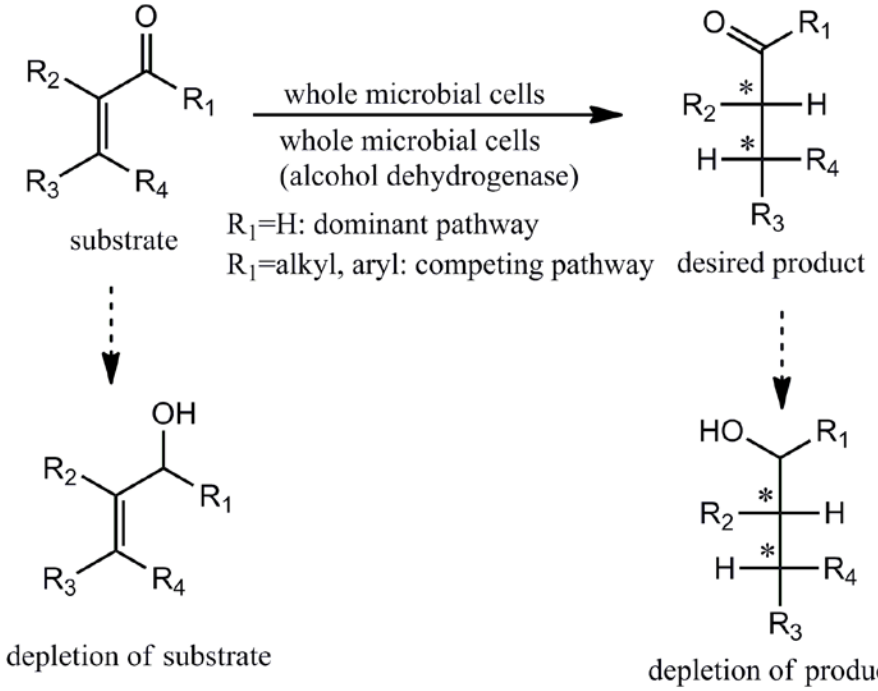


Figure 2-3. Asymmetric bioreduction of α,β-unsaturated aldehydes and ketones using whole microbial cells often shows undesired carbonyl reduction.

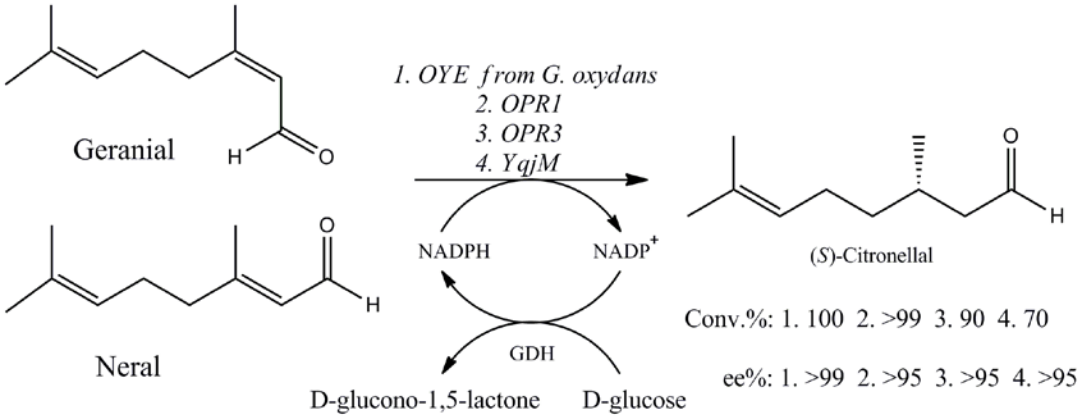


Figure 2-4. The asymmetric enzymatic reduction of the isomers neral and geranial (citral) to (*S*)-citronellal by different OYEs.

Asymmetric Cyclohexenone Bioreductions

Cyclohexenone is an organic compound which is a versatile intermediate used in the synthesis of a variety of chemical products such as pharmaceuticals and fragrances.

When α or β position of cyclohexenone is substituted, C=C bond reduction leads to chiral cyclohexanones, which are one of the most interesting synthons due to their applications in the production of biologically active substances. Up to now, both chemical and biochemical reductions for the cyclohexenone have been developed. One is the conjugate additions of organometallic reagent to cycloalkenone, the other is the asymmetric conjugate reduction of conjugate enones. Also, enzymatic reduction using P90 and P44 reductases from *Nicotiana tabacum* demonstrates that this enzyme can enantiotropically reduce the C=C double bond of enones to afford optically active 2-alkylated cyclic ketones with high enantiomeric excess (Fig 2-5, Table 2-1, Table 2-2).[49]

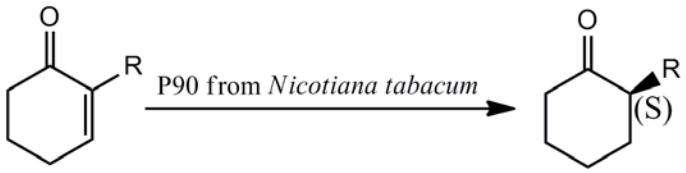


Figure 2-5. Reduction of enone with P90 from *Nicotiana tabacum.*

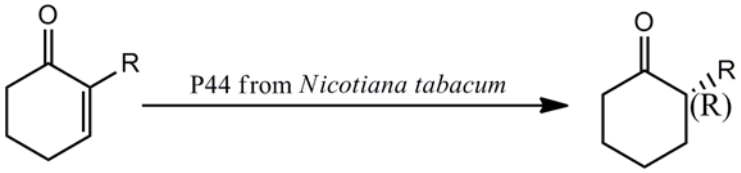


Figure 2-6. Reduction of enone with P44 from *Nicotiana tabacum.*

Table 2-1. Conversion and ee value for reduction of different enone with P90

R	conv.%	ee%
Me	95	99
Et	57	98
n-Pr	35	95

Table 2-2. Conversion and ee value for reduction of different enone with P44

R	conv.%	ee%
Me	80	>99
Et	45	>99
n-Pr	37	>99

A variety of flavin-containing old yellow enzyme catalyzed bioreduction for cyclic ketones have also been studied, and the catalytic mechanism has been investigated in great detail. Faber used OPR1, OPR3, YqjM to reduce α-substituted cyclopentenone and cyclohexenone, and found that the ring size of the substrate had a tremendous effect on the stereochemical outcome of the bioreduction (Fig 2-7, Table 2-3).[47] However, this transformation only applied to the α-substituted compounds. For the corresponding β-substituted cycloalkenones, conversions were not observed for both substrates using OPR1 and YqjM. OPR3 showed low activities, but with excellent stereoselectivity. Stewart used OYE of *Saccharomyces carlsbergensis* expressed in *Escherichia coli* cells, and conducted the chemo- and stereoselective alkene reductions using a series of both α and β-substituted 2-cyclohexenones (Fig 2-8). The proposed mechanism includes an anti addition of H_2, hydrogen bonds contributed by His 191 and Asn 194 position activate the carbonyl oxygen, then hydride transfer from the flavin with concomitant protonation by the side-chain of Tyr 196 (Fig 2-9).[50] Chemo- and stereoselectivity are generally excellent, and the product configuration depends on substituted pattern of the 2-cyclohexenone (Table 2-4).

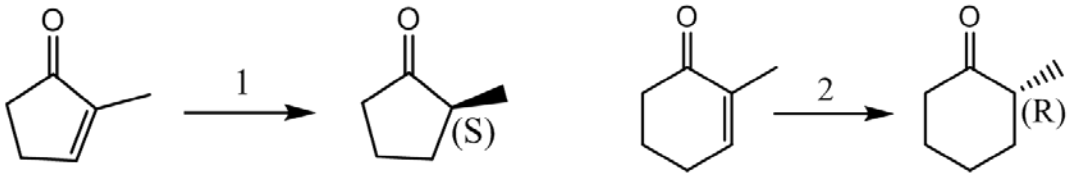


Figure 2-7. Asymmetric bioreductions of cyclic alkenes by OPR1, OPR3 and YqjM.

Table 2-3. Asymmetric bioreduction of α-substituted cyclopetenone and cyclohexenone using OPR1, OPR3 and YqjM.

Entry	Cofactor	OPR1		OPR3		YqjM	
		Conv.%	ee%	Conv.%	ee%	Conv.%	ee%
1	$NADP^+$/G6PDH	14	(S)61	10	(S)58	72	(S)94
2	$NADP^+$/G6DPH	82	(R)66	92	(R)71	95	(R)93

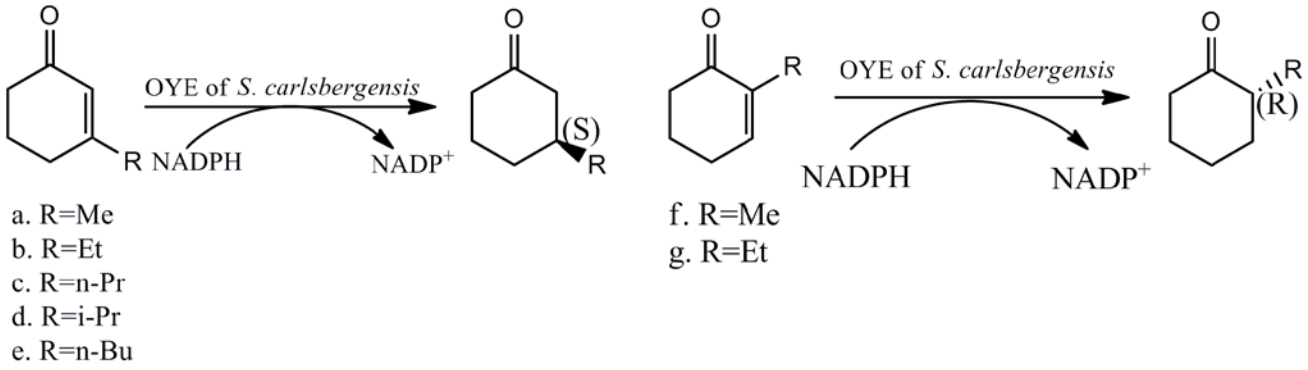


Figure 2-8. A series of 2- and 3-alkyl-substituted cyclohexenones in the asymmetric bioreductions by OYE from *S. carlsbergensis.*

Table 2-4. Reductions of alkyl-substituted 2-cyclohexenones by engineered *E. coli* cells overexpressing *S. carlsbergensis* old yellow enzyme.

ketone	*R*	Conversion(%)	ee(%)	Configuration
a	Me	100	94	S
b	Et	76	94	S
c	*n*-Pr	25	89	S
d	*i*-Pr	18	90	S
e	*n*-Bu	-	-	-
f	Me	100	96	R
g	Et	16	90	R

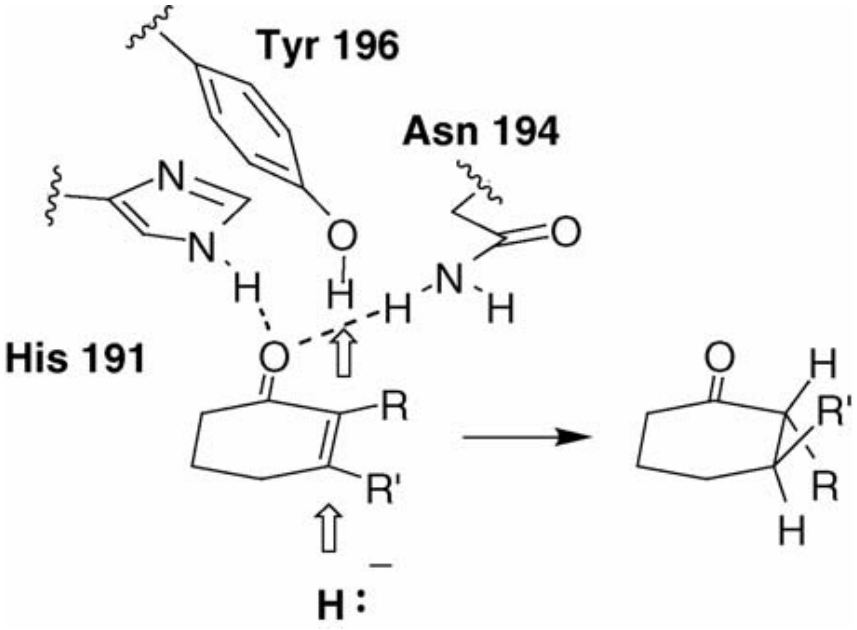


Figure 2-9. Schematic diagram of cyclohexenone OYE bioreduction.

Asymmetric Nitroalkene Bioreductions

Optically active β-nitroalkanes are valuable building blocks in synthetic chemistry; they could be easily converted into chiral amines, which are very useful compound in organic synthesis,[51] they are valuable precursors to a variety of intermediates, some of which are summarized in Fig. 2-10.

Commonly used reduction methods for nitroalkene include borohydride derivative based reduction,[52] catalytic transfer hydrogenation[53] and metal halides based reduction.[54] However, the selective reduction of the conjugated double bond is rather difficult because nitro-group reduction often takes place simultaneously. Sodium borohydride is an effective reductant , but the reaction is often accompanied by the formation of polymeric side products through Michael addition of the nitronate intermediate to the starting nitroalkene. Metal catalyzed hydrogenations and transfer hydrogenation are also very effective, but disposal of the toxic metal ions aroused another difficulty.[55] Whole cell bioreduction using baker's yeast can avoid the drawbacks. Kawai used two different nitroalkene reductases YNAR-I and YNAR-II that were isolated from baker's yeast to reduce a trisubstituted nitroalkene, and the products showed excellent enantioselectivity, moderate diastereoselectivity, and good yield (Fig. 2-11).[56]

β-Nitroacrylates are a class of electron-poor nitroalkenes having two electron-withdrawing groups in α- and β-positions. This peculiarity makes their chemistry behavior more interesting with respect to the classical conjugated nitroalkenes. The flavin-containing old yellow enzyme with the NADPH cofactor recycling system was used in the reduction step of β-nitroacrylate as a route to chiral β^2-amino acids. These enzymatic reductions occur with 87-96% ee, with larger substrate providing greater

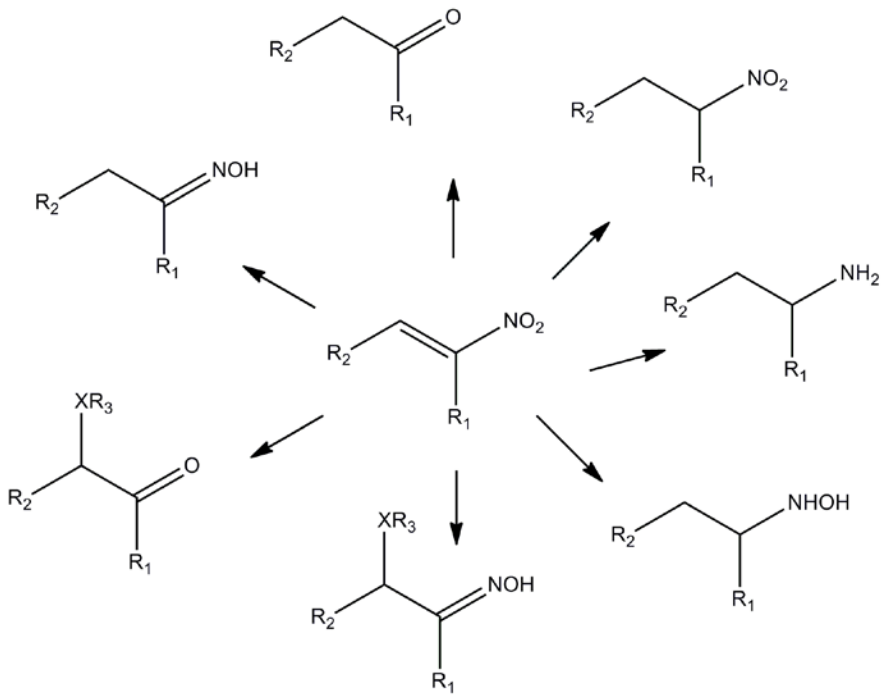


Figure 2-10. Nitroalkene as a precursor to a variety of intermediates.

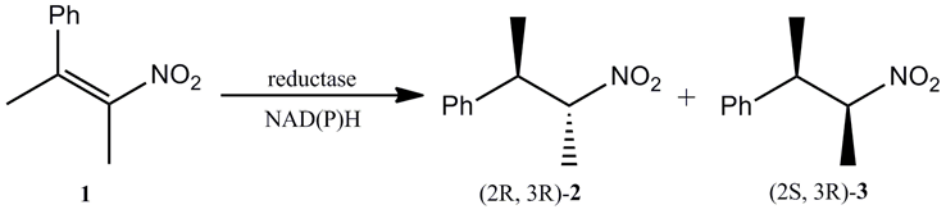


Figure 2-11. Enzymatic reduction of (*Z*)-3-phenyl-2-nitro-2-butene with YNAR-I and YNAR-II[a]

Table 2-5. Enzymatic reduction of (*Z*)-3-phenyl-2-nitro-2-butene with YNAR-I and YNAR-II[a]

Enzyme	Coenzyme	Relative activity	d.e.[b](%)	e.e.[c](%)	e.e.[d](%)
YNAR-I	NADPH	1.00	31	>98	97
	NADH	0.60	29	>98	97
YNAR-II	NADPH	1.00	35	>98	97
	NADH	0.54	34	>98	97

[a] Condition: acetate buffer, 100mM, pH=5.0.
[b] Excess of 2 over 3.
[c] e.e. of 2
[d] e.e. of 3

stereoselectivities (Fig 2-12, Table 2-6).[57] A mechanism for the reduction was also proposed. Compared with 2-cyclohexenone, one nitro oxygen occupies the same

location as the carbonyl oxygen and the alkene is positioned similarly. Hydrogen bonds contributed by His 191 and Asn 194 position activate the nitro oxygen, then hydride transfer happens from the flavin with concomitant protonation by the side-chain of Tyr 196, the net result is that H_2 trans-addition was achieved (Fig 2-13).[57]

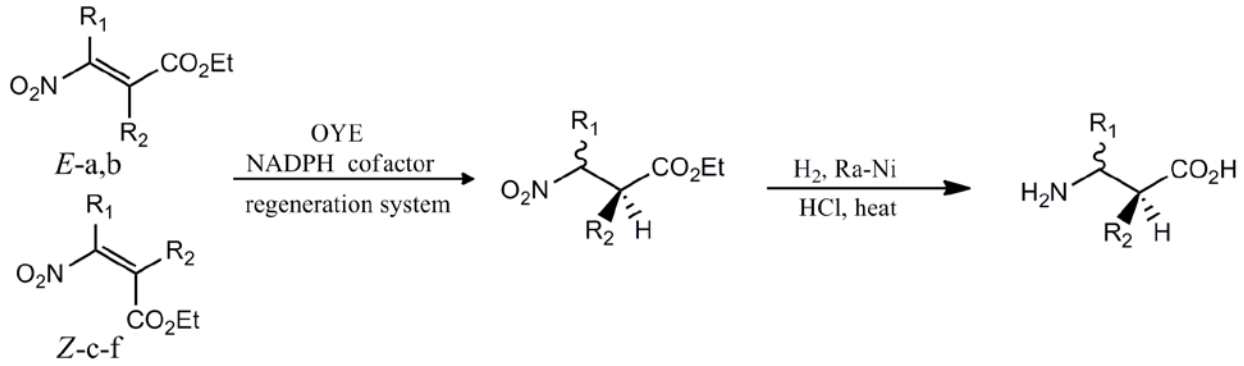


Figure 2-12. Asymmetric bioreduction of β-nitroacrylates to chiral β^2-amino acid.

Table 2-6. Reductions of β-nitroacrylates by *S. carlsbergensis* old yellow enzyme.

	R1	R2	Starting Cmpd	Conv. after 8h(%)	e.e.[c](%)	Config.
a	Me	H	(*E*)-a	>98	8	R
b	Et	H	(*E*)-b	50	-	-
c	H	Me	(*Z*)-c	>98	98	R
d	H	Et	(*Z*)-d	>98	91	R
e	H	*n*-Pr	(*Z*)-e	>98	94	R
f	H	*i*-Pr	(*Z*)-f	>98	96	R

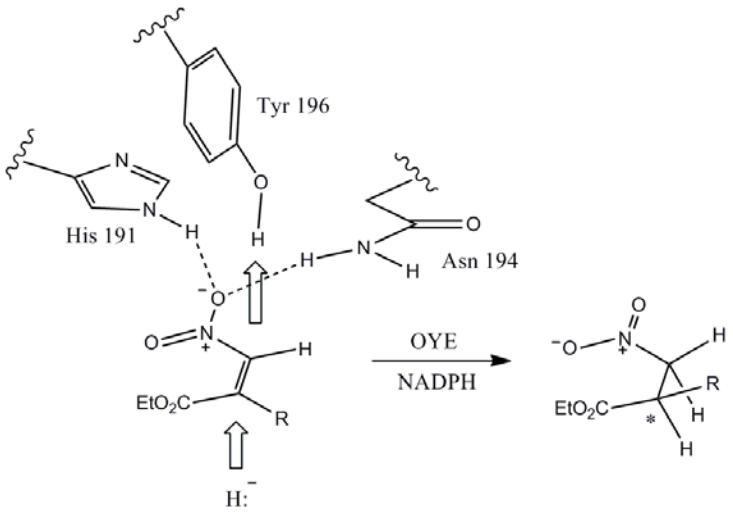


Figure 2-13. Schematic diagram of β-nitroacrylates OYE bioreductions.

CHAPTER 3
OYE: A POTENTIAL CATALYST FOR ENANTIOSELECTIVE HENRY REACTION

Introduction

The addition reaction between nitroalkanes and carbonyl compounds to yield a nitroalcohol, namely the nitroaldol or Henry reaction, it provides chemists a powerful strategy for C-C bond formation. In addition, the nitro alcohol products obtained from Henry reaction could be transformed into many important derivatives, such as niroalkene, amino alcohol, amino acids etc. Although significant success has been achieved in asymmetric versions of the Henry reaction, control of the stereoselectivity still remains difficult. The use of chiral metal catalysts has proved to be effective in the stereocontrol, but the long reaction times, the cost of the complex catalysts, and their disposal has prompted chemists to find simpler and environmental friendly ways. Based on the mechanism of the Henry reaction, as well as the asymmetric bioreduction of nitroalkenes, we hypothesized that the same enzymatic catalysis could also be applied to Henry reaction. Since the nitronate carbanion intermediate formed after the hydride was delivered to the β carbon has a long life time, if we add a small carbonyl compound like acetylaldehyde, nucleophilic attack might happen to the acetylaldehyde within the OYE, with a subsequent protonation by the solvent. By contrast, the nitronate carbanion protonation should be inhibited. The Henry reaction product is interesting because it contains three different chiral centers, we can see if the formation of one chiral center influences the chirality of another, also, due to the fact that enzymatic reduction is highly selective, we could see whether OYE catalyzed Henry reaction can improve the stereoselectivity or not. Fig. 3-1 shows the catalytic cycle for biocatalytic Henry reaction using old yellow enzyme.

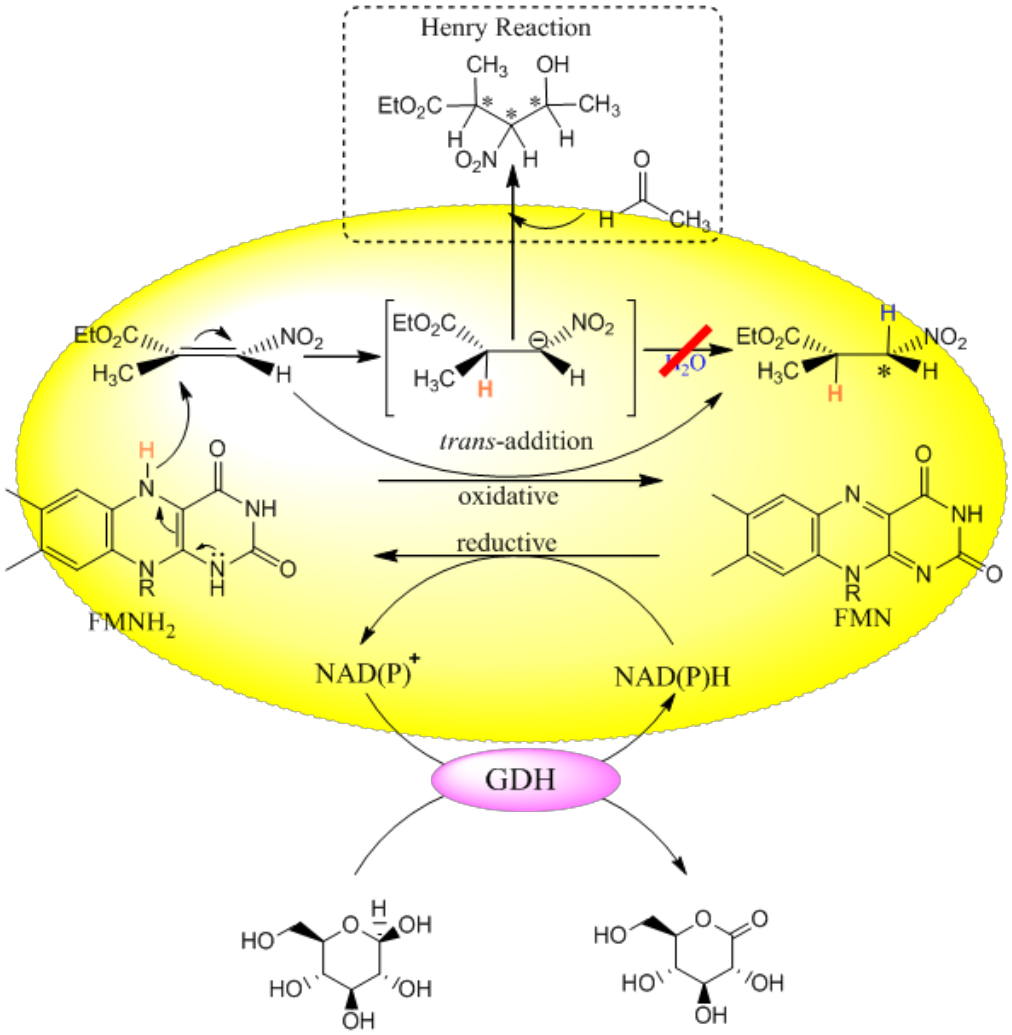


Figure 3-1. Catalytic cycle for biocatalytic Henry reaction using old yellow enzyme.

Results and Discussions

To test the hypothesis, we first needed to prepare a β-nitro acrylic ester. After comparing several synthetic methods, we decided to follow Ballini's protocol since this method avoids drastic conditions, low yields and low selectivity (Fig 3-2).[58] The first step of the sequence is a nitroaldol Henry reaction between nitromethane and ethyl pyruvate, performed under heterogeneous catalysis using ion exchange resin (Amberlyst A-21). The reaction proceeds under room temperature without the protection of argon, and the yield after purification can vary from 64%-88%. NMR spectra of the nitroaldol product

matched the previous reports.[58] This alcohol was further converted to the dehydration product β-nitroacrylate through mesylation and elimination.[59] Since this step usually lead to a relatively low yield of β-nitroacrylate, we explored the reaction conditions, including temperatures of -70°C, -20°C, 0°C along with a variety of reactant stoichiometries. The optimal reaction condition proved to be nitroalcohol, MsCl and Et_3N with a 1:3:3 ratio under 0°C for 2-4 hours (Table 3-1).

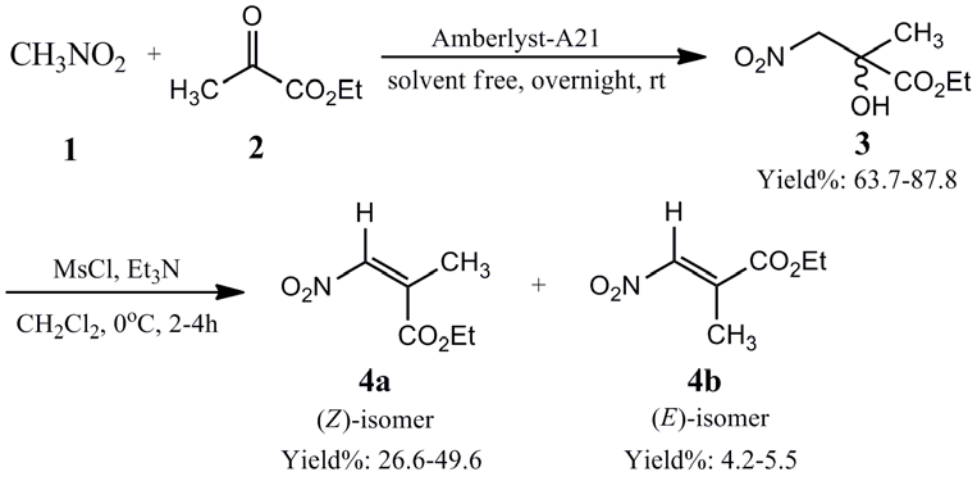


Figure 3-2. Synthetic route of mono-substituted β-nitroacrylate (**4a,b**).

Table 3-1. Several different reaction conditions for the synthesis of β-nitroacylate (**4a,b**).

Starting Material	Reagent	Ratio (SM:MsCl:Et_3N)	Temp	Rxn Time	Total Yield%	Yield(*E*)%	Yield(*Z*)%
Alcohol	MsCl,Et_3N	1:3:3	0°C	2-4h	53.8	4.2	49.6
Alcohol	MsCl,Et_3N	1:3:3	-20°C	2-4h	32.1	5.5	26.6
Alcohol	MsCl,Et_3N	1:3:3	-70°C	2-4h	49.2	4.8	44.4

The hydrogenation of β-nitroacrylate is a necessary step in making the standard. We have used $NaBH_4$ as a reducing agent to make the β-nitroalkane but the ester group was overreduced at the same time. After purification, the yield of the nitroalkane was only 20%-32%. A milder reducing agent $NaBH(OAc)_3$ was substituted. Unfortunately there was no significant improvement and the yield of the nitroalkane was

23%-30% (Fig 3-3). The organocatalytic transfer hydrogenation using Hantzsch ester as the hydride source and thiourea catalyst can effectively increase the yield (up to 77% in our case, Fig. 3-4),[60,61] but purification was difficult since the pyridine by-product and the thiourea catalyst were always detected in the purified product. After weighing the advantages and disadvantages of different reducing agents, $NaBH_4$ was finally chosen due to its low cost, product purity and the facile reaction process.

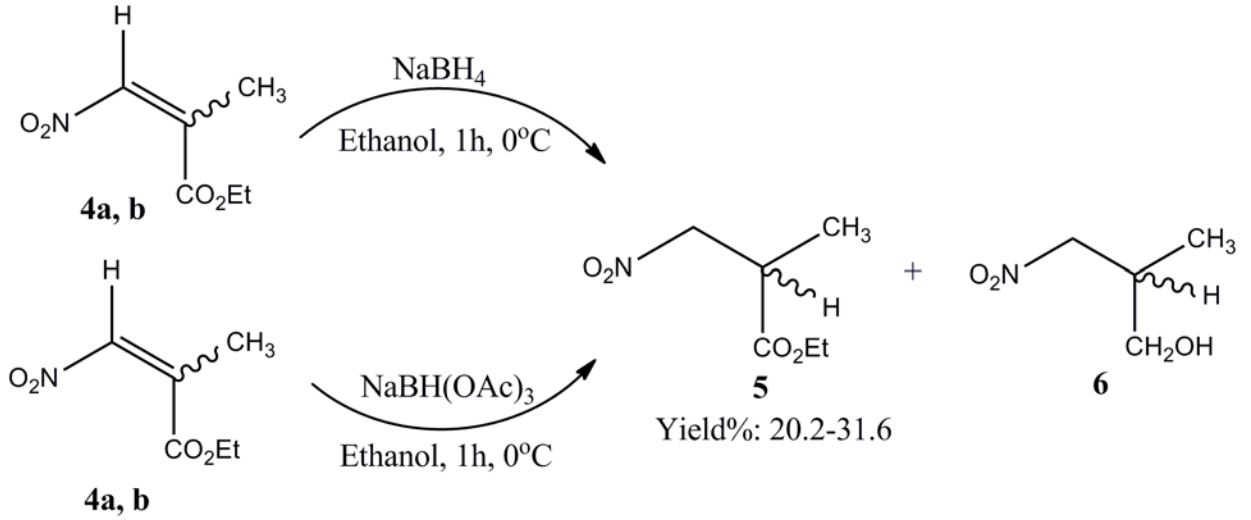


Figure 3-3. β-nitroacylates (**4a,b**) reduction using $NaBH_4$ and $NaBH(OAc)_3$

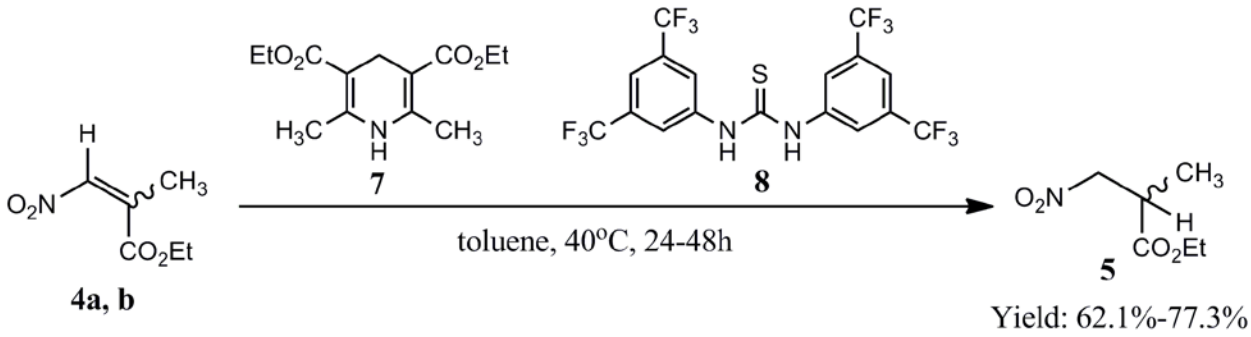


Figure 3-4. Organocatalytic transfer hydrogenation of β-nitroacylate (**4a,b**).

The last step to make the standard is the Henry reaction between the β-nitroalkane and aldehyde. Acetylaldehyde was chosen because bigger molecule might

impede the enzymatic reduction that occurs in the enzyme. Several bases were used to deprotonate the acidic proton, and the addition of 4Å molecular sieve was helpful, but the ion exchange resin (Amberlyst A-21) proved to be the best choice (Table 3-2). Gas chromatography-Mass spectrometry demonstrated the existence of four stereoisomers in the product and they are proved to be the Henry reaction products (**10**). There is also one impurity among these peaks, which we were unable to remove. The ratio of each isomer is #1: 20.5%, #2: 13.9%, #3: 23.2% and #4: 42.4% individually. The total conversion of this reaction was 48.2%.

Table 3-2. Different conditions of Henry reaction between β-nitroalkane (**5**) and acetylaldehyde (**9**).

Reagent	Solvent	Temp.	Rxn Time	Yield%
TBAF	THF	rt	10h	No reaction
NaOEt	EtOH	0°C/rt	4h	No reaction
Et_3N, MS 4Å	CH_2Cl_2	0°C/rt	6-10h	41.7%
Et_3N, MS 4Å	Solvent free	0°C/rt	overnight	54.3%
Amberlyst A-21	Solvent free	5°C	overnight	81.3%

The enzymatic reduction was conducted using flavin-containing well-type OYE1 in the presence of NADPH cofactor and a glucose-based regeneration system in KP_i 100mM, pH~7.0. The reaction was monitored by GC-MS every hour until it went to completion after 12 hours (**10a**), four stereoisomers were observed on GC with conversion of 48.1%, the ratio of each stereoisomer is: #1: 23.7%, #2: 2.6%, #3: 21.0% and #4: 52.8%. To see if the reaction was also catalyzed by buffer, β-nitroalkane instead of β-nitroacrylate was used in the same reaction. The same four stereoisomers were still observed on GC with a conversion of 53.3% (**10b**), and the ratio of each isomer is: #1: 18.4%, #2: 4.7%, #3: 21.2% and #4: 55.7%. Then, a reaction medium without enzyme and regeneration system was created, β-nitroacrylate and acetylaldehyde was dissolved directly into the buffer and the reaction was monitored,

after 12 hours, four stereoisomers were observed again (**10c**), and the ratio is: #1: 18.1%, #2: 3.3%, #3: 22.5% and #4: 56.1%, with a total conversion of 48.2%. Based on these results summarized in Table 3-3, it appears that the Henry reaction was actually catalyzed by the buffer. OYE reduced the β-nitroacrylate and formed a carbanion, which was protonated. After release from the active site, the subsequent Henry reaction occurred in solution. To testify this explanation, we carried out the same reaction, with all conditions the same except that it was conducted at pH 6.0. No Henry product was formed when β-nitroacrylate and acetylaldehyde were used. By contrast, using β-nitroalkane and acetylaldehyde, the Henry product was detected, but at much lower conversion (15.7%). Therefore, we came to the conclusion that this Henry reaction was catalyzed by buffer instead of OYE1. Presumably, protonation of the carbanion is faster than the nucleophilic attack. Further work might focus on the studies on the rate of carbanion protonation and nucleophilic attack, optimization of reaction media and exploration of other substrates.

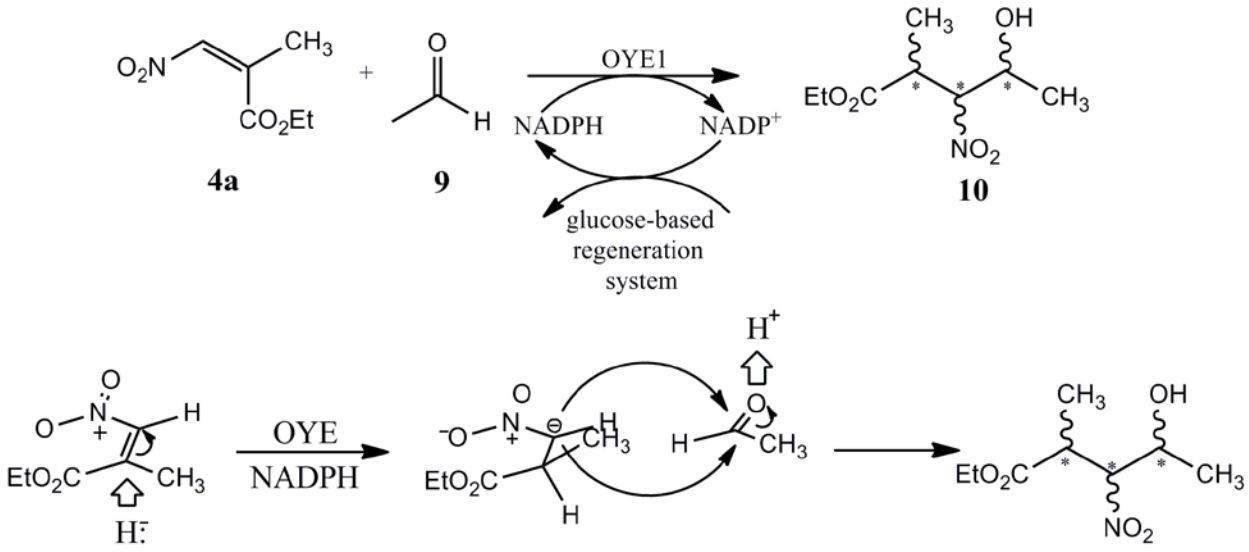
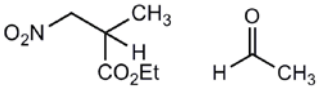
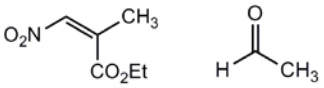
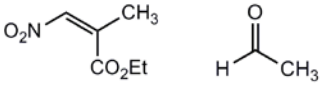
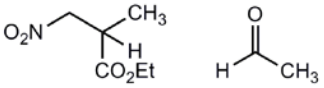
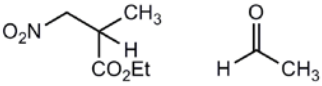


Figure 3-5. Reaction and mechanism of OYE catalyzed Henry reaction between β-nitroacrylate (**4a**) and acetylaldehyde (**9**).

Table 3-3. Conversion and product percentage of chemical and enzymatic Henry reaction products

Reagent	KP_i 100mM, PH~7.0	OYE1, GDH, $NADP^+$	Conv. %	Product Percentage
	No, chemical synthesis	No, Amberlyst A-21	48.2	
	Yes	Yes	48.1	
	Yes	No	No	
	Yes	Yes	53.3	
	Yes	No	48.2	

Experimental

Reagents and Instrumentations

Solvents were purchased from Fisher Scientific and dichloromethane and tetrahydrofuran were dried on an MBRAUN solvent purification system using a double 4.8 L activated alumina columns type A2. Triethylamine was distilled from CaH_2 under atmospheric pressure and methanesulfonyl chloride was distilled from CaH_2 under vacuum. Most of the reactions were performed under argon, except for those that are not sensitive to water and air. Reactions were monitored by thin layer chromatography (Whatman, with fluorescent indicator) and gas chromatography (DB-17 column, 0.25 mm x 25m x 0.25 µm thickness) with mass spectrometric detection, products were

purified by column chromatography (Purasil silica gel 230-400 mech, Whatman). NMR spectra were measured in $CDCl_3$ solution and recorded at room temperature on a Varian Gemini 300 spectrometer operating at 300 MHz for 1H with chemical shifts (δ, ppm) reported relative to tetramethylsilane. Infrared Radiation (IR) spectra were obtained from Perkin-Elmer Spectrum One FT-IR spectrophotometer. Mass spectrometry were run on ThermoFinnigan (San Jose, CA) LCQ with electrospray ionization (ESI).

Synthesis of β-Nitroalcohol (3).

In a 50 mL 2 necked flask equipped with a mechanical stirrer, 60mmol (3.66 g) nitromethane was charged and cooled with ice bath. Amberlyst A-21 5-7 g was added and the mixture was stirred for 10 minutes, then 60 mmol (6.97 g) ethyl pyruvate was added. The reaction temperature was raised to room temperature and the mixture was stirred overnight. The reaction mixture was filtered and the Amberlyst resin was washed with CH_2Cl_2 (4 x 25 mL), then the solvent was evaporated in vacuo to yield β-nitroalcohol. The crude product was purified by flash chromatography. (ethyl acetate: hexane= 1:3, yield: 64-88%), NMR data was consistent with Ballini's report. [58] ^{1}H NMR ($CDCl_3$): δ 4.83 (d, *J*=6 Hz, 1H), 4.54 (d, *J*=6 Hz, 1H), 4.35 (q, *J*=6 Hz, 2H), 3.73 (s, 1H), 1.46 (s, 3H), 1.34 (t, *J*=6 Hz, 3H) ppm.

Synthesis of β-Nitroacrylate (4a,b).

The method of McMurry's was used with some modifications.[59] β-nitroalcohol (**3**)(17 mmol, 3.0 g) was dissolved in 17 mL CH_2Cl_2 at 0^{o}C under argon atmosphere. Dry methanesulfonyl chloride (51 mmol, 3.95 mL) was added dropwise, and then the mixture was stirred under 0^{o}C for 2 hours. Dry triethylamine (51 mmol, 7.1 mL) was added to the mixture dropwise and the reaction mixture was stirred for another 2 hours

at 0°C. The reaction mixture was then transferred to a separatory funnel with the aid of 40 mL CH_2Cl_2, and then washed with water, 5% HCl, and brine. The mixture was dried over anhydrous $MgSO_4$ and gravity filtered. After concentration at the rotary evaporator, the crude product was purified by flash chromatography. (ethyl acetate: hexane=1:10). NMR data was consistent with Stewart's report.[57] (*Z*)-isomer ^{1}H NMR ($CDCl_3$): δ 6.88 (q, *J*=3 Hz, 1H), 4.35 (q, *J*=6 Hz, 2H), 2.11 (d, *J*=3 Hz, 3H), 1.35 (t, *J*=6 Hz, 3H) ppm. (*E*)-isomer ^{1}H NMR ($CDCl_3$): δ 7.73 (q, *J*=3 Hz, 1H), 4.31 (q, *J*=6 Hz, 2H), 2.32 (d, *J*=3 Hz, 3H), 1.36 (t, *J*=6 Hz, 3H) ppm.

Synthesis of β-Nitroalkane (5).

The method of List's was used with some modifications.[61]

Reduction with $NaBH_4$: To a solution of β-nitroacrylate (**4a,b**) (2.67 mmol, 0.425 g) in ethanol (0.6 mL) at 0°C, $NaBH_4$ (4.0 mmol, 0.151 g, 3.0 equiv) was added in one portion. The reaction mixture was stirred at 0°C for 1h and then treated at 0°C with a saturated aqueous NH_4Cl solution. The two phases were separated and the aqueous phase extracted with EtOAc. The combined organic phases were dried over anhydrous $MgSO_4$. The volatile compounds were removed in vacuo and the crude product was purified by flash chromatography (1-5 % Et_2O in hexane, yield : 20-28%).

Reduction with Hantzsch ester : To a solution of β-nitroacrylate (**4a,b**)(1.98 mmol, 0.315 g) in toluene (1.6 mL), thiourea catalyst (0.396 mmol, 0.186 g, 0.2 equiv) and Hantzsch ester (0.6 g, 2.38 mmol, 1.2 equiv) were added. The reaction mixture was stirred at 40°C for 24-48 h under argon atmosphere until completion of the reaction. The solvent was removed in vacuo and the resulting mixture purified by flash column chromatography (1-5 % Et_2O in hexane, yield: 62-77%). NMR data was consistent with List's report.[61] ^{1}H NMR ($CDCl_3$): δ 4.72 (dd, *J*=15 and 9 Hz, 1H), 4.42 (dd, *J*=15 and 9

Hz, 1H), 4.19 (q, *J*=6 Hz, 2H), 3.25 (m, 1H), 1.28 (t, *J*=6 Hz, 3H), 1.30 (d, *J*=9 Hz, 3H) ppm.

Synthesis of Thiourea Catalyst (8).

The method of making Schreiner's catalyst was used.[62]

In a dry 100 ml round-bottom flask, a mixture of 3,5-bis(trifluoromethyl)aniline (10 mmol, 2.34 g, 1,59 mL) and triethylamine (11.9 mmol, 1.66 mL) in THF (72 mL) was prepared. Under Argon atmosphere, a mixture of thiophosgene (4.3 mmol, 0.33 mL) in THF (7 mL) was added dropwise to the stirred solution at -5^{o}C-0^{o}C. After addition, the yellow suspension was allowed to stir at room temperature for 24 hours. After the reaction, the bulk of the solvent was removed in a rotary evaporator, the concentrated blown color residue was added to 45 mL water and the aqueous layer was extracted with ether (2 x 15 mL). The combined organic layers were washed with brine (1 x 10 mL), and dried over anhydrous $MgSO_4$. After filtration and evaporation of the solvent. The red brown solid crude product was purified by recrystallization from chloroform once, and the resulting slightly yellow solid was dissolved in ether to be re-precipitated by addition of hexane as newly white solid that was dried over Sicapent in a dessicator. (yield: 35%). Melting Point, IR and MS data were consistent with Schreiner's report.[62] MP: 170-171^{o}C; IR (KBr): 3205, 3048, 2985, 1550, 1464, 1371, 1326, 1287, 1180, 1131, 932, 887, 711, 698.; HRMS calcd for [$C_{17}H_8N_2SF_{12}$]: 500.02; found [$C_{17}H_8N_2SF_{12}+H^+$]: 501.0210.

Synthesis of Ethyl 4-Hydroxy-2-Methyl-3-Nitropentanoate (10).

In a 5 mL tip-bottom flask, 0.68 mmol β-nitroalkane (**5**) was charged and cooled with ice bath. Amberlyst A-21 0.05-0.07 g was added and the mixture was stirred for 10 minutes, then 2.0 mmol acetylaldehyde (**9**) was added. The reaction temperature was

raised up to 5^{o}C and stirred overnight, the reaction mixture was filtered and the Amberlyst resin was washed with CH_2Cl_2 (4 x 2 mL), then the solvent was evaporated in vacuo to yield the desired product. (yield: 80-98%) (HRMS: calcd for [$C_8H_5NO_5$]: 205.10 and found [$C_8H_5NO_5+H^+$]: 206.1013. GC-MS showed the existence of four different stereoisomers with reasonable fragmentations.)

Enzymatic Reduction of β-Nitroacrylate Using Old Yellow Enzyme

A reaction mixture contained 25 mM β-nitroacrylate (**4a**), 25 mM acetyladehyde (9), $NADP^+$ (4 µM, 3 mg), glucose (0.254 mmol, 85.8 mg), glucose dehydrogenase (100 µg), and purified OYE1(5 mg) in a total volume of 10 mL of 100 mM $KP_{i,}$ pH 7.0. The mixture contained 5% isopropanol in order to increase substrates solubility. The reaction mixture were separated in 10 centrifuge tubes (1.5 mL) with 1 mL each, and left on a rotisserie under 30^{o}C. Samples were extracted every hour with EtOAc (3 x 0.5 mL), and the combined organic layers were washed with brine, water, dried over $MgSO_4$, and concentrated in vacuo. The sample was diluted with EtOAc for GC-MS analysis.

CHAPTER 4
CONCLUSION AND FUTURE WORK

The flavin-containing old yellow enzyme was used in the Henry reaction between β-nitroacrylate and acetylaldehyde in the presence of $NADP^+$ cofactor and glucose regeneration system. Because the oxygen on nitro group could form hydrogen bond with His 191 and Asn 194, as well as the requirement that the β-carbon lies above N5 of the flavin, reduced FMN always delivers hydride to the *re* face of the bound β-nitroacrylate, and form a reactive nitronate carbanion. We expected this carbanion could proceed through Henry reaction when acetylaldehyde was introduced and achieve a high stereoselectivity, however, protonation by the solvent-derived hydrogen always seems to be predominant, with a subsequent buffer catalyzed Henry reaction. Future work might be focused on the kinetic study on the rate of carbanion protonation and reaction between carbanion and acetylaldehyde, modifying the reaction media to inhibit the carbanion protonation, and choosing better substrates.

APPENDIX A
GC ANALYSIS OF HENRY REACTION PRODUCTS

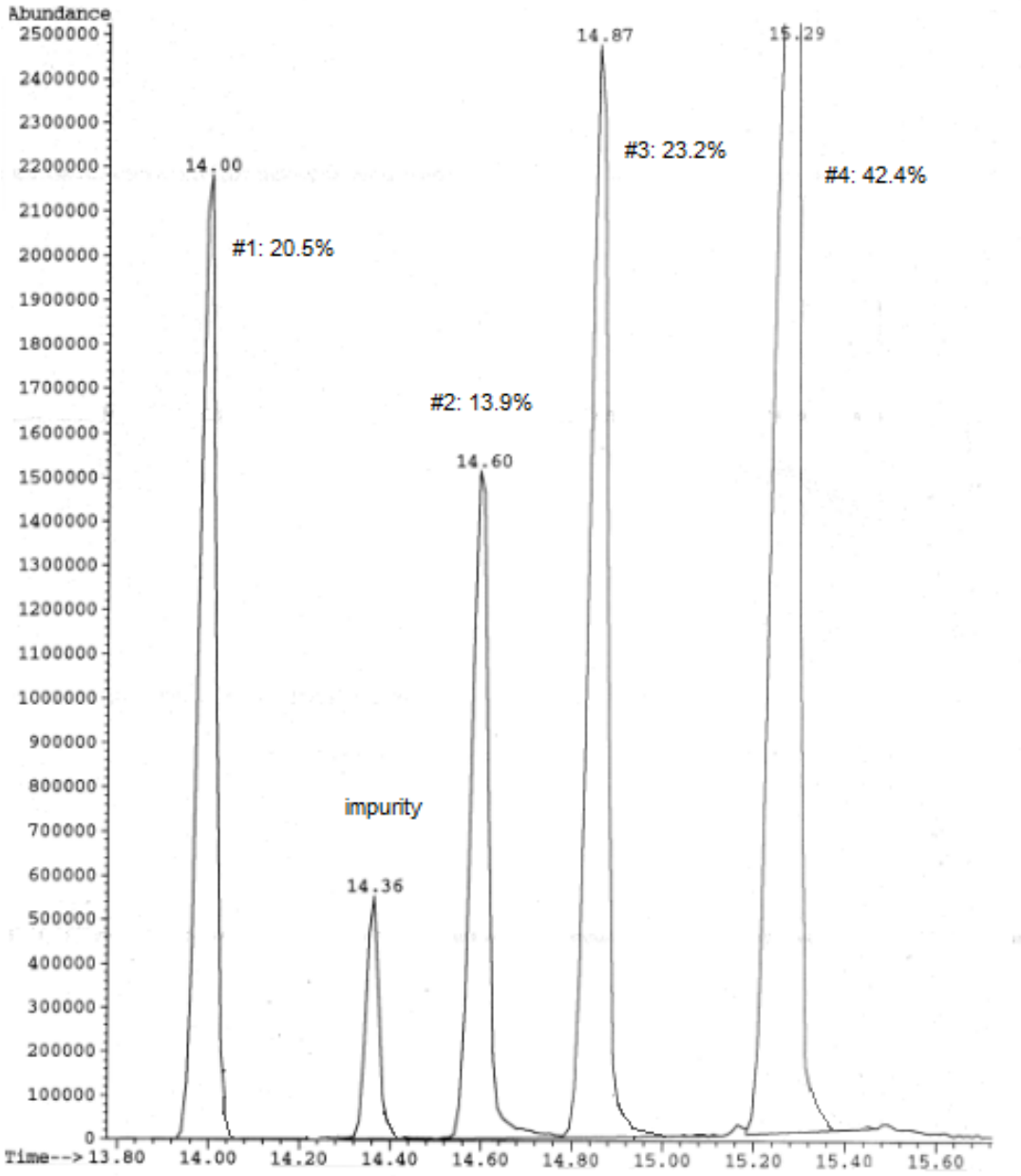


Figure 1-A. GC chromatogram of **10**.

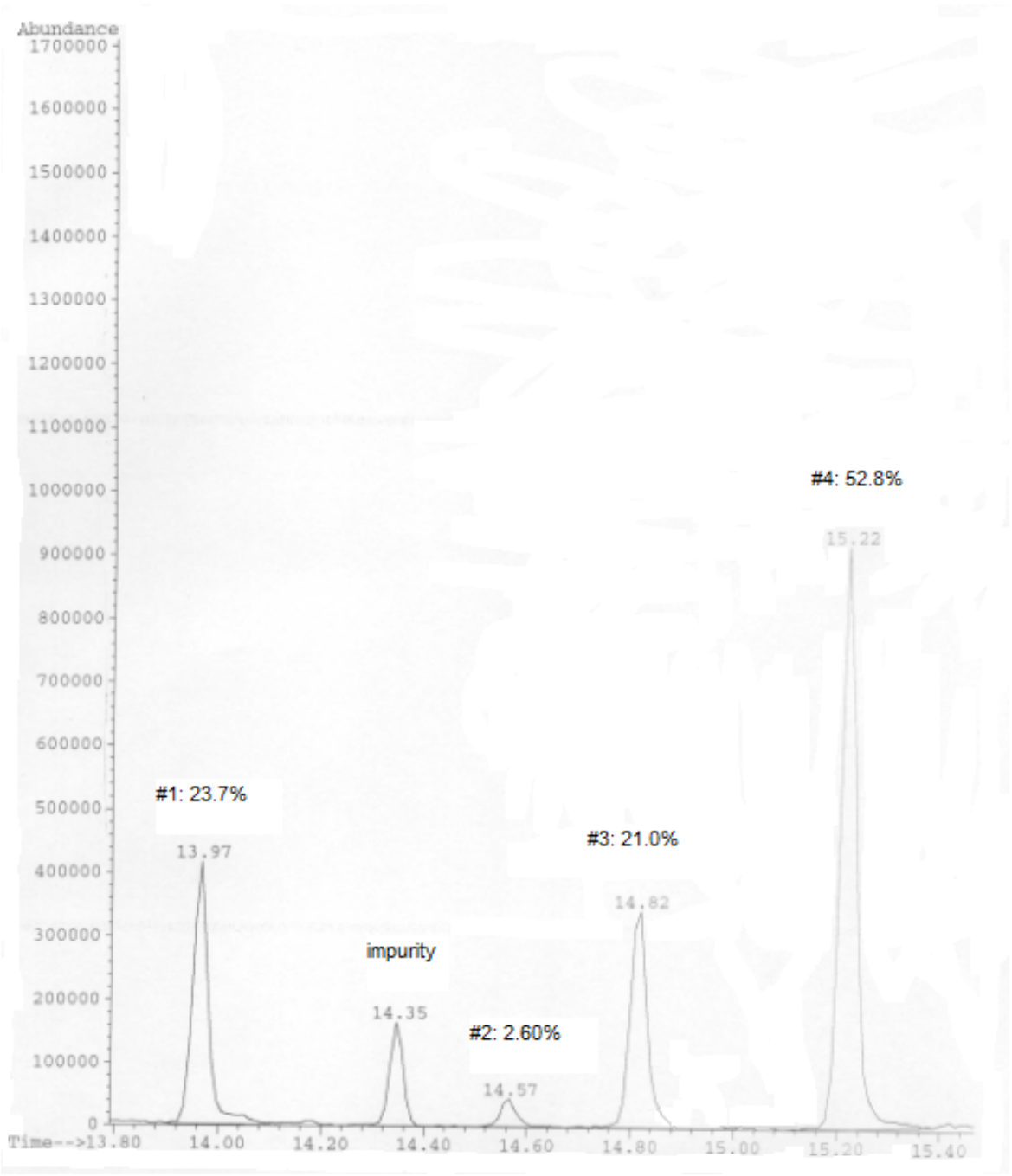


Figure 2-A. GC chromatogram of **10a**.

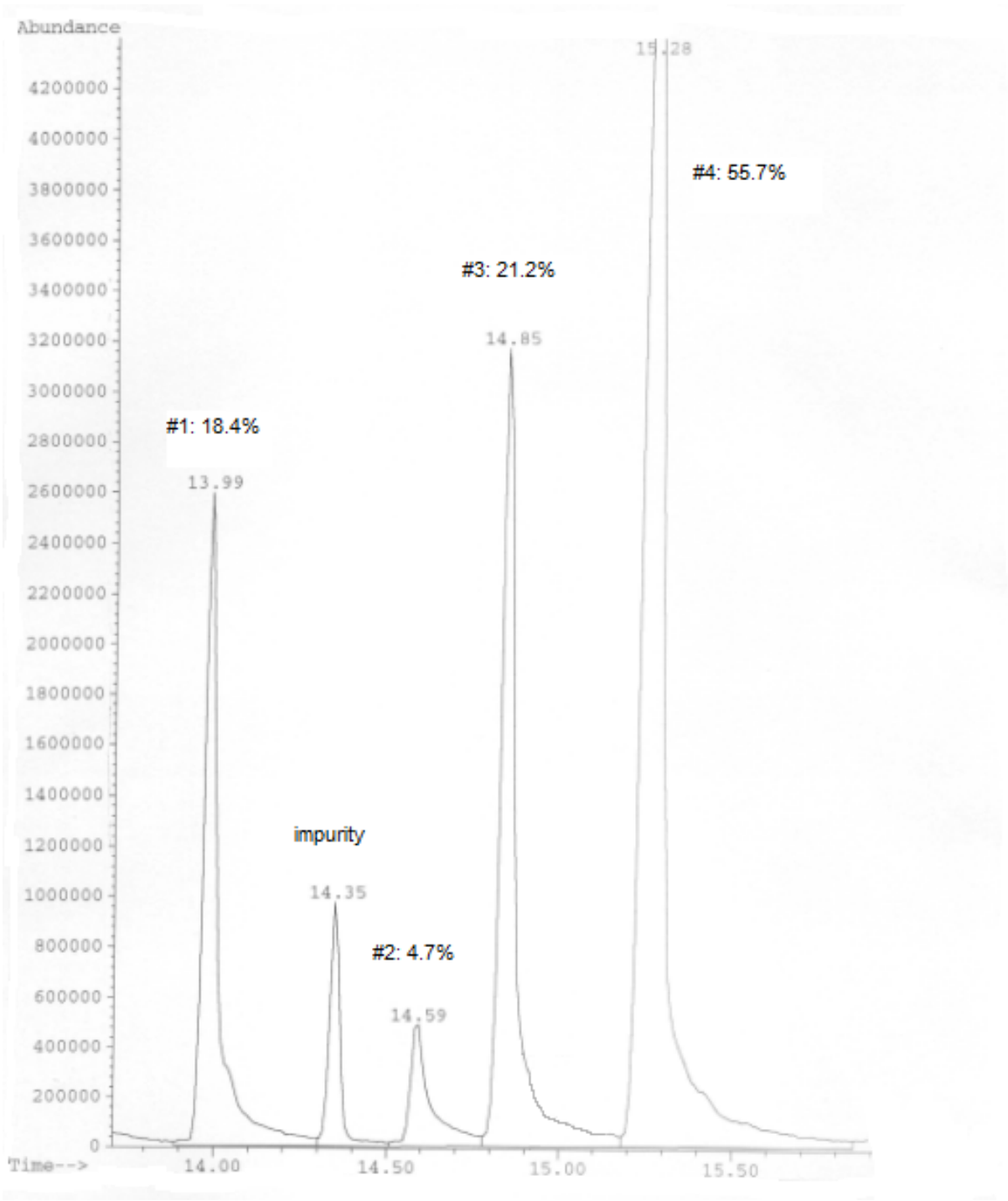


Figure 3-A. GC chromatogram of **10b**.

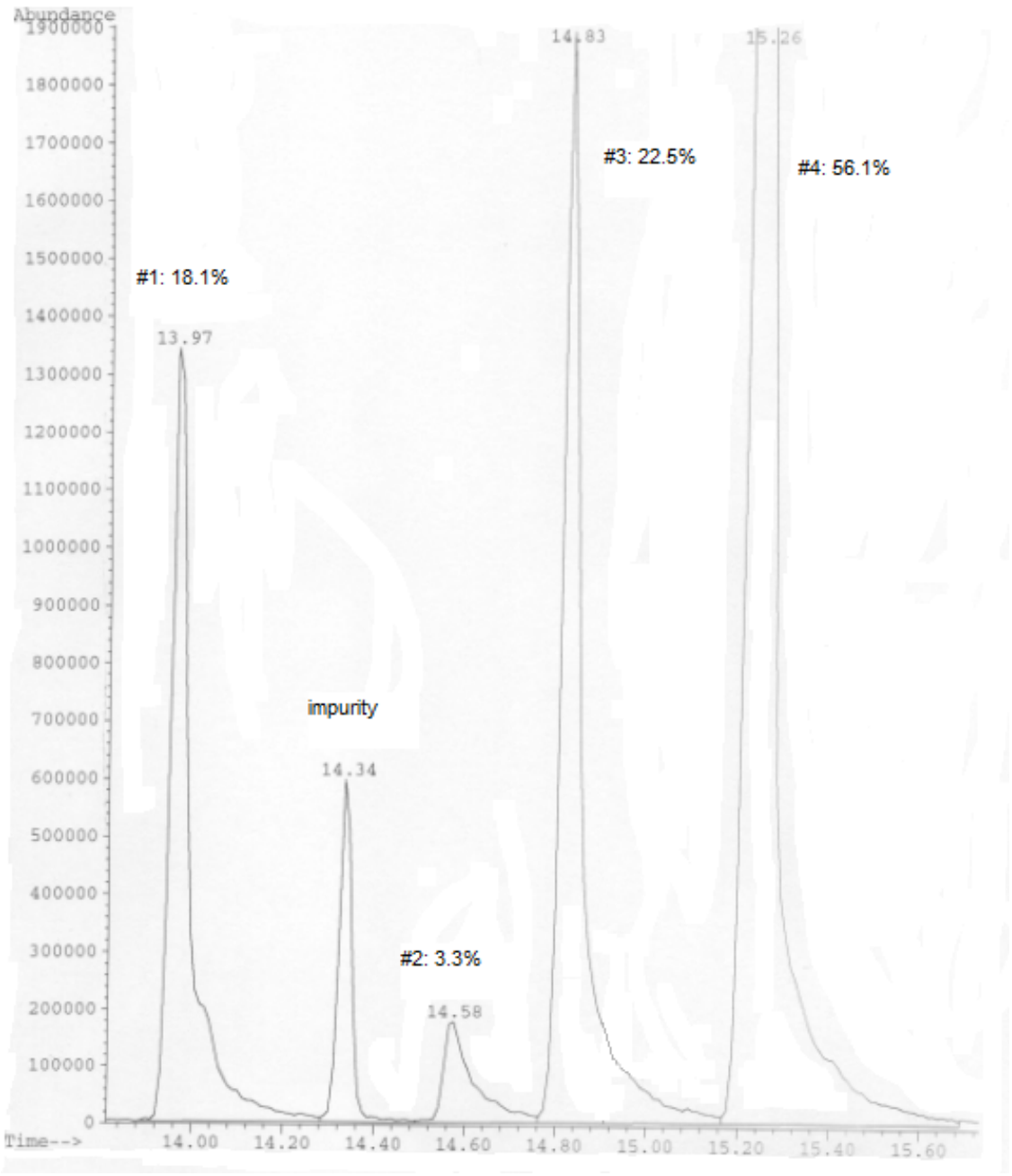


Figure 4-A. GC chromatogram of **10c**.

APPENDIX B

[1]H-NMR SPECTRA OF NITROALCOHOL, B-NITROACRYLATE AND NITROALKANE

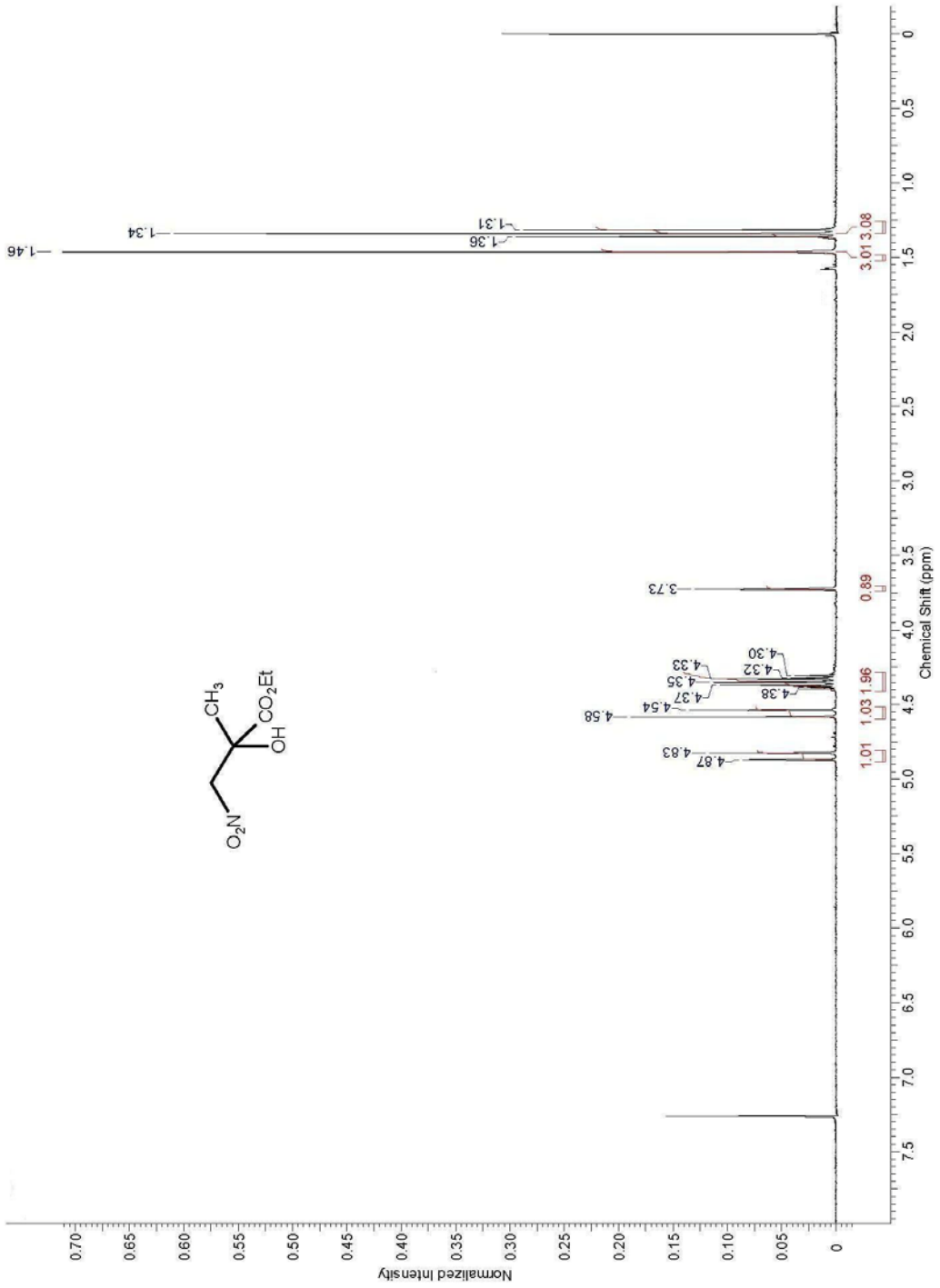


Figure 1-B. [1]H-NMR spectra of nitroalcohol (**3**).

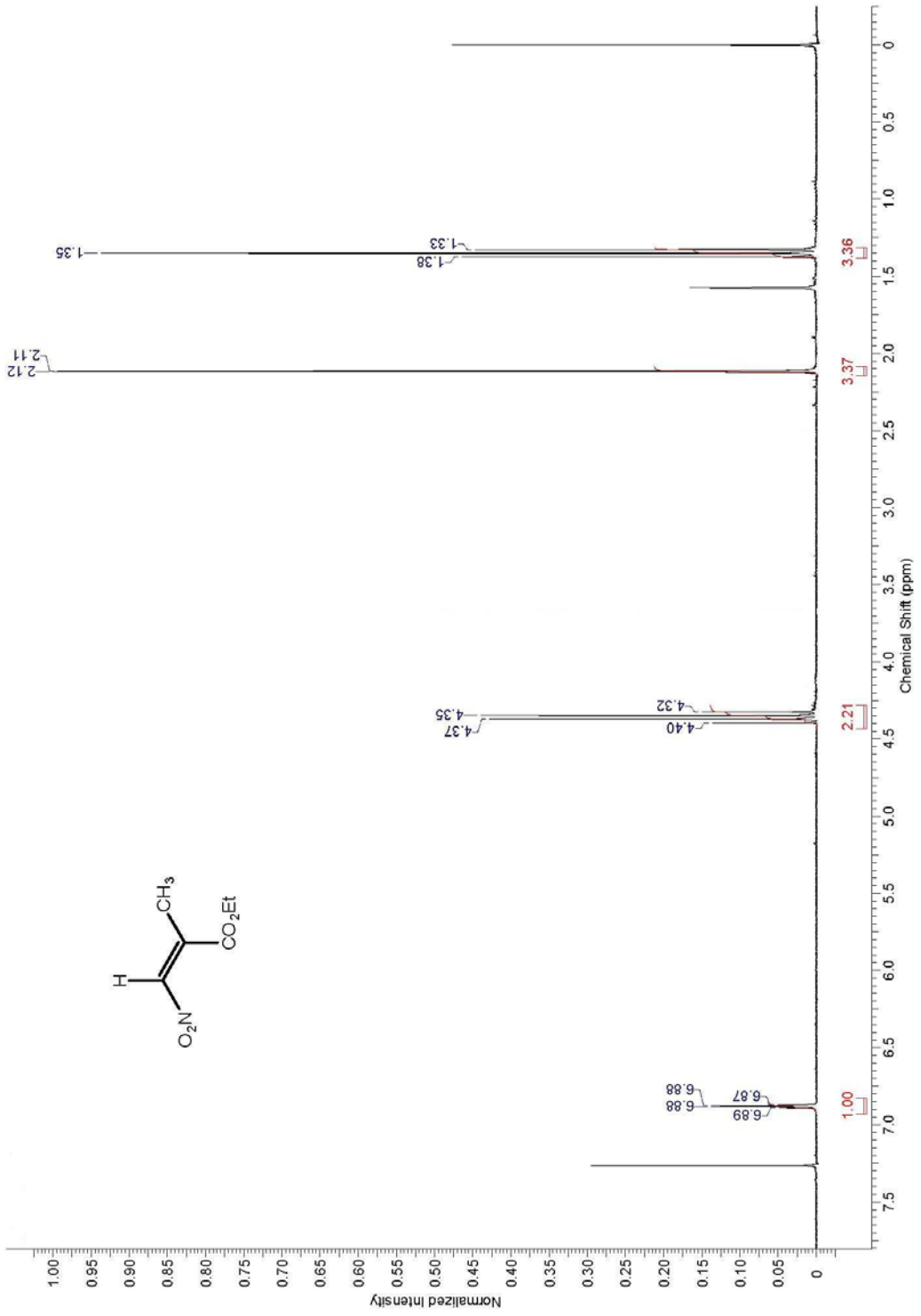


Figure 2-B. ^{1}H-NMR spectra of β-nitroacrylate (**4a**,*Z*-isomer).

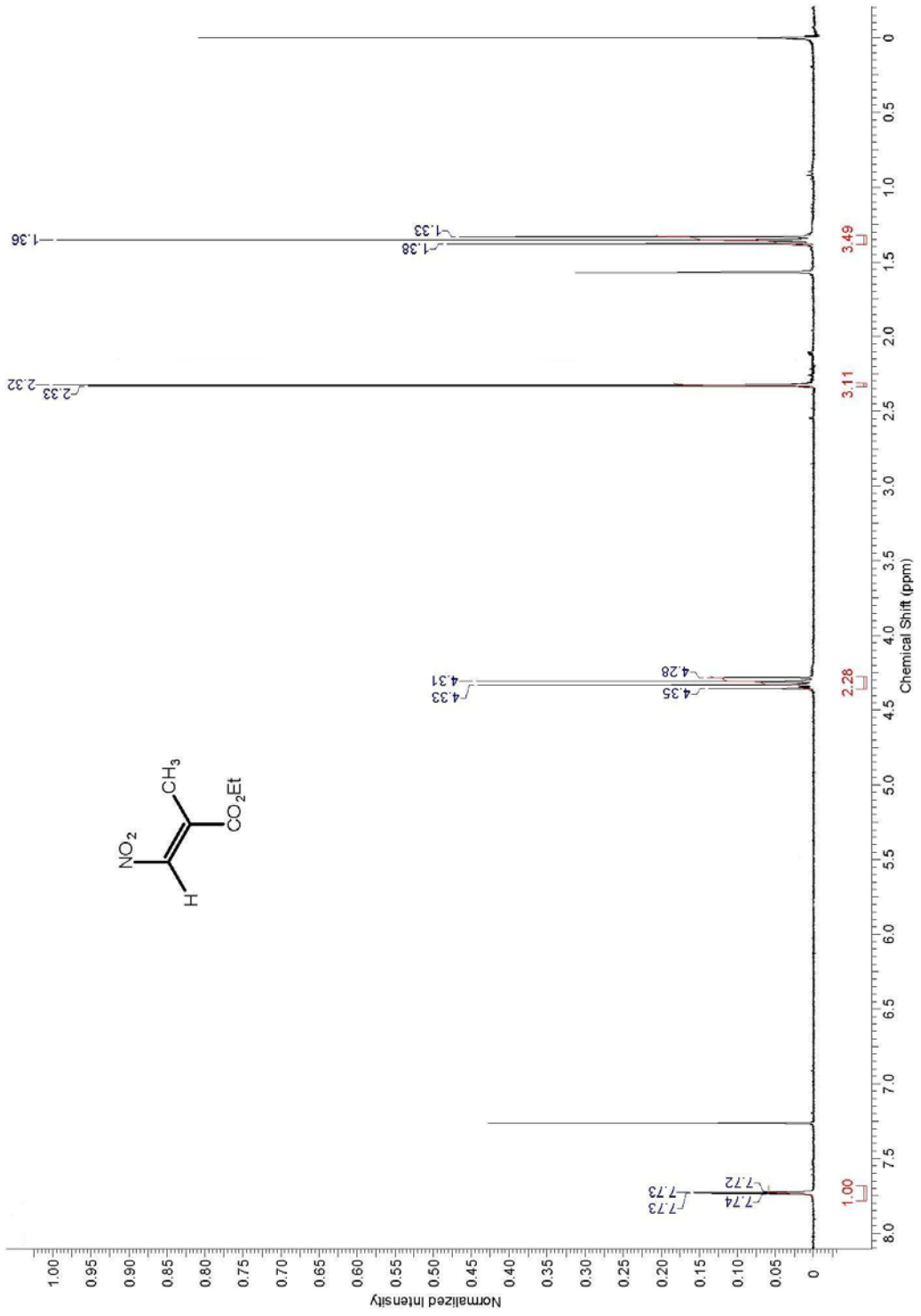


Figure 3-B. ^{1}H-NMR spectra of β-nitroacrylate (**4b**,*E*-isomer).

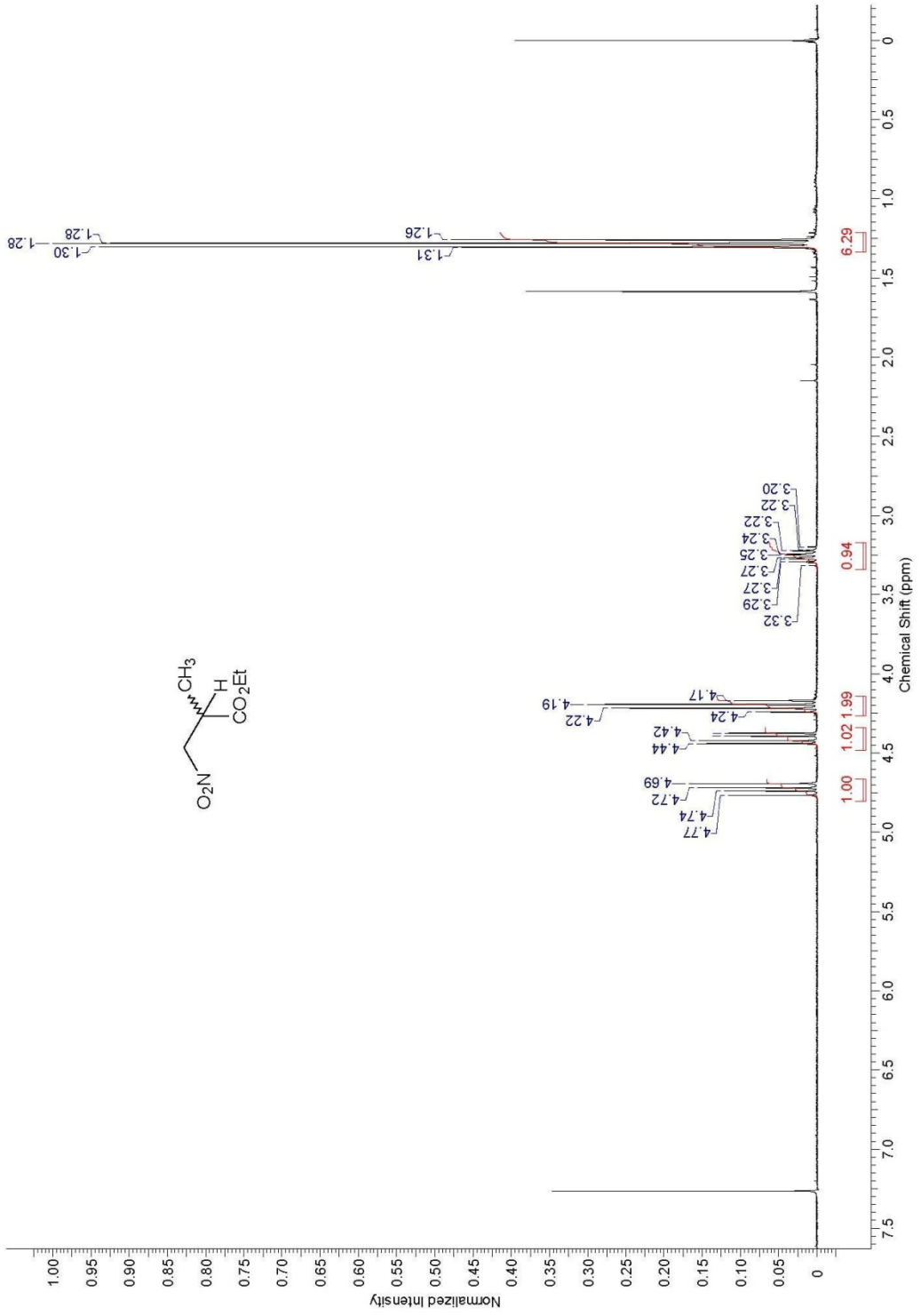


Figure 4-B. ^{1}H-NMR spectra of β-nitroalkane (**5**).

APPENDIX C
MASS SPECTROMETRY OF THE HENRY REACTION PRODUCT

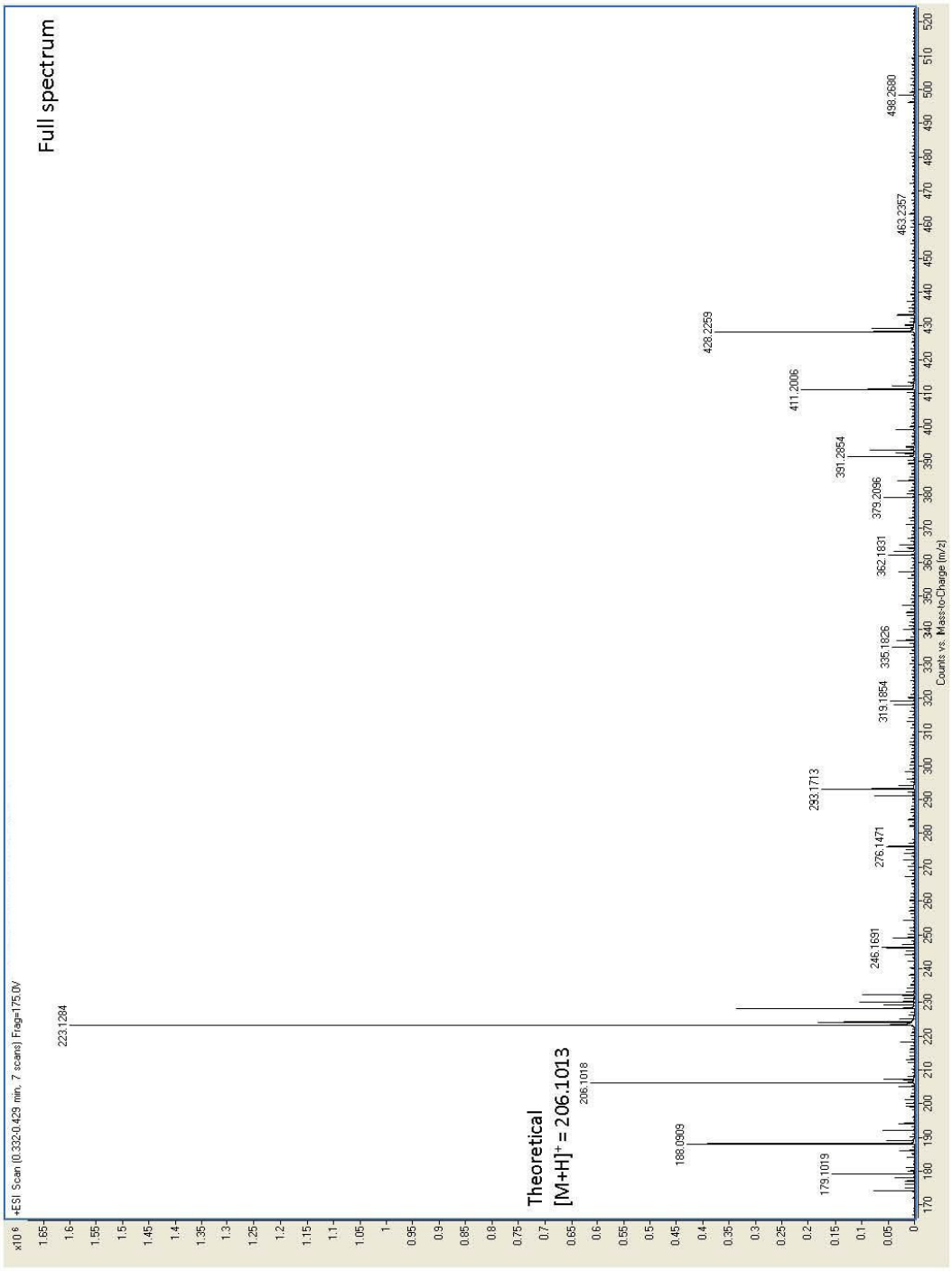


Figure 1-C. Mass spectrometry of Henry product through chemical synthesis (**10**).

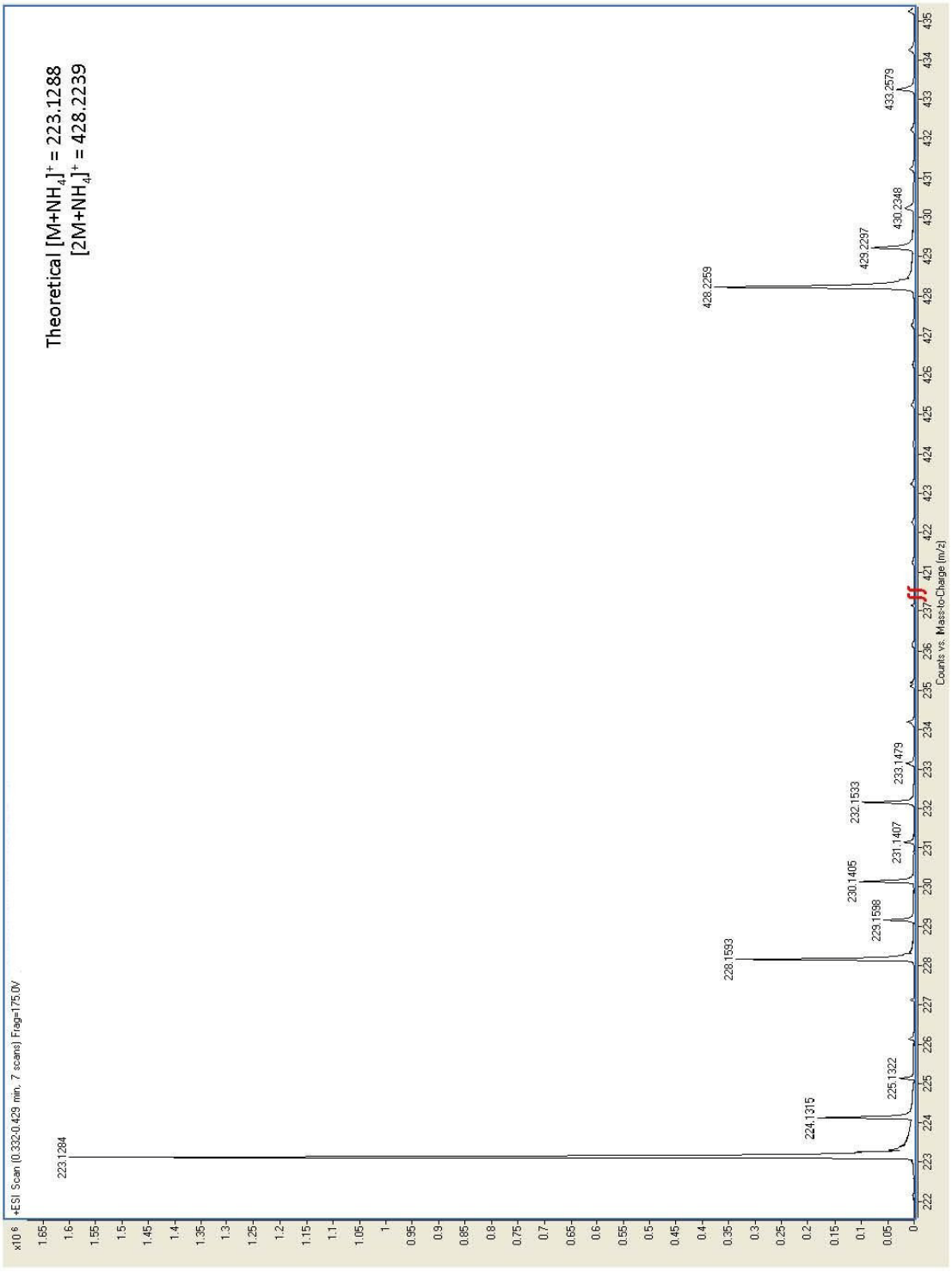


Figure 2-C. Another mass spectrometry of Henry product through chemical synthesis (**10**).

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BIOGRAPHICAL SKETCH

Di Zhou was born and brought up in Beijing, China. In 2004, he attended Beihang University (formerly Beijing University of Aeronautics and Astronautics) for his undergraduate studies in chemistry. After graduation in 2008, he moved to University of Florida for graduate studies and joined the Stewart's group. His research focused on the organic and biocatalytic chemistry.