TECHNISCHE UNIVERSITÄT WIEN

VIENNA UNIVERSITY OF TECHNOLOGY

DISSERTATION

APPLICATION OF ENE-REDUCTASES IN THE CHIRAL SYNTHESIS

Conducted at

Institute of Applied Synthetic Chemistry

Faculty of Technical Chemistry
Vienna University of Technology

Under the supervision of

A.o. Univ. Prof. Dipl.-Ing. Dr. techn.
Marko D. Mihovilovic

Performed by

Naseem IQBAL

Matr. No. 0727892

Brigittenauer Lande 224, 1200, Vienna, Austria

Dedicated to

“My Beloved Family”

"Read in the name of your Lord who created - Created man from a clot - Recite, and your Lord is the most Generous - Who taught by the pen - Taught man which he knew not"

The Holy Quran (al-Alaq 96:1-5)

“I light myself on fire, people come to see me burn”

Wesley, John

I owe my sincere gratitude to my supervisor, Professor Marko D. Mihovilovic, at first place for giving me an opportunity to work in his group at Institut für Angewandte Synthesechemie, Technische Universität Wien. My journey of PhD started with miles apart correspondence from Pakistan, as I showed interest in his work. The approval of my PhD application, is more than a good reason to incite me for special thanks to Professor Marko for having trust in me. Your incredible passion for science, encouragement and optimistic attitude has been a driving force to me during my PhD studies. Our discussions over the years have been food for me to widen my knowledge in the field of biocatalysis. I do not only flourish my chemistry of Science, but also chemistry of life, spending three year under your supervision.

My special thanks goes to Professor Peter Stanetty for his scientific consultation and discussions about confronting problems in synthetic chemistry and Dr. Michael Schnürch for his valuable comments and sharing knowledge during my project.

Professor Jon D. Stewart, at Department of Chemistry and Biomedical Engineering, University of Florida, USA is greatly acknowledged for his scientific collaborations for this project. Prof. Peter Macheroux from Technische Universität Graz is greatly acknowledged for providing vital expression system OPR1, OPR3 and YqjM to my research. I also acknowledge the generous donation of expression systems for *Shewanella* EREDs by Dr. Ann Brigé, University of Ghent, Belgium.

A special note of thanks to all my colleagues and friends in our research group, Muhammad Farooq Zia, Saima Feroz, Moumita Koley, Navid Dastbaravardeh, Michael Fink , Birgit Morzek, Max Haider, Thomas Fischer (for HPLC and GC measurements), Michael Schoen, Andrej Kolarovic, Mohammed Abdel Hameid, Alex Winninger, Laurin Wimmer, Birgit Waldner, Michael Ghobrial, Lisa Recnik, Johanna Hölbling and Thomas Linder for their support, assistance and providing friendly working environment during my stay in TU Wein.

It is a pleasure to acknowledge my friends and previous group members, Dr. Ather Farooq, Dr. Radoslav Flasik, and Dr. Johanna Hämmerle, who facilitate me to adjust in a new environment of our laboratory, but also inspired me to explore versatile Austrian culture. Special Thanks to my all friends in Vienna for nice company during the weekends and visits.

This work would have not been made possible without the financial support from the Higher Education Commission of Pakistan and Vienna University of Technology, Austria. I acknowledge them a big deal.

My parents, family and friends miles away in Pakistan, but who have always been with me through their love, prayers and encouragement. I would have never gone so far in life without them.

Finally, I would like to thank my beloved wife Tayyaba Noor, for always being there with me and making me think something else than chemistry. Happy new times to come in our life. I am looking forward to share them with you.

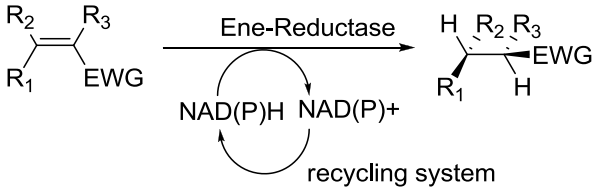
Legend

All compounds prepared or used as starting materials in this thesis are numbered in bold Arabic numbers. Compounds unknown to the literature or prepared according to a new route are additionally underlined. General structures are numbered in bold Roman numbers.

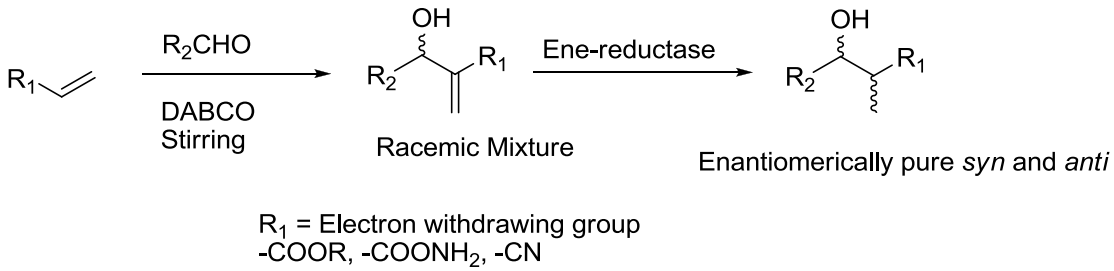
Literature citations are indicated by superscript Arabic numbers.

Abstract

In first part we studied the substrate acceptance of Shewanella yellow enzyme (SYE-3 & SYE-4). Biotransformations with recombinant whole cell expression systems and cell protein of SYE-3 & SYE-4 were performed with different substrates (α,β-unsaturated cyclic ketones, unsaturated diesters, N-substituted imides). The results (conversion and ee) were rationalized by chiral GC. Absolute configuration of these products was determined by comparing the values and sign of optical rotation with reference compounds. In all cases R-enantiomers were obtained. Unsaturated diesters and N-substituted imides were the best substrates and gave reduced products with high enantioselectivity. SYE-4 enzyme showed greater activity as compared to SYE-3 enzyme.



Products of Baylis-Hillman addition and subsequent hydrogenation have importance in drugs with antiproliferative activity. Due to the already established stereocentre in the substrate the hydrogenation with chiral catalysts should provide interesting information as to whether the stereochemistry in the final product is governed by the substrate or by the catalyst. Principally, the hydrogenation of racemic material with an achiral catalyst may afford a mixture of two pairs of racemic diastereomers where the hydroxy group and the newly created methyl substituent are placed either in a syn- or an anti-arrangement.

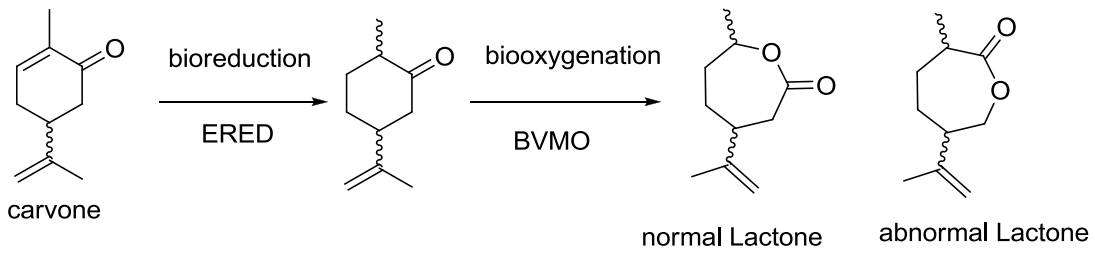


These Baylis Hillman products having a double bond attached with an electron withdrawing group fulfill the requirement for bioreduction using ene-reductases. Theoretically in the presence of a chiral catalyst reduction products will be enantiomerically pure syn and anti

diastereoisomers in 50:50 mixture. EREDs as chiral biocatalysts are particularly appealing for bioreducing these types of substrates having a racemic centre. Applying general Baylis Hillman conditions we were able to synthesize different adducts using aldehyde (aromatic and aliphatic), acrylate (methyl acrylate and acrylamide) and DABCO in good to moderate yields. The bioreduction of Baylis Hillman products was studied with EREDs. In case of YqjM enzyme *syn* selectivity was observed in bioreduction of all Baylis Hillman products. The reason for *syn* selectivity lies in the epimerization around the carbinol carbon during the reaction.

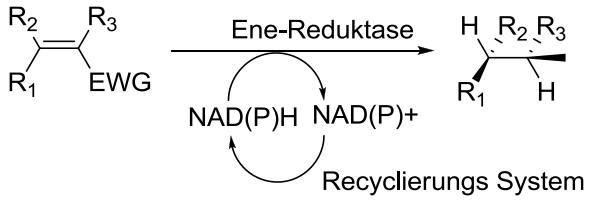
On the other hand, only anti products were isolated from the bioreduction of aza Baylis Hillman products. The bioreduction of aza Baylis Hillman products led to the stereoselctive synthesis of β-amino acids.

Multi-step one-pot reactions have caught attention of synthetic chemists in recent years as such transformations allow for increasing dramatically the structural and functional complexity of a chemical entity in a single operation. The combination of bioreduction with biooxygenation is an important strategy. The microbial Baeyer–Villiger biooxidation has become a highly attractive methodology to access optically pure lactones in both kinetic resolution and desymmetrization processes. ERED mediated bioreductions of enones was coupled with subsequent biotransformations. A particularly appealing combination is the reduction of an activated C=C double bond with subsequent Baeyer-Villiger oxidation. We have recently identified suitable Baeyer-Villiger monooxygenases (BVMOs) to access all enantiomers and regioisomers (“normal” and “abnormal” lactones) for the biooxidation of various terpenones depending on the absolute configuration of the substrate ketone. By linking this biotransformation with a preceding reduction step, simple and commercially available terpenes were directly converted to chiral lactones of significant interest to the fragrance industry. Herein, we are reporting a study on regiodivergent and enantiocomplementary one pot redox biotransformations of enantiomerically pure carvones into lactones.

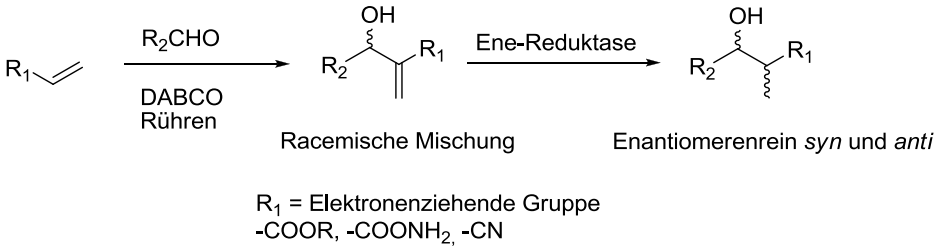


Deutshe Kurzfassung

Im ersten Teil untersuchten wir die Substratakzeptanz des gelben Shewanella Enzyms (SYE-3 & SYE-4). Biotransformationen mit rekombinanten Ganzzellen Expressionssystemen und Zellproteinen von SYE-3 & SYE-4 wurden mit verschiedenen Substraten (α,β-ungesättigten zyklischen Ketonen, ungesättigten Diestern, N-substituierten Imiden) durchgeführt. Die Ergebnisse (Umsatz und EE) wurden durch chirale GC ermittelt. Die Absolutkonfiguration der Produkte wurde durch Vergleich von Größe und Vorzeichen der Optischen Rotation mit Referenzverbingungen festgestellt. In allen Fällen wurden die R-Enantiomere erhalten. Ungesättigte Diester und N-substituierte Imide eigneten sich am besten als Substrate und ergaben die reduzierten Produkte mit hoher Enantioselektivität. Im Vergleich zeigte das Enzym SYE-4 die größere Aktivität.



Produkte der Baylis-Hillman Addition und nachfolgender Hydrierung haben wegen ihrer antiproliferativen Wirkung große Bedeutung als Wirkstoffe. Aufgrund des im Substrat vorhandenen stereogenen Zentrums kann erwartet werden, dass die Hydrierung mit einem chiralen Katalysator interessante Informationen liefert, ob die Stereochemie des Endprodukts durch Substrat oder Katalysator bestimmt wird. Grundsätzlich ergibt die Hydrierung der racemischen Ausgansmaterialien mit einem achiralen Katalysator Mischungen von zwei Paaren von racemischen Diastereomeren wobei die Hydroxygruppe und der neu hinzugekommene Methylsubstituent entweder syn oder anti zueinander stehen.



Jene Baylis-Hillman Produkte, welche einen elektronenziehenden Rest verbunden mit der Doppelbindung enthalten, erfüllen die Bedingungen für die Bioreduktion mittels Ene-Reduktase (EREDs). Theoretisch sollten in Anwesenheit eines chiralen Katalysators enantiomerenreine syn- und anti-Diasteriomere als 50:50 Mischung erhalten werden. EREDs sind hier als chirale

Biokatalysatoren besonders attraktiv, da sie die als Racemat auftretenden Baylis-Hillman Produkte als Substrat akzeptieren. Unter allgemeinen Baylis-Hillman Bedingungen erhielten wir Produkte aus verschiedenen Aldehyden (aliphatische und aromatische) Acrylaten (Methacrylat und Acrylamid) und DABCO in moderaten bis guten Ausbeuten. Die Bioreduktion der Baylis-Hillman Produkte wurde mit ERED's untersucht. Im Falle des Enzyms YqjM wurde syn-Selektivität in der Reduktion der Baylis-Hillman Produkte beobachtet. Die Ursache dieser syn-Selektivität liegt in der Epimerisierung um den Carbinolkohlenstoff während der Reaktion. Im Gegensatz dazu wurden bei der Bioreduktion der aza-Baylis-Hillman Produkte nur anti-Produkte erhalten. Die Bioreduktion dieser Substanzen führte zur stereoselektiven Synthese von β-Aminosäuren.

Mehrstufige Eintopfsynthesen haben in den letzten Jahren unter Synthesechemikern viel Aufmerksamkeit auf sich gezogen, da sich auf diese Weise die strukturelle und funktionelle Komplexität eines Moleküls in einem einzigen Schritt dramatisch erhöhen läßt. Die Komnination von Bioreduktion und Biooxidation ist eine bedeutende Strategie. Die Mikrobielle Bayer-Villiger-Oxidation hat sich zu einer höchst attraktiven Mehtode entwickelt um zu optisch reinen Lactonen zu kommen, sowohl über kinetische Racematspaltung als auch Desymmetrisierung. Die ERED-vermittelte Bioreduktionen von Enonen wurde mit einer nachfolgenden Biortransformation gekoppelt. Besonders attraktiv ist die Kombination der Reduktion von C-C-Doppelbindungen mit einer nachfolgenden Bayer-Villiger Oxidation. Wir haben unlängst geeignete Bayer-Villiger Monooxygenasen (BVMO) identifiziert. Bei der Biooxidation von verschiedenen Terpenonen konnten wir, durch Verwendung von Substratketonen der jeweiligen Konfiguration, alle Enantiomere und Regioisomere "normales" und "abnormales" Lacton) gezielt zugänglich machen. Durch die Kopplung dieser Biotransformation mit einem vorhergehenden Reduktionsschritt konnten einfache und kommerziell verfügbare Terpene direkt in chirale Lactone umgewandelt werden, welche für die Geruchsstoffindustrie eine bedeutende Rolle spielen. An dieser Stelle berichten wir eine Untersuchung der regiodivergenten und enantiokomplementären Eintopf-Redox-Biotransformation enantiomerenreiner Carvone in Lactone.

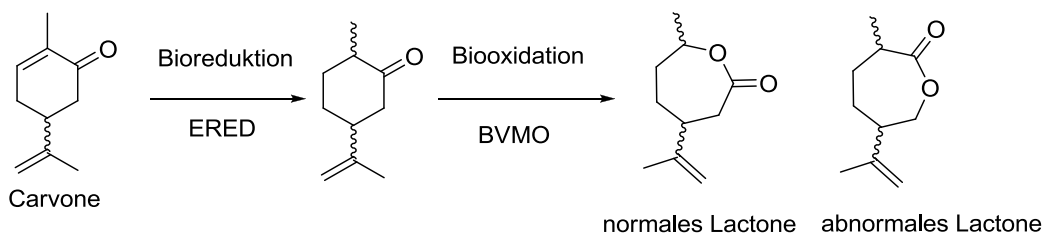
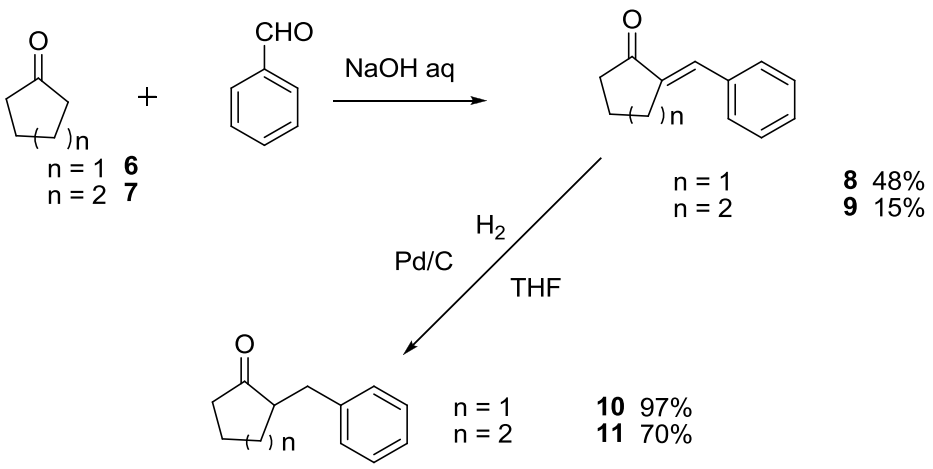
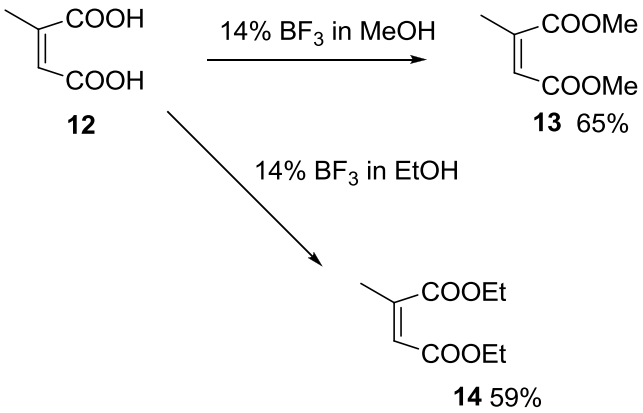
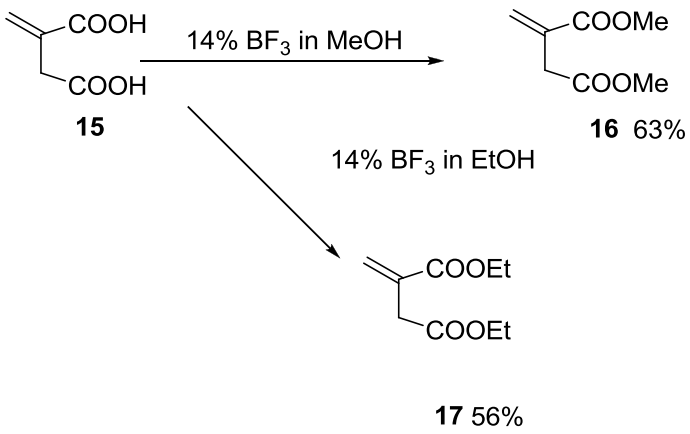


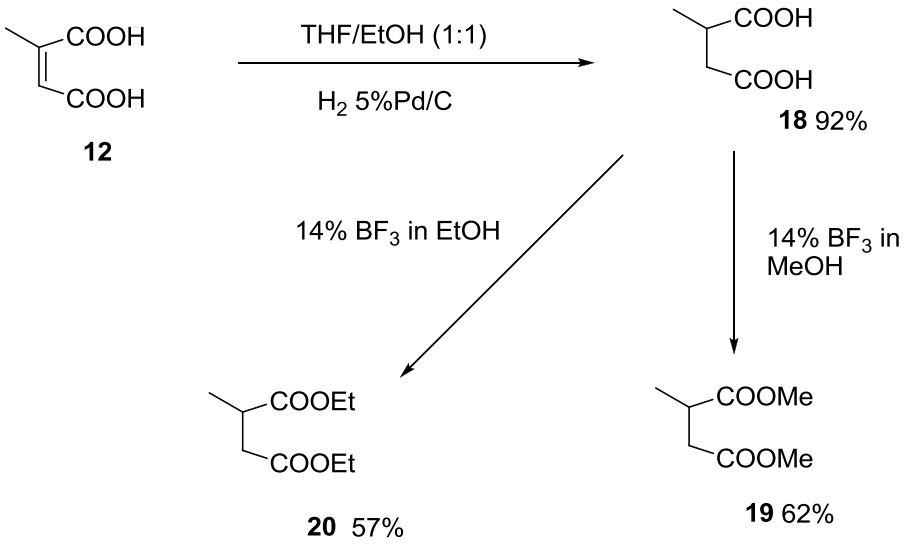
Table of Contents

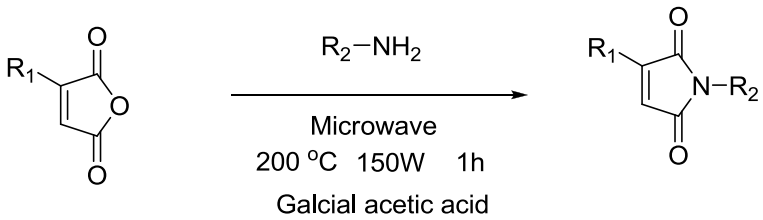
General Schemes and Substrates



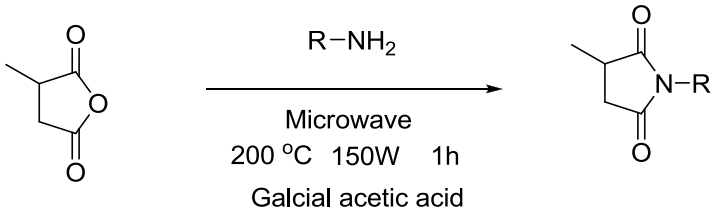




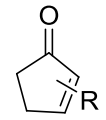




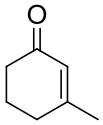
R_2 = Me	R_1 = Me	**23**	45%
R_2 = Et	R_1 = Me	**24**	43%
R_2 = Propyl	R_1 = Me	**25**	78%
R_2 = Butyl	R_1 = Me	**26**	54%
R_2 = Allyl	R_1 = Me	**27**	64%
R_2 = Phenyl	R_1 = Me	**28**	71%
R_2 = Allyl	R_1 = H	**29**	48%
R_2 = Phenyl	R_1 = H	**22**	52%



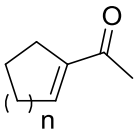
R = Benzyl	**30**	38%
R= Allyl	**31**	42%
R = Methyl	**32**	83%
R = Ethyl	**33**	77%
R = Propyl	**34**	75%
R = Butyl	**35**	80%



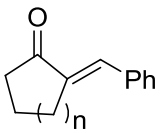
R = 2-Methyl **1**
R = 3-Methyl **2**



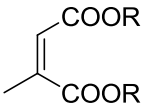
3



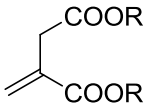
n = 1 **4**
n = 2 **5**



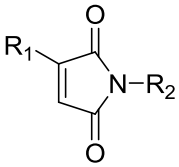
n = 1 **8**
n = 2 **9**



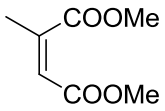
R = Me **13**
R = Et **14**



R = Me **16**
R = Et **17**

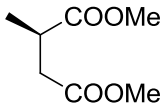


R_2 = Me	R_1 = Me	**23**
R_2 = Et	R_1 = Me	**24**
R_2 = Propyl	R_1 = Me	**25**
R_2 = Butyl	R_1 = Me	**26**
R_2 = Allyl	R_1 = Me	**27**
R_2 = Phenyl	R_1 = Me	**28**
R_2 = Allyl	R_1 = H	**29**
R_2 = Phenyl	R_1 = H	**22**



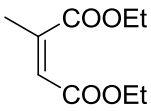
13

SYE-4



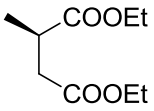
(*R*)-**19**

85%



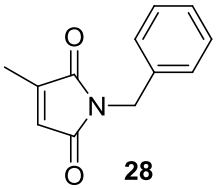
14

SYE-4



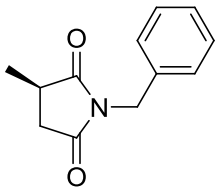
(*R*)-**20**

83%



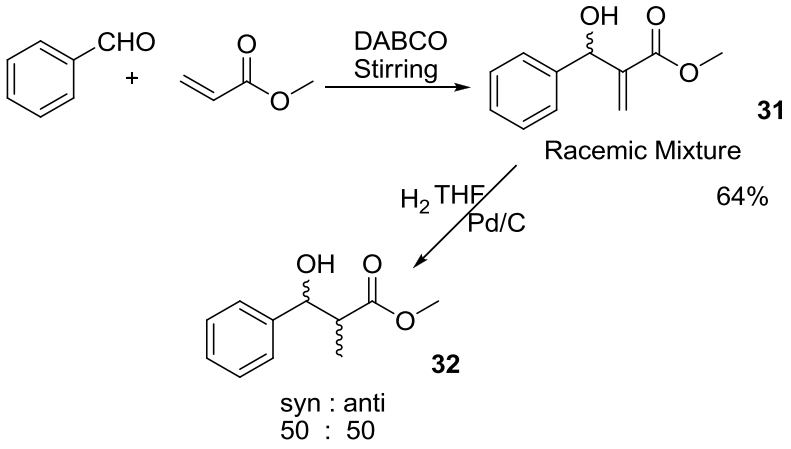
28

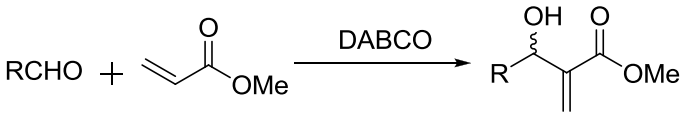
SYE-4



(*R*)-**30**

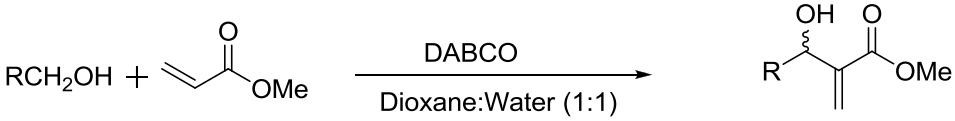
76%





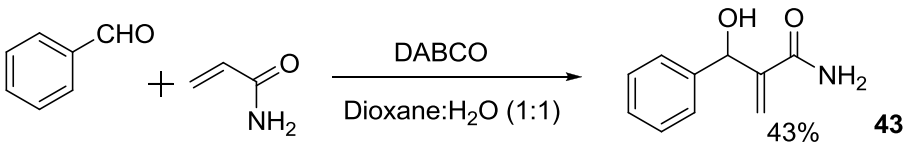
R = -CH_3	53%	**33**
R = -CH_2CH_3	58%	**34**
R = -$CH_2CH_2CH_3$	49%	**35**
R = -$CHCH_3CH_3$	61%	**36**

R = 4-MeO-C_6H_4	47%	**37**
R = 4-Me-C_6H_4	76%	**38**
R = 4-Cl-C_6H_4	69%	**39**

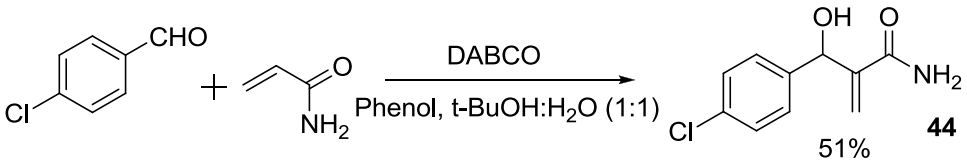


R = 2-pyridyl	53%	**40**
R = 2-furyl	61%	**41**

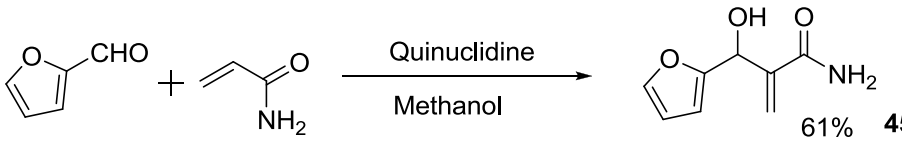
79% **42**



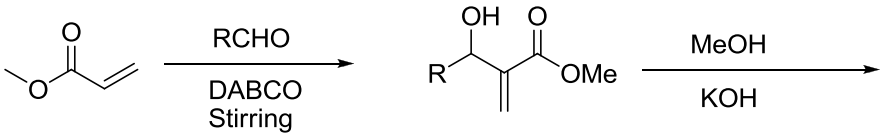
43% **43**



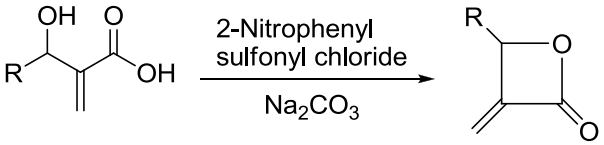
51% **44**



61% **45**

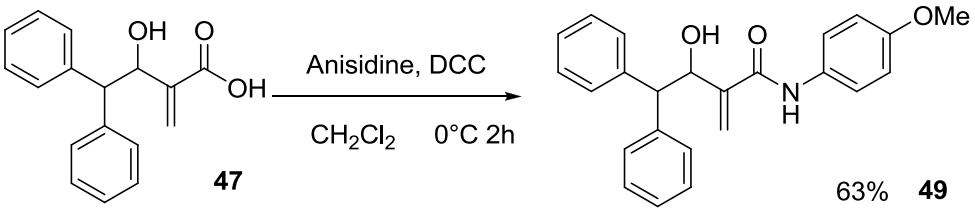


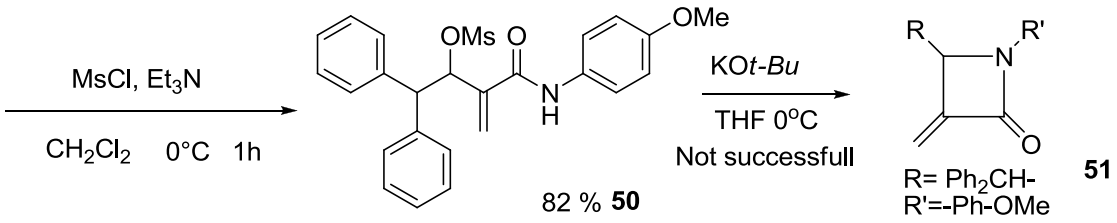
R= Ph_2CH 46% **46**

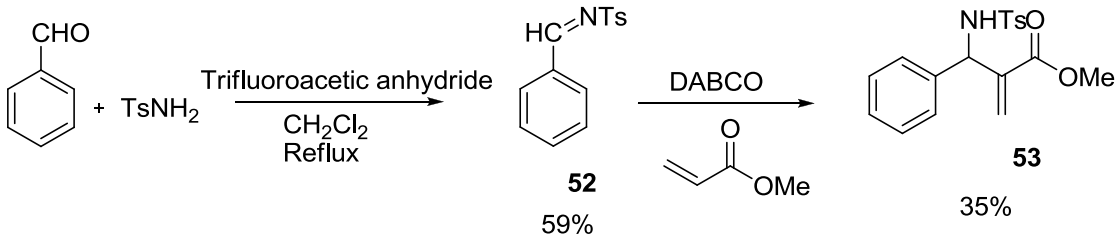


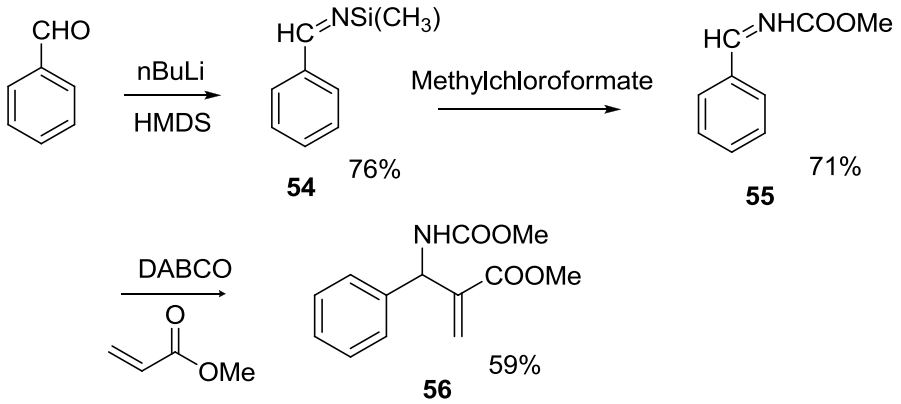
R= Ph_2CH 74% **47**

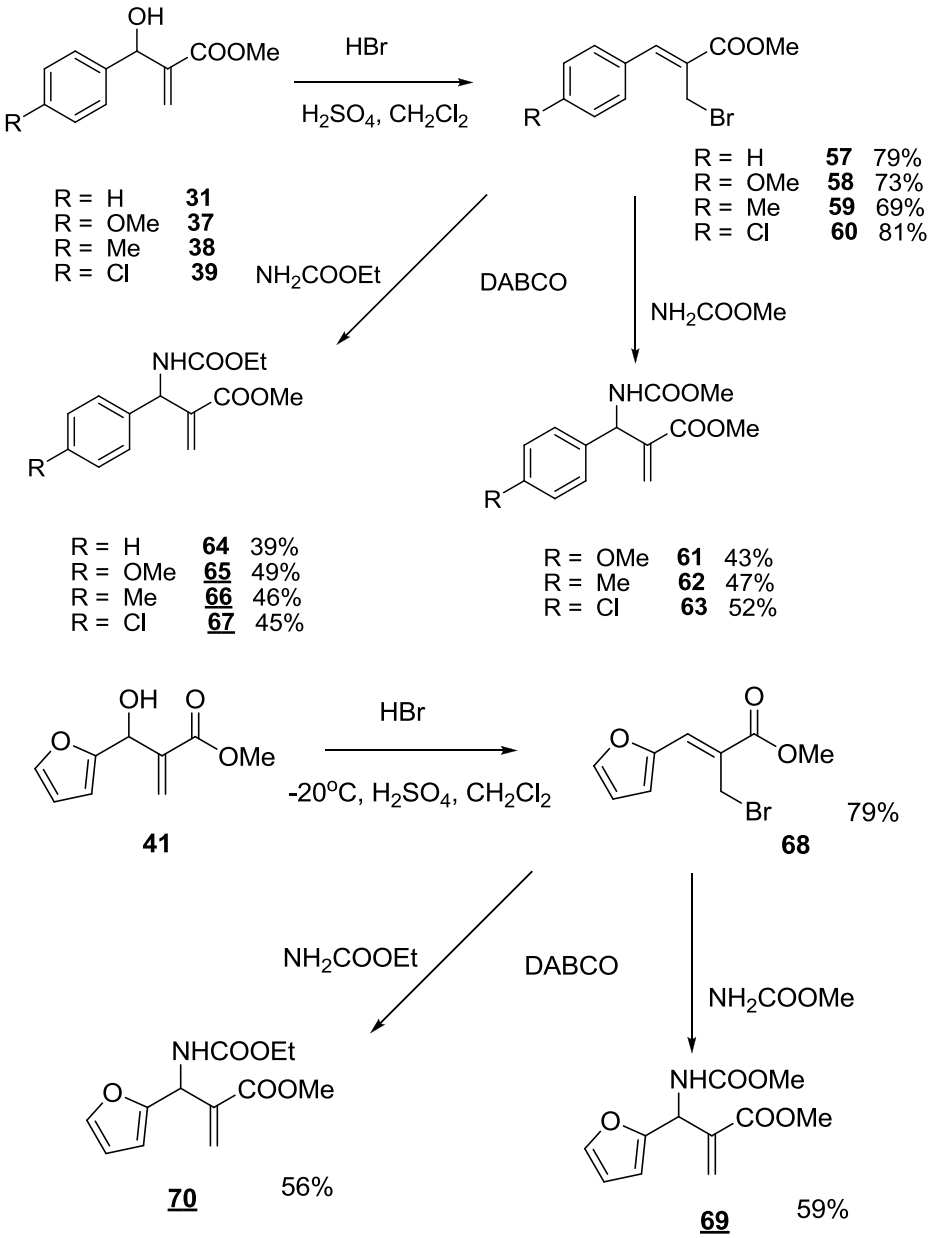
R= Ph_2CH 45% **48**

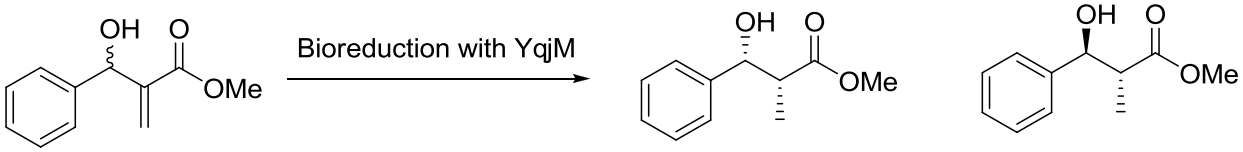

OH
O
OH
47
Anisidine, DCC
CH_2Cl_2 0°C 2h
OH
O
OMe
N
H
63% 49


MsCl, Et_3N
CH_2Cl_2 0°C 1h
OMs
O
OMe
N
H
82 % 50
KO*t-Bu*
THF 0°C
Not successfull
R
R'
N
O
R= Ph_2CH-
R'=-Ph-OMe
51


CHO
+ $TsNH_2$
Trifluoroacetic anhydride
CH_2Cl_2
Reflux
HC=NTs
52
59%
DABCO
O
OMe
NHTs
O
OMe
53
35%


CHO
nBuLi
HMDS
HC=NSi(CH_3)
54
76%
Methylchloroformate
HC=NHCOOMe
55
71%
DABCO
O
OMe
NHCOOMe
COOMe
59%
56





R and S

31

2R, 3R

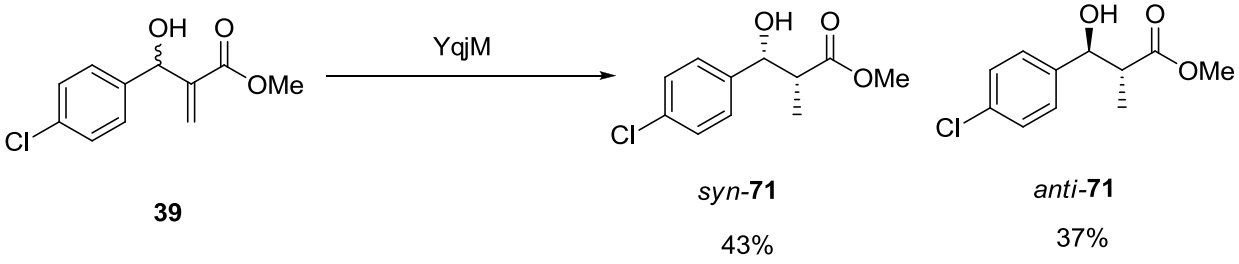
syn-**32**

50%

2R, 3S

anti-**32**

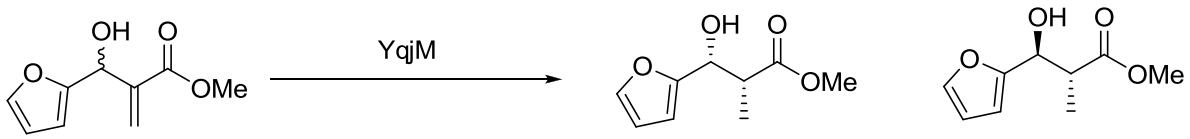
29%



39

syn-**71**
43%

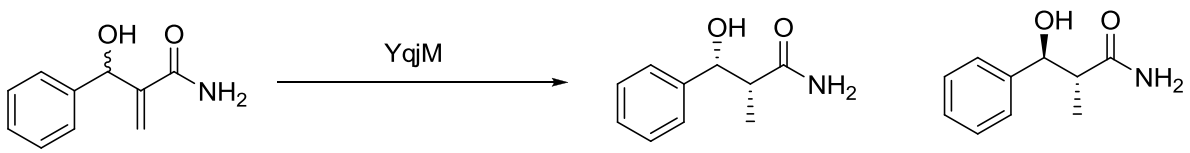
anti-**71**
37%



41

syn-**72**
79%

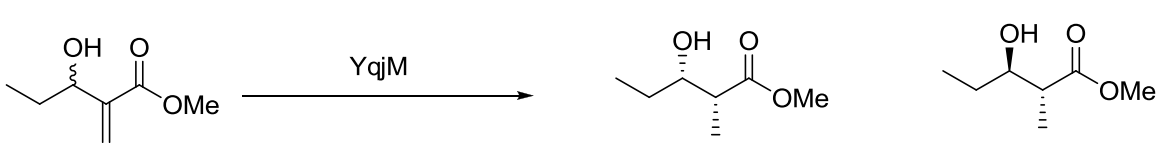
anti-**72**
9%



43

syn-**73**
23%

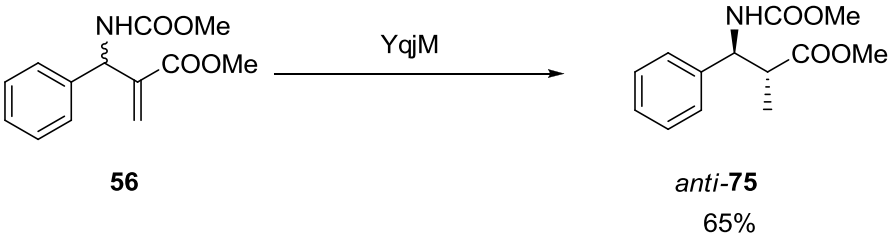
anti-**73**
25%



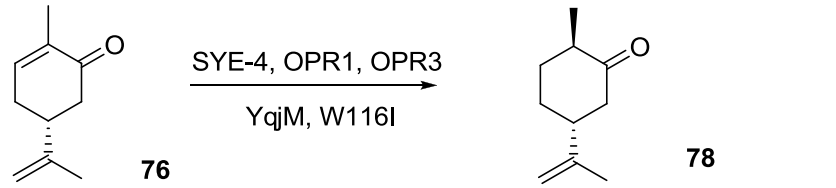
34

syn-**74**
73%

anti-**74**
11%

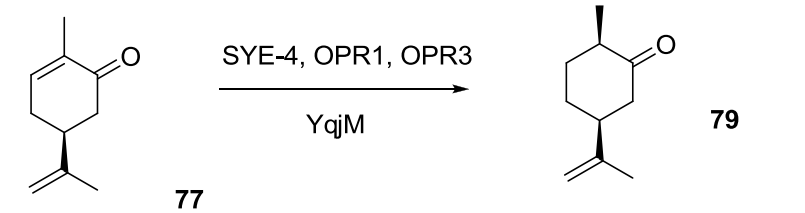


56 *anti*-**75** 65%



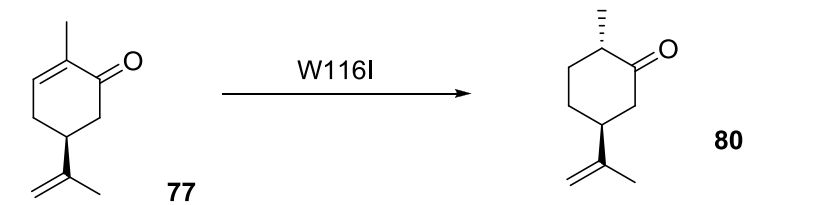
76 **78**

(-)-Carvone (+) *trans*-Dihydrocarvone



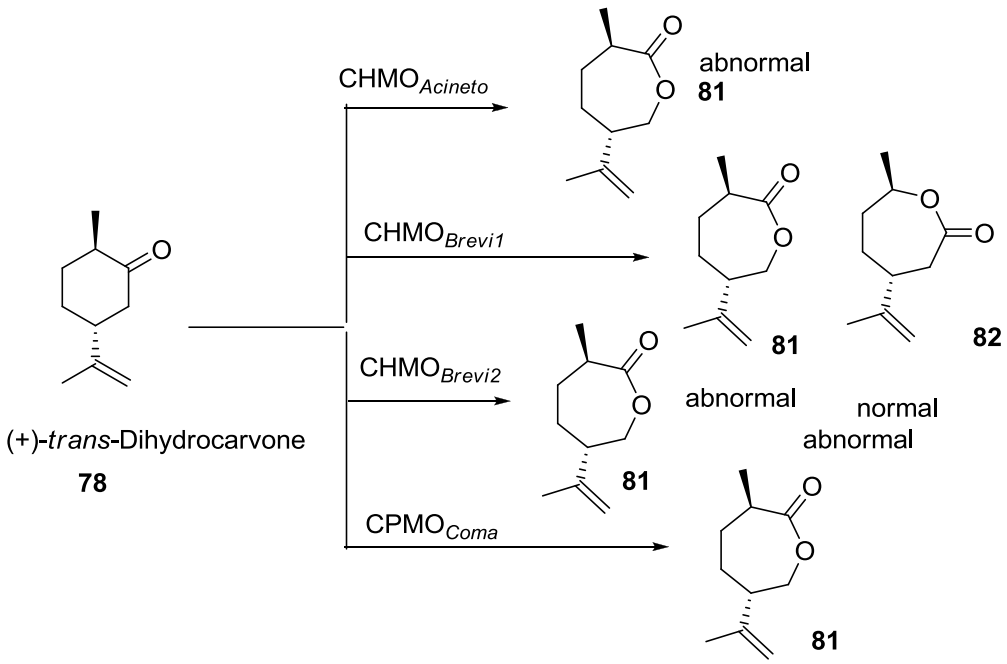
77 **79**

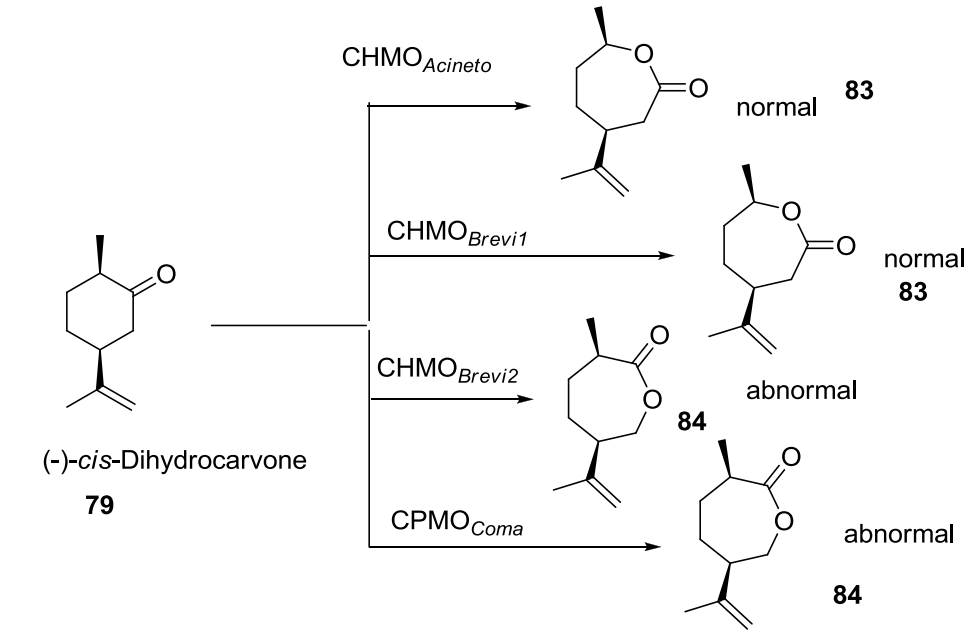
(+)-Carvone (-) *cis*-Dihydrocarvone

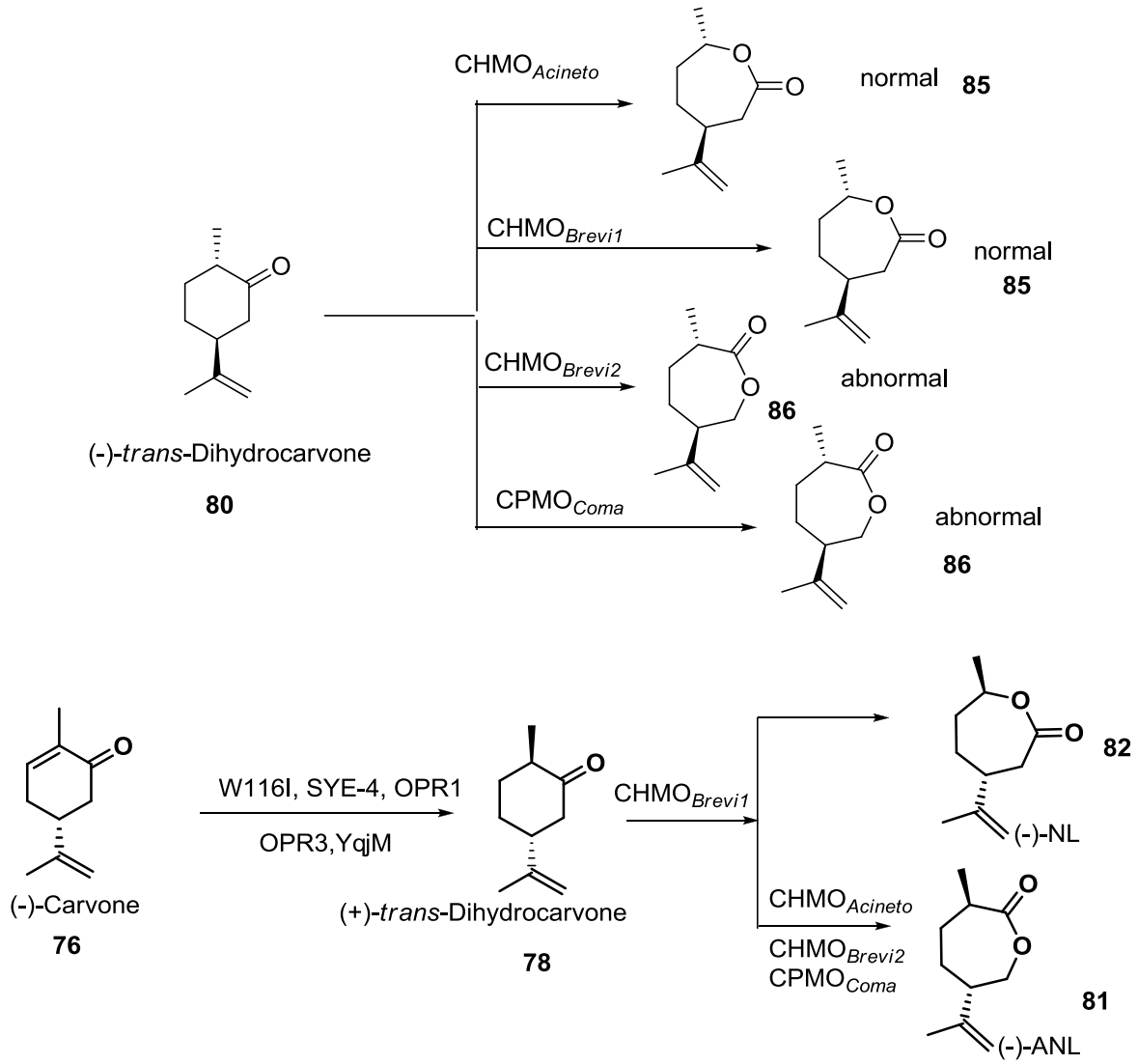


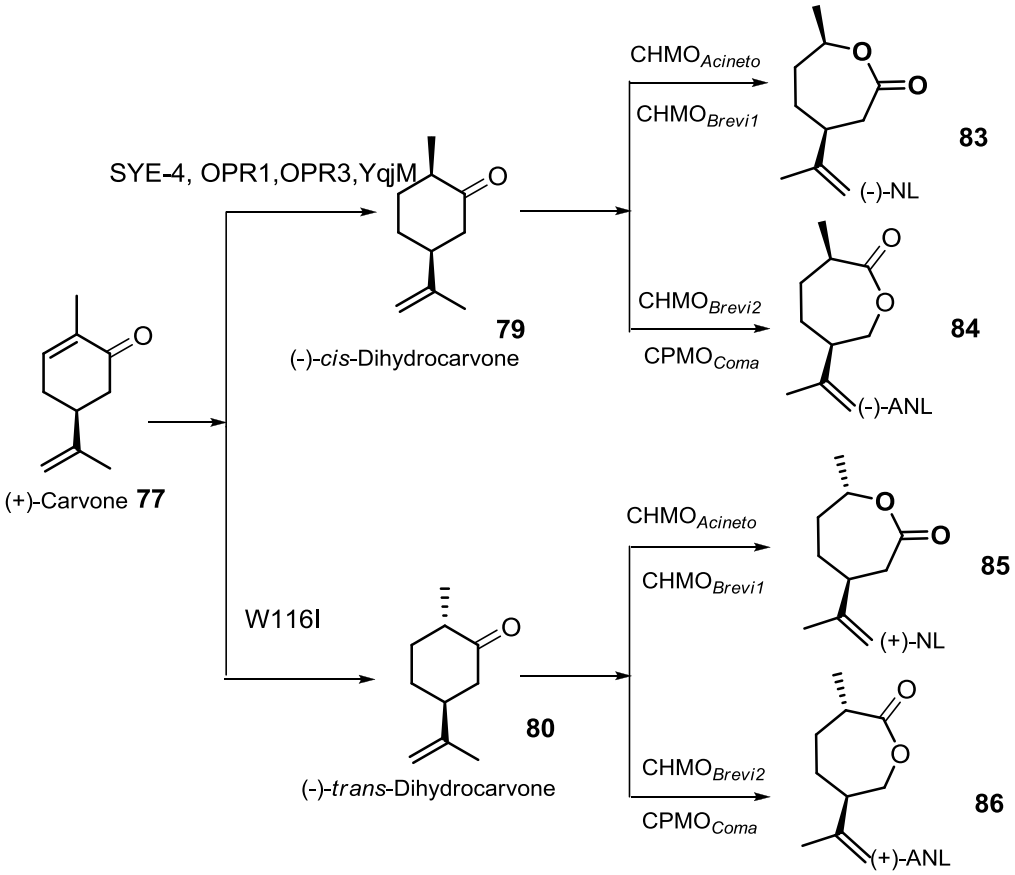
77 **80**

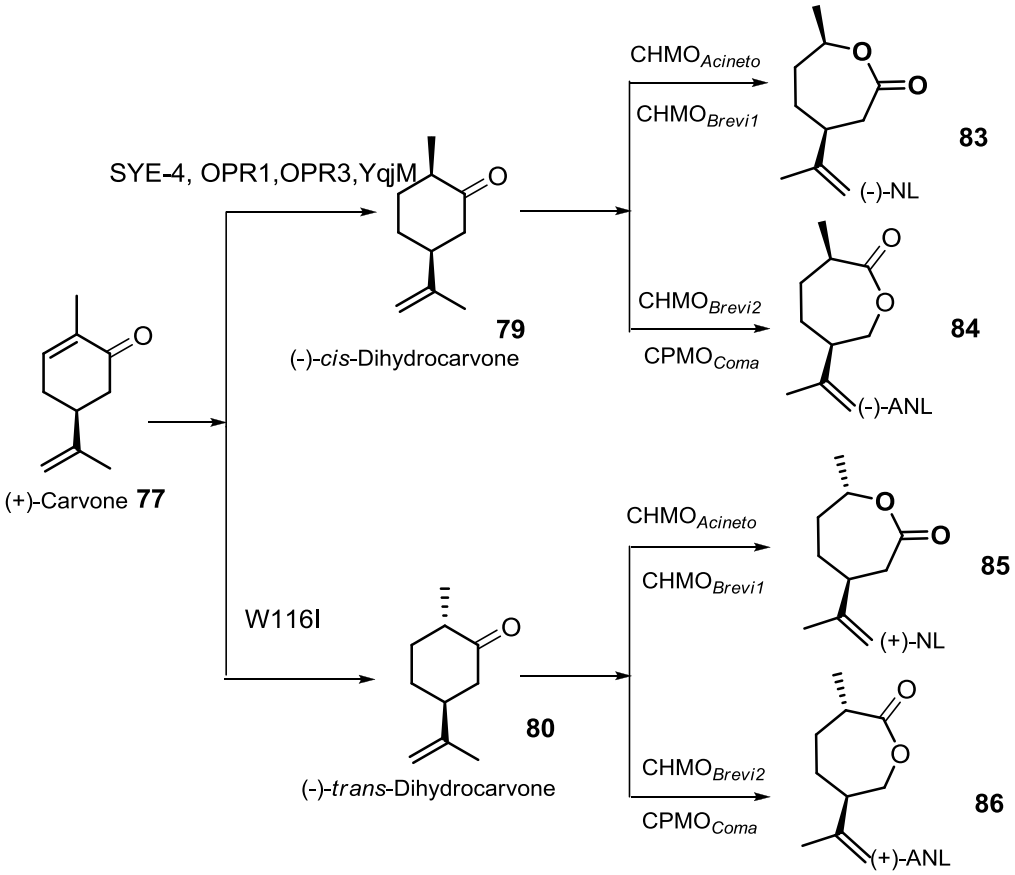
(+)-Carvone (-) *trans*-Dihydrocarvone

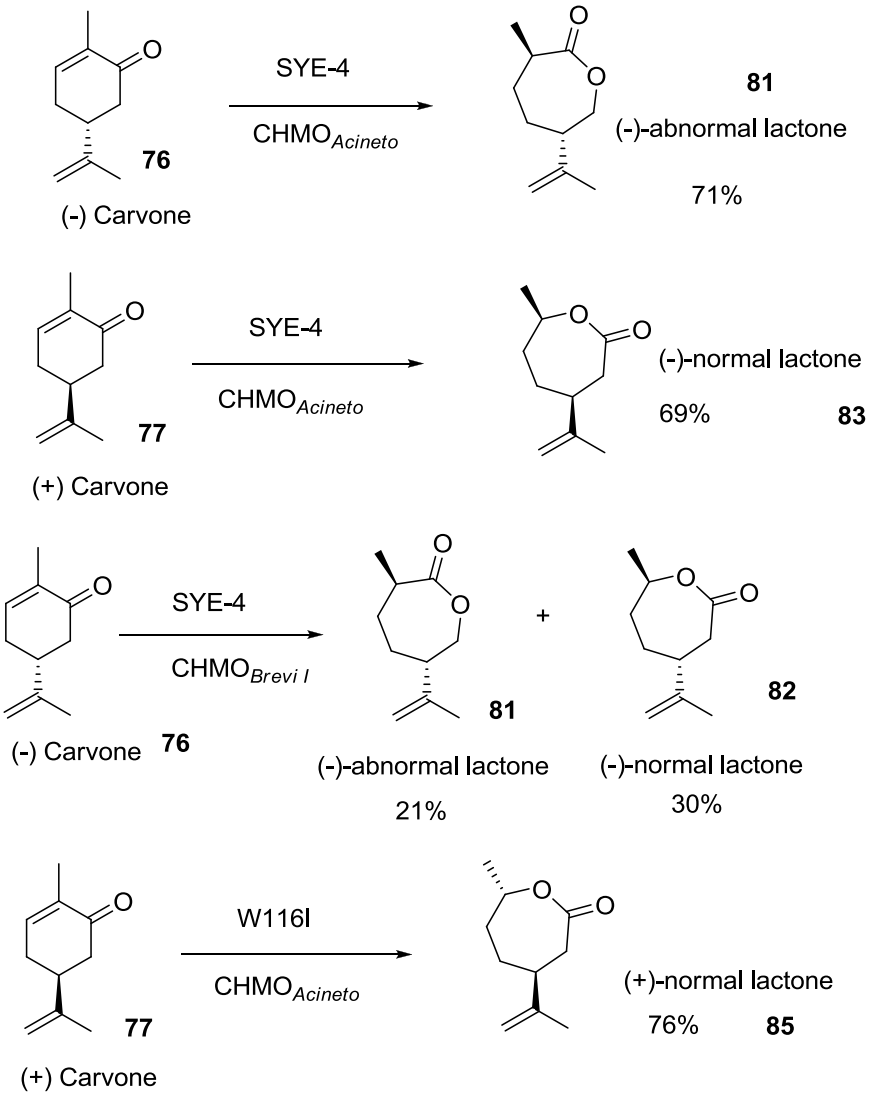

CHMO$_{Acineto}$
abnormal
81
CHMO$_{Brevi1}$
81
82
normal
abnormal
CHMO$_{Brevi2}$
abnormal
81
CPMO$_{Coma}$
81
(+)-*trans*-Dihydrocarvone
78


CHMO$_{Acineto}$
normal
83
CHMO$_{Brevi1}$
normal
83
CHMO$_{Brevi2}$
abnormal
84
CPMO$_{Coma}$
abnormal
84
(-)-*cis*-Dihydrocarvone
79


CHMO$_{Acineto}$
normal 85
CHMO$_{Brevi1}$
normal
85
CHMO$_{Brevi2}$
86
abnormal
CPMO$_{Coma}$
abnormal
86
(-)-*trans*-Dihydrocarvone
80
(-)-Carvone
76
W116I, SYE-4, OPR1
OPR3,YqjM
(+)-*trans*-Dihydrocarvone
78
CHMO$_{Brevi1}$
82
(-)-NL
CHMO$_{Acineto}$
CHMO$_{Brevi2}$
CPMO$_{Coma}$
81
(-)-ANL


SYE-4, OPR1,OPR3,YqjM
W116I
(+)-Carvone 77
79
(-)-cis-Dihydrocarvone
80
(-)-trans-Dihydrocarvone
CHMOAcineto
CHMOBrevi1
CHMOBrevi2
CPMOComa
83
(-)-NL
84
(-)-ANL
85
(+)-NL
86
(+)-ANL


SYE-4, OPR1,OPR3,YqjM
W116I
(+)-Carvone 77
79
(-)-cis-Dihydrocarvone
80
(-)-trans-Dihydrocarvone
CHMOAcineto
CHMOBrevi1
CHMOBrevi2
CPMOComa
83
(-)-NL
84
(-)-ANL
85
(+)-NL
86
(+)-ANL


SYE-4
CHMO Acineto
76
(-) Carvone
81
(-)-abnormal lactone
71%
SYE-4
CHMO Acineto
77
(+) Carvone
(-)-normal lactone
69%
83
SYE-4
CHMO Brevi I
76
(-) Carvone
81
(-)-abnormal lactone
21%
+
82
(-)-normal lactone
30%
W116I
CHMO Acineto
77
(+) Carvone
(+)-normal lactone
76%
85

Introduction

1.1 Biocatalysis

Biocatalysis is the use of natural catalysts, such as enzymes, to perform chemical transformations on organic compounds. Both enzyme preparations of various types (crude cell extracts, isolated proteins, fused proteins, chemically modified proteins) and enzymes still residing inside living cells are employed for this task.[1,2,3]

Biocatalysis underpins some of the oldest chemical transformations known to humans, for brewing predates recorded history.

Enzymes are the subset of proteins that catalyze the chemistry of life, transforming both macromolecular substrates and small molecules. The precise three-dimensional architecture of enzymes permits almost unerring selectivity in physical and chemical steps to impose remarkable rate accelerations and specificity in product-determining reactions. Many enzymes are members of families that carry out related chemical transformations and offer opportunities for directed in vitro evolution, to tailor catalytic properties to particular functions.

The biggest advantage of enzymes is their often unsurpassed selectivity, while enzymes are used beneficially to increase chemical selectivity or regioselectivity of a reaction. With enzymes, enantioselectivities of > 99% e.e. can be achieved routinely, although by no means in every case. This fact becomes increasingly important for using biocatalysts in the synthesis of advanced pharmaceutical intermediates, as regulatory agencies require separate toxicological studies for every impurity comprising above 1% of the content.[4]

The fact that enzymes are active mostly at mild, near-ambient conditions of temperature and pH, and preferentially in aqueous media is further regarded as another advantage. Goals for industrial processing such as “sustainable development”, “green chemistry”, or “environmentally benign manufacturing”, an increasingly important boundary condition for

---

[1] Anthonsen, T. In *Applied Biocatalysis*; Adlercreutz. P.; Straathof, A. J. J. Eds.; Harwood Academic Publishers: UK, **1999**, 18-53.
[2] Faber, K. *Biotransformations in Organic Chemistry*, 4th ed., Springer-Verlag, Berlin, **2000**.
[3] Jayasinghe L. Y.; Smallridge A. J.; Trewhella M. A. *Tetrahedron Letters*, **1993**, *34*, 24, 3949-3950.
[4] Crossley, R. *Chirality and the Biological Activity of Drugs*, CRC Press, Boca Raton, **1995.**

industrial activity in a large part of the world, would be much harder to attain without the availability of biocatalysts which tolerate and require such conditions.

The use of enzyme instead of whole cells has many advantages. Firstly it restricts the number of reactions, especially to eliminate down stream of the product. Enzymes are more stable for long time as compare to whole cells. Eznyme turn over number is high i.e. the same amount of enzyme catalyses more conversions. Enzymes can tolerate conditions (high substrate concentration; organic phase; inhibitory substrate; elevated temperature; permeability barriers) which are not suitable for cell growth.

The use of isolated enzymes in a bioreactor offers several advantages over the use of whole cells. For example, substrate and enzyme concentration can be set to optimal values in an enzyme reactor while at the same time; transport limitations by cellular membranes can be excluded. Furthermore, yield reductions by side reactions of the cellular metabolism are avoided. However, it is crucial to address typical drawbacks such as efficient enzyme production, *in vitro* stability or cofactor dependency at process engineering as well as at biochemical levels.

Biocatalytic processes increasingly penetrate the chemical industry. In a recent study, 134 industrial-scale biotransformations, on a scale of > 100 kg with whole cells or enzymes starting from a precursor other than a C-source, were analyzed[5]. In pharmaceutical industry, the reason for the drive for enantiomeric purity is that the vast majority of novel drugs are chiral targets, favoring biocatalysis as the technology with the best selectivity performance.[6] Biocatalytic processes for producing enantiomerically pure pharmaceutical intermediates or active ingredients are of growing importance. Recent advances in molecular biological methods such as recombinant enzyme expression, high-throughput DNA sequencing, and enzyme-evolution technologies, make biocatalysis a viable option for producing single enantiomers. Even two- and three-step biotransformation can be accomplished by combining and adapting enzymes from different sources. Recombinant microbial whole-cell biocatalysts, or so-called “designer cells,” which provide each enzyme at the optimum amount, are a particularly efficient approach. Under

---

[5] Andreas, S. B.; Bettina, R. R. *Biocatalysis*, **2004** WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.
[6] Straathof, J. J.; Panke, S.; Schmid, A. *Curr. Opin. Biotechnol*. **2002**, *13*, 548–556.

this approach, all of the required enzymes can be produced by one single fermentation. Cell disruption, clarification, and concentration of the enzyme solution are dispensable. The separation of the biocatalyst after biotransformation can easily be conducted by flocculation and filtration of the biomass. Substantial substrate and product-transfer limitations through the cell membranes have not been observed using frozen or dried biocatalysts.

From different disciplines, biotechnology and biocatalysis are seen from very different angles and perspectives (Figure 1.1). Chemistry and chemists emphasize a *molecularly-oriented* perspective dominated by compounds and transformations, whereas chemical engineering and thus chemical engineers favor a *process-oriented* perspective of reactions and processes; lastly, biology and its practitioners contribute a *systems-oriented* perspective of description at the organism level as well as in their view of evolution.

Different parts of each of the three disciplines are needed for the successful practice of biocatalysis: biochemistry and organic chemistry from chemistry; molecular biology, enzymology, and protein (bio) chemistry from biology; and catalysis, transport phenomena, and reaction engineering from chemical engineering are indispensable. Both biotechnology and biocatalysis are interdisciplinary areas; as most practitioners tend to hail from one of the three major contributing disciplines, hardly anybody has an equally strong command of all the sub-disciplines of biocatalysis.

There are not only many contributing disciplines for biotechnology and biocatalysis, but also many *areas of application:*

1. production and transformation of compounds, mainly in the chemical and pharmaceutical industry,
2. analytics and diagnostics, mainly in medicine, and
3. environmental protection and bioremediation (reconstruction of the environment).

The areas of application differ from the *industries* which apply them; the most important ones are pharmaceutical, food, fine chemicals, basic chemicals, pulp and paper, agriculture, medicine, energy production, and mining industries (Figure 1.1).

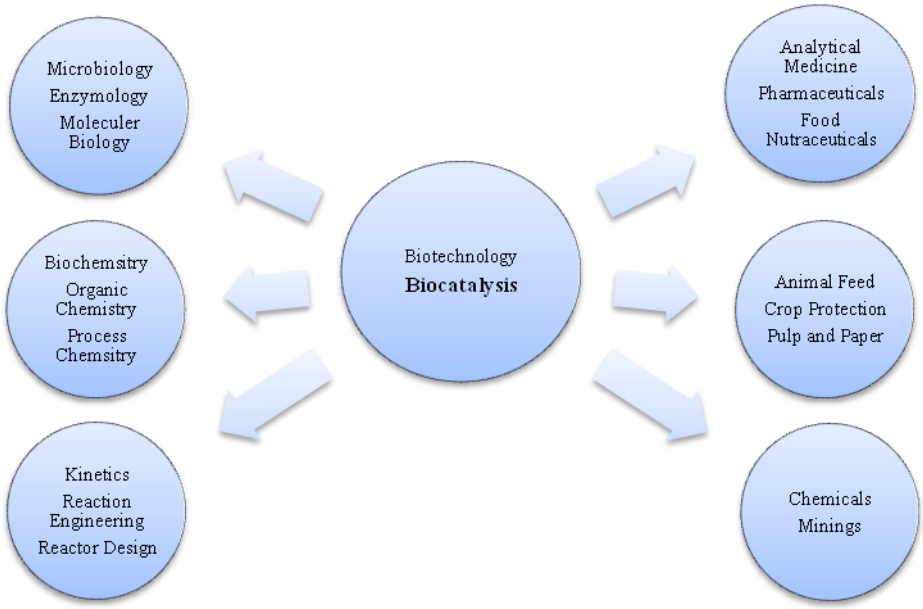


Figure 1.1 Central roles of biocatalysis and biotechnology between interdisciplinary feeder sciences (biology, chemistry, chemical engineering science) and multiple user industries[5].

1.2 Redox Biocatalysis

Enzyme mediated reactions have become a powerful methodology in asymmetric synthesis and this area has received considerable attention within the past decade.[7] Currently, biocatalysis offers complementary solutions to metal-assisted transformations in chiral catalysis and is considered one of the important strategies of "green chemistry". In particular the high sustainability of protein based catalytic entities represents a major advantage. Recent trend studies indicate a significant potential for growth of the contribution by "white biotechnology" to the industrial scale synthesis of chiral compounds *via* biotransformations at the expense of classical chiral auxiliary strategies (with recently re-discovered "organocatalysis" as yet to be adopted methodology for industrial applications).[8]

---

[7] a) *Biocatalysis in the Pharmaceutical and Biotechnology Industries*, Patle, R.N. (ed.) CRC Press, Boca Raton, **2007**. b) *Enzyme Catalysis in Organic Synthesis*, Drauz, K.; Waldmann, H. (eds.) Wiley-VCH, Weinheim **2002**.
[8] Frost. Sullivan. *Business assessment, Chem. Eng. News* **2003**, *81*, 18, 45-55.

Initial activities had focussed on hydrolytic biotransformations due to readily available thermostable enzymes from the detergent industry that tolerate organic solvents. The success story of biocatalysis is based on reactions utilizing lipase, esterase, and protease in the resolution of racemates by selective reaction of one substrate enantiomer[9] or by desymmetrization to form chirality in a *de novo* fashion.[10]

Recent progress in the field shifted to other conversions, in particular C-C bond formation and redox processes. The latter type of reaction usually requires cofactors (e.g. NADH, NADPH etc.), which have to be recycled in order to enable an economically reasonable process. Hence, an auxiliary substrate has to be added in order to regenerate the cofactor in the required oxidation state ultimately closing the catalytic cycle. This problem is often solved by creating a closed-loop system with an additional enzyme for cofactor regeneration. Suitable systems are available for recycling of the most abundant cofactors NADH and NADPH.[11] In general, this complicates the application in synthetic chemistry by increasing the preparative efforts; a more complicated experimental set-up is required and product isolation is more demanding. In addition, redox-enzymes often display a limited stability (e.g. membrane bound biocatalysts) and are therefore more difficult to isolate and manipulate.

An appealing strategy to overcome these challenges both on laboratory and industrial scale is the application of whole-cells.[12] As long as a cell is living and the required nutrients are added all native cofactor recycling systems are operational and there is no need to take additional measures. However, considering the genetic background of a native microorganism with 4000+ genes (*E.coli*: approx. 4500 genes, *S.cerevisiae*: approx. 6500 genes), there is a significant likelihood of unwanted side reactions either decreasing the yield of the reaction or/and decreasing the stereoselectivity of the biotransformation. Such problems are nowadays fixed by

---

[9] Faber, K. *Biotransformations in Organic Chemistry*, Springer, Berlin, **1997**.
[10] Garcia-Urdiales, E.; Alfonso, I.; Gotor, V. *Chem. Rev.* **2005**, *105*, 313-354.
[11] a) Wichmann, R.; Wandrey, C.; Bückmann, A.F.; Kula, M.R. *Biotechnol. Bioeng.* **1981**, *23*, 2789-2802. b) Wong, C.H.; Whitesides, G.M. *J. Am. Chem. Soc.* **1981**, *103*, 4890-4899. c) Tishkov, V.I.; Galkin, A.G.; Marchenko, G.N.; Tsyganokov, Y.D.; Egorov, H.M. *Biotechnol. Appl. Biochem.* **1993**, *18*, 201-207. d) Kataoka, M. *Appl. Microbiol. Biotechnol.* **1999**, *51*, 486-490. e) Vrtis, J.M.; White, A.K.; Metcalf, W.W.; vam der Donk, W.A. *Angew. Chem. Int. Ed.* **2002**, *41*, 3257-3259.
[12] a) Arrigo, D. P.; Pedrocchi-Fantoni, G.; Servi, S. *Stereoselect. Biocatal.* **2000**, 365-396. b) Stewart, J.D. *Curr. Opin. Biotechnol.* **2000**, *11*, 363-368. c) Stewart, J.D. *Biotechnol. Genet. Engin. Rev.* **1997**, *14*, 67-143.

creating recombinant overexpression systems of the required protein in which a strong promoter upstream of the gene encoding the biocatalyst is responsible for producing large quantities of the enzyme. Consequently, the desired catalytic entity becomes the dominant fraction in the cell's proteome and side-reactions by competing enzymes become negligible. Eventually, specific gene knock-out strategies can be applied to completely delete unwanted biotransformations. However, when achieving such high redox-biocatalyst concentrations within the cell by using strong promoters, cofactor recycling may again become a bottle-neck for the overall process.

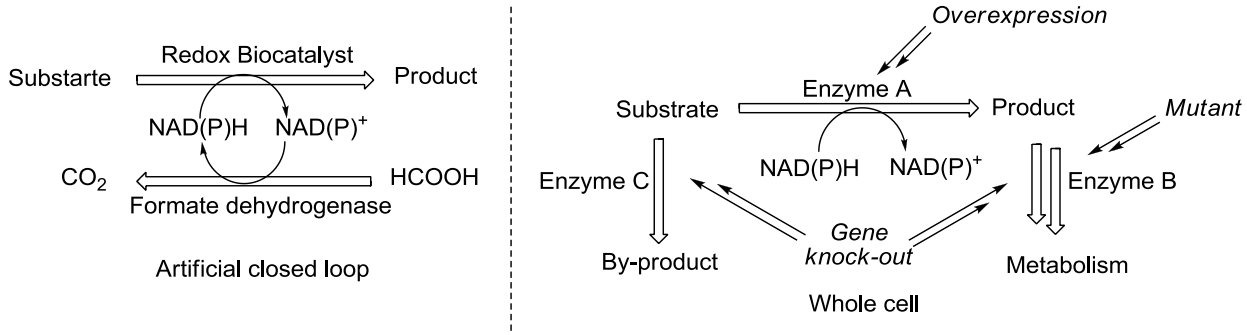


Figure 1.2 Strategies for cofactor recycling.

1.3 Multistep Biotransformations

Multi-step one-pot reactions have caught attention of synthetic chemists in recent years as such transformations allow for increasing dramatically the structural and functional complexity of a chemical entity in a single operation.[13] Catalyzed domino, tandem, or cascade processes with highly selective catalytic species are particularly attractive since such transformations can be combined in a highly modular manner. From a mechanistic point of view several sub-types of catalyzed multi-component reactions can be distinguished depending on the action of the catalytic entity.[14]

---

[13] a) Dömling, A.; Ugi, I. *Angew. Chem.* **2000**, *112*, 3300-3344. b) Tietze, L.F.; Modi, A. *Med. Res. Rev.* **2000**, *20*, 304322. c) Zhu, J. *Eur. J. Org. Chem.* **2003**, 1133-1144. d) Wangelin, A.J.; Neumann, H.; Gördes, D.; Klaus, S.; Strübing, D.; Beller, M. *Chem. Eur. J.* **2003**, *9*, 4286-4294. e) Tempest, P.A. *Curr. Opin. Drug Discovery Dev.* **2005**, *8*, 776-788.

[14] Fogg, D.E.; dos Santos, E.N. *Coord. Chem. Rev.* **2004**, *248*, 2365-2379.

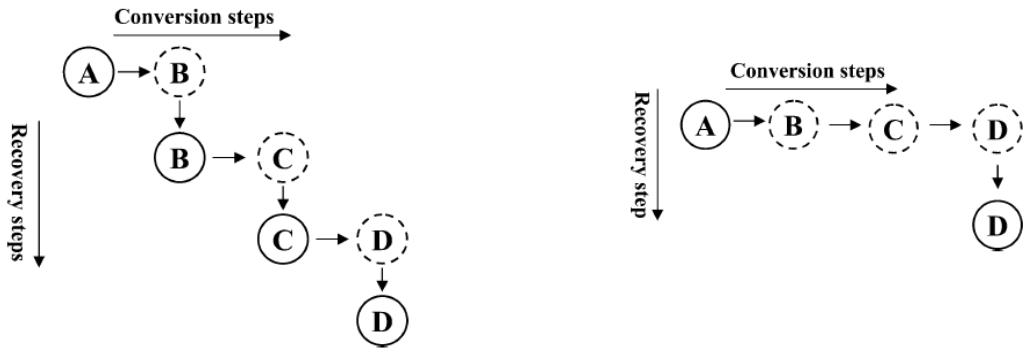


Figure 1.3 Multi-step transformations.

While this strategy is rather new in synthetic organic chemistry with an emphasis on the application of metal-based catalysts, nature is using this principle since its beginnings. All metabolic pathways are essentially cascade reactions which are self-organized into steady state equilibrium. However, in contrast to demands in synthetic chemistry for a highly diverse process applicable to a large diversity of compounds, nature usually optimized catabolism and anabolism to a particular compound or target. Hence, natural metabolic pathways are difficult to adapt towards the conversion of diverse starting materials. Nonetheless, highly successful utilizations are reported from metabolic engineering taking advantage of already established pathways, predominantly of the secondary metabolism.[15]

The difference between biocatalysis and classical fermentation is the extension of enzymatic transformations to non-natural substrates. The promiscuity of enzymes is utilized in biocatalysis like a classical methodology in synthetic chemistry. Consequently, the extension of single-step biotransformations, which are a particularly powerful tool in asymmetric synthesis today, is just a logical development.[16] Due to the intrinsic chemoselectivity of enzymes and the highly similar operational environment for biocatalysts (pH, temperature, etc.), proteins are particularly promising catalysts in multi-step transformations. This is not only limited to all-enzyme cascade reactions, but can also be realized in a hybrid fashion combining the advantages of biocatalysis with metal-assisted reactions.[17]

---

[15] Frost, J. *Chem. Engineer* **1996**, *611*, 32-33.
[16] a) Mayer, S.F.; Kroutil, W.; Faber, K. *Chem. Soc. Rev.* **2001**, *30*, 332-339. b) Bruggink, A.; Schoevaart, R.; Kieboom, T. *Org. Proc. Res. Develop.* **2003**, *7*, 622-640. c) Pellissier, H. *Tetrahedron* **2006**, *62*, 2143-2173.
[17] Simons, C.; Hanefeld, U.; Arends, I.W.C.E.; Maschmeyer, T.; Sheldon, R.A. *Topics Catal.* **2006**, *40*, 35-44.

Nature is using this design principle highly successfully, as all metabolic pathways are interconnected and conducted within the "single-vessel" environment of a cell. However, this approach is largely limited to already existing metabolic reaction sequences not necessarily delivering the structural diversity of compounds utilized by chemical industry.

Adopting this strategy by combining usually unrelated biotransformations resembles the usual approach in synthetic organic chemistry, where typical reaction protocols are combined in elaborate routes. While conventional catalytic systems in synthesis often operate under highly diverse conditions, the combination of biocatalysis is much more straight forward based on the narrow operational window of enzymes. As major difference to natural metabolic pathways, which were usually optimized for the production of single compounds, *de novo* biocatalytic reaction sequences should be based on promiscuous enzymes capable to convert a defined range of substrates.

In this context, the combination of bioreductions with biooxygenations represents a particularly appealing approach. A rapidly emerging target for application of the outlined strategy, in the area of bioreductions, is the area of formal biocatalytic olefin hydrogenation, which has progressed significantly in recent years. The asymmetric Baeyer-Villiger oxidation has proven to be a highly valuable transformation for the synthesis of chiral lactones as building blocks in natural product and bioactive compound synthesis. Baeyer-Villiger monooxygenases (BVMOs) represent most versatile and stereoselective catalytic entities with remarkable capabilities to also affect the regiochemistry of this oxygen insertion process.[18] The biocatalytic approach to realize this transformation with high regio- and enantioselectivity takes advantage of the high promiscuity of flavin dependent monooxygenases to accept a large diversity of non-natural substrates. Moreover, this "green chemistry" strategy utilizes molecular oxygen for the oxidation process as most sustainable oxidant. An increasing number of such Baeyer–Villiger monooxygenases (BVMOs) have become available in recent years and the microbial Baeyer–Villiger biooxidation has been developed towards a highly versatile biocatalyst platform.

---

[18] a) Mihovilovic, M.D. *Curr. Org. Chem.* **2006**, *10*, 1265-1287. b) Kamerbeek, N.M.; Janssen, D.B.; van Berkel, W.J.H.; Fraaije, M.W. *Adv. Synth. Catal.* **2003**, *345*, 667-678. c) Mihovilovic, M.D.; Müller, B.; Stanetty, P. *Eur. J. Org. Chem.* **2002**, 3711-3730.

1.4 Bioreduction of C=C Bonds with Ene-Reductases

The asymmetric (bio)catalytic reduction of C=C bonds goes in hand with the creation of (up to) two chiral centres and, thus, represents a highly appealing strategy for the production of chiral materials. Whereas *cis*-hydrogenation using transition-metal-based homogeneous catalysts has been developed to an impressive standard, stereo-complementary asymmetric *trans*-hydrogenation is still at the stage of development.[19]

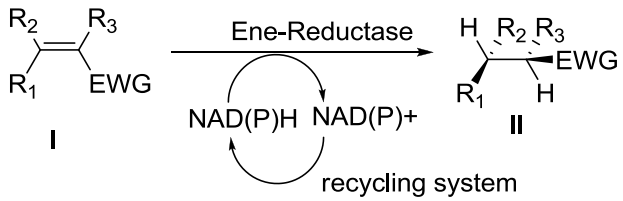


Figure 1.4 Asymmetric bioreduction of activated double bond

The old yellow enzyme (OYE) family of enzymes as a whole has been shown to reduce an extensive library of α,β-unsaturated activated alkene compounds. Substrates typically contain electron-withdrawing or 'activating groups' such as aldehydes, acyclic and cyclic ketones, carboxylic acids, esters, and nitro functionalities.[20]

1.5 Mechanism of Ene-Reductase

Bioreductions represent the emerging field of formal biocatalytic olefin hydrogenation. This biotransformation is merely a nucleophilic attack by hydride originating from NAD(P)H across an activated C=C double bond *via* a Michael-type reaction. The mechanism of asymmetric reduction of activated alkenes by OYEs has been studied extensively.[21] The overall reaction

---

[19] Yang, J. W.; Hechavarria, M. T.; Vignola, N.; List, B. *Angew Chem Int Ed Engl.* **2005**, 44, 108-110.
[20] Stuermer, R., Hauer, B., Hall, M., Faber, K., *Curr. Opin. Chem. Biol.* **2007,** *11*, 203–213.
[21] a) Khan, H.; Harris, R. J., Barna, T.; Craig, D. H.; Bruce, N. C.; Munro, A. W.; Moody, P. C.;. Scrutton, N. S. *J. Biol. Chem.* **2002**, *277*, 21906 – 21912. b) Karplus, P. A.;. Fox, K. M.; Massey, V. *FASEB J.* **1995,** *9*, 1518 –1526. c) Khan, H.; Barna, T.; Bruce, N. C.; Munro, A. W.; Leys, D.; Scrutton, N. S. *FEBSJ.* **2005**, *272*, 4660 –4671. d) Kohli, R. M.; Massey, V. *J. Biol. Chem*. **1998,** *273*, 32763 – 32770. e) Messiha, H. L.; Munro, A. W.; Bruce, N. C.; Barsukov, I.; Scrutton, N. S. *J. Biol. Chem.* **2005,** *280*, 10695 –10709. f) Nakamura, T.; Yoshimura, J.; Ogura, Y. *J. Biochem.* **1965**, *57*, 554 –564.

catalyzed by the OYE family is the NAD(P)H-dependent reduction of activated alkenes. The reaction proceeds in two stages: NAD(P)H oxidation by hydride transfer to the FMN cofactor (reductive half reaction) of the OYE followed by the reduction of activated alkenes by hydride transfer from the reduced flavin (oxidative half reaction).[22] These steps are performed by a bi-bi ping pong mechanism, with both reductive and oxidative substrates binding within the same active site.[23] (Figure 1.5)

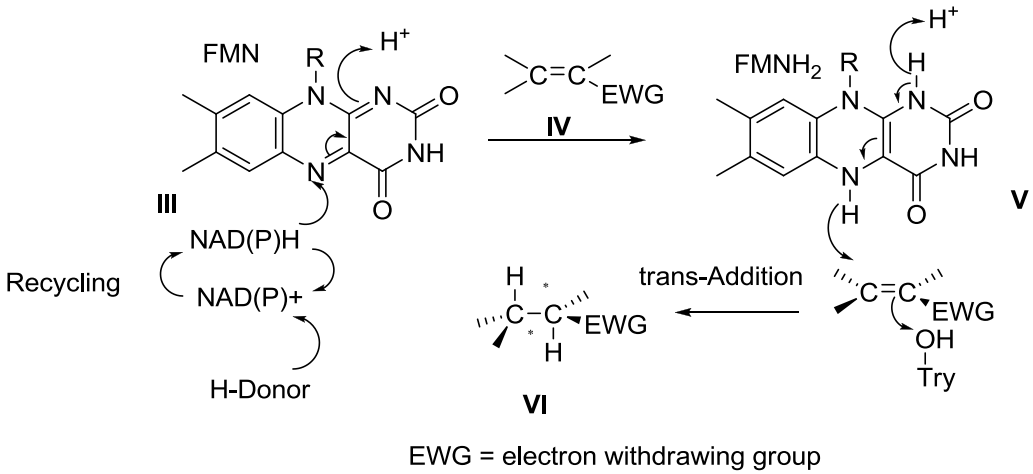


Figure 1.5 Catalytic mechanism of Ene-Reductases

1.6 Stereochemistry of C=C Bioreduction

Stereochemistry of compounds is very important in medicinal chemistry. The affluence of structural information on the binding of substrates and inhibitors in the active site of OYEs helps in rationalizing the known stereochemical result of the reactions, as well as predicting both substrate selectivity and enantiopreference of the product.[24] This structure-driven approach facilitates the targeting of specific residues for mutagenesis studies in the generation of novel biocatalysts with improved substrate specificity and/or product enantiopurity.[25] However, low product ee's acquired with some substrates (excluding product racemization) suggests there may be more than one substrate binding mode in these enzymes, given the absolute stereospecificity

---

[22] Breithaupt, C.; Strassner, J.; Breitinger, U.; Huber, R.; Macheroux, P.; Schaller, A.; Clausen, T. *Structure* **2001**, *9*, 419– 429.
[23] Kohli, R. M.; Massey, V. *J. Biol. Chem.* **1998**, *273*, 32763 – 32770.
[24] Toogood, H. S.; Fryszkowska, A.; Hare, V.; Fisher, K.; Roujeinikova, A.; Leys, D.; Gardiner, J. M.; Stephens, G. M.; Scrutton, N. S. *Adv. Synth. Catal.* **2008,** *350*, 2789 –2803.
[25] Brown, B. J.; Hyun, J. W.; Duvvuri, S.; Karplus, P. A.; Massey, V. *J. Biol. Chem.* **2002,** *277*, 2138 –2145.

with which they transfer [2H].[26] Moody and coworkers[27] explained that near atomic resolution crystal structure of the 2-nitrocyclohexene-bound PETNR (pentaerythritol tetranitrate reductase) enabled the detection of two highly overlapping conformations of the substrate in the active site, with one existing in a 'flipped' orientation. The two binding modes are related by a near 180^{o} rotation about the cyclohexene ring, with an inversion of the half-chair conformation. This 'flipped' conformation places the olefinic bond in a position not preferential for hydride transfer. Co-crystal structures of PETN reductase bound to steroid substrates (1,4-androstadiene-3,17-dione and prednisone) and an inhibitor (progesterone) showed in all cases an inactive 'flipped' binding conformation, which prevented finest arrangement of the reducible unsaturated bonds with the FMN N5[28]. (Figure 1.6)

Although structural, mechanistic, and enantioselectivity information is available for OYEs, it is likely that catalysis may involve a metastable enzyme–substrate complex, with the most stable complex observed crystallographically not necessarily a hydride-transfer competent state. Successful catalysis may be followed by multiple substrate binding events, until optimal Cβ-N5 overlap is achieved. Some of these 'inactive' binding sites may be more highly occupied due to steric clashes between the ligand and the protein in the 'active state', as predicted by the PETN reductase-progesterone structure[29] and the inhibitor 9S,13S-OPDA-bound LeOPR1 structure.[30] This diversity of binding modes, including multiple 'active' states, may be due to the relatively few interactions existing between the ligands and the protein combined with the relatively large and open active site of OYEs.[31] Therefore, the formation of less enantiomerically pure products may reflect, to a certain extent, the binding of the substrate in more than one 'active' conformation.

---

[26] Kohli, R. M.; Massey, V. *J. Biol. Chem.* **1998**, *273*, 32763– 32770.
[27] a) Barna, T. M.; Khan, H.; Bruce, N. C.; Barsukov, I.; Scrutton, N. S.; Moody, P. C. *J. Mol. Biol.* **2001,** *310*, 433–447. b) Khan, H.; Barna, T.; Bruce, N. C.; Munro, A. W.; Leys, D.; Scrutton, N. S. *FEBS J.* **2005,** *272*, 4660 –4671.
[28] Toogood, H. S.; Gardiner, J. M.; Scrutton, N. S. *ChemCatChem* **2010**, *2*, 892 – 914.
[29] Barna, T. M.; Khan, H.; Bruce, N. C.; Barsukov, I.; Scrutton, N. S.; Moody, P. C. *J. Mol. Biol.* **2001,** *310*, 433–447.
[30] Breithaupt, C.; Strassner, J.; Breitinger, U.; Huber, R.; Macheroux, P.; Schaller, A.; Clausen, T. *Structure* **2001,** *9*, 419– 429.
[31] Kitzing, K.; Fitzpatrick, T. B.; Wilken, C.; Sawa, J.; Bourenkov, G. P.; Macheroux, P.; Clausen, T. *J. Biol. Chem.* **2005,** *280*, 27904 – 27913.

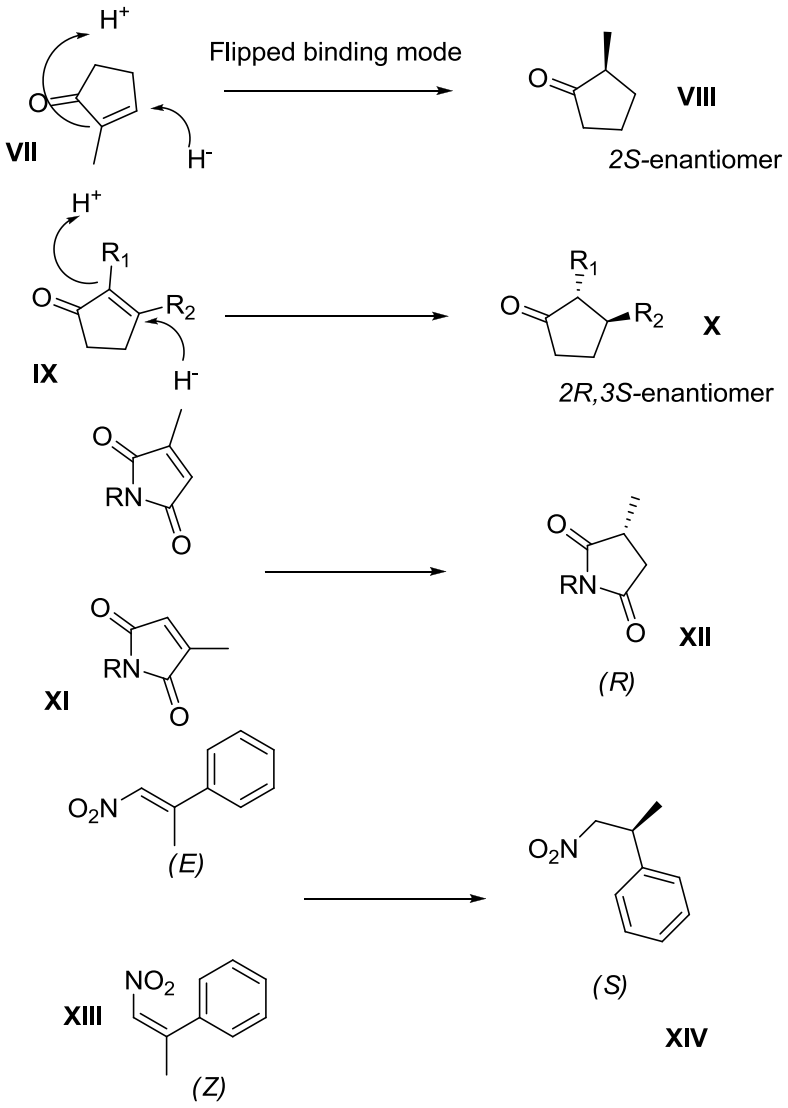


Figure 1.6 Model of the postulated 'normal' *vs* 'active flipped' binding mode of three classes of alkene substrates to PETN reductase and the prediction of product enantiospecificity. Based on the binding of CH to PETN reductase.[32]

Models of 'normal' substrate binding of α,β-unsaturated substituted cyclic ketones with 5- and 6-membered rings to OYEs predict the formation of either a (*2R*)- and/or (*3S*)-enantiomeric product, depending on the position of the substituent. These models are based on the crystal structures of CH- and 1-nitrocyclohex-1-ene-bound PETN reductase, which indicates the most favourable substrate overlap with Cβ-FMN N5 indicative of an 'active' conformation. The

[32] Khan, H., Harris, R. J.; Barna, T.; Craig, D. H.; Bruce, N. C.; Munro, A.W.; Moody, P. C.; Scrutton, N. S. *J. Biol. Chem.* **2002,** *277,* 21906 – 21912.

models are further supported by biocatalysis studies that showed the generation of (*R*)-2-methyl-cyclohexanone, (*S*)-3-methyl-cyclohexanone, and (*S*)-3-methyl-cyclopentanone from their corresponding alkene precursors by many OYEs at high enantiopurity. However, the reduction of 2-methyl-cyclopenten-1-one (**VII**) was found consistently to produce the (*S*)-enantiomeric product (**VIII**) with inferior ee's, indicating substrate binding mainly in an 'active flipped' binding mode.[33] Interestingly, the bioreduction of *N*-phenyl-2-methyl maleimide (**XI**) by PETN reductase was investigated by ^{1}H NMR of reduced products in the presence of either (*R*)-[4-2H]-NADPH or deuterated solvent and indicated that the substrate binds to the enzyme in two 'active' conformations in an approximate 60:40 ratio.[34] Both binding conformations lead to the formation of the (*R*)-enantiomeric product (**XII**) with high enantiopurities due to the almost symmetrical nature of the substrate.[35] A similar situation is predicted for the reaction with another di-ketone cyclic alkene ketoisophorone, although the low product enantiopurities often obtained has been shown to be due to water-mediated product racemization.

The bioreduction of a variety of α- or β-alkyl-β-arylnitroalkenes by OYEs typically generates the equivalent (*R*)- and (*S*)-enantiomeric products, respectively.[36] This reaction is enantioconvergent, as the reduction of both (*E*)- and (*Z*)-stereoisomers of the substrates produces the same enantiomeric product. This is in contrast to the reduction of α,β-unsaturated dicarboxylic acids by OYEs (OPR1, OPR3, and YqjM) in which the enantiomeric product obtained was dependent on the combination of the enantiopreference of the enzyme and the stereoisomer of the substrate (enzyme- and substrate-based stereocontrol).[37] Typically both α-alkyl-β-arylnitroalkenes isomers and the (*E*)-isomeric α-alkyl-β-arylnitroalkenes gave less enantiomerically pure products, indicating the multiple 'active' binding conformations.[38]

---

[33] a) Corpet, F. *Nucleic Acids Res.* **1988,** *16*, 1088–10890. b) Gramatica, P.; Manitto, P.; Poli, L. *J. Org. Chem.* **1985,** *50*, 4625–4628.
[34] Fryszkowska, A.; Toogood, H. S.; Sakuma, M.; Gardiner, J. M.; Stephens, G. M.; Scrutton, N. S. *Adv. Synth. Catal.* **2009,** *351*, 2976–2990.
[35] a) Hall, M.; Stueckler, C.; Kroutil, W.; Macheroux, P.; Faber, K. *Angew. Chem.* **2007**, *119*, 4008–4011. b) *Angew. Chem. Int. Ed.* **2007,** *46*, 3934 –3937. c) Mueller, N. J.; Stueckler, C.; Hauer, B.; Baudendistel, N.; Housden, H.; Bruce, N. C.; Faber, K. *Adv. Synth. Catal.* **2010,** *352*, 387– 394.
[36] Fryszkowska, A.; Fisher, K.; Gardiner, J. M.; Stephens, G. M. *J. Org. Chem.* **2008,** *73*, 4295–4298.
[37] Stueckler, C.; Hall, M.; Ehammer, H.; Pointner, E.; Kroutil, W.; Macheroux, P.; Faber K. *Org. Lett.* **2007,** *9*, 5409– 5411.
[38] Fryszkowska, A.; Toogood, H. S.; Sakuma, M.; Gardiner, J. M.; Stephens, G. M.; Scrutton, N. S. *Adv. Synth. Catal.* **2009**, *351*, 2976 –2990.

1.7 Cofactor Recycling System

An external cofactor NAD(P)H for enzyme reduction is one of the problems of biocatalytic versus synthetic hydrogenation of activated alkenes. One way of avoiding this problem is the use of whole (fermenting) cells which include all the necessary cofactor recycling systems.[20] Whole cells have been used in the asymmetric bioreductions, primarily with Baker's yeast, which have been successful in the formation of products with high enantiopurities.[39] However, there are some problems with the whole cell biotransformations. Firstly, some of the organisms used for whole cell biotransformations are oxygen sensitive (e.g. *Clostridia*, *Proteus* and *Acetobacterium* species),[37] which made the growth and handling of the organisms difficult due to current limitations in the industrial- scale growth of anaerobes. Secondly, in addition, there was often a competing reaction of reduction of the activating carbonyl group to the corresponding alcohol due to the presence of alcohol dehydrogenases (Figure 1.5).[40] In this example of whole cell biotransformations of (E/Z)-citral (**XV**), using organisms such as *Candida parapsilosis* DSM 70125 and *Pichia angusta* DSM 70277[40], the relative product yields of (*R*)-citronellal (**XVIII**) and (*R*)-citronellol (**XXI**) was dependent on the relative activities of the competing enoate reductases and *prim*-alcohol dehydrogenase(s). There are some further competing reactions found in some whole cell biotransformations included citral lyase (loss of acetaldehyde to form sulcatone (**XVII**)) and the oxidation of the aldehyde to produce geranic or neric acid (**XVI**)[40]. The enzyme(s) responsible for the reduction of nerol and geranol (**XIX**) to citronellol (**XXI**) have not been identified yet. As alcohols are not typical activating groups for OYEs, a different enzyme class may be responsible for this enoate reductase activity.

---

[39] a) Fronza, G.; Fuganti, C.; Mendozza, M.; Rigoni, R.; Servia, S.; Zucchi, G. *Pure Appl. Chem.* **1996,** *68*, 2065–2071. b) Takabe, K.; Hiyoshi, H.; Sawada, H.; Tanaka, M.; Miyazaki, A.; Yamada, T.; Katagiri, T.; Yoda, H. *Tetrahedron: Asymmetry* **1992,** *3*, 1399 – 1400. c) Gramatica, P.; Manitto, P.; Monti, D.; Speranza, G. *Tetrahedron* **1987,** *43*, 4481–4486.

[40] Hall, M.; Hauer, B.; Stuermer, R.; Kroutil, W.; Faber, K. *Tetrahedron: Asymmetry* **2006,** *17*, 3058–3062.

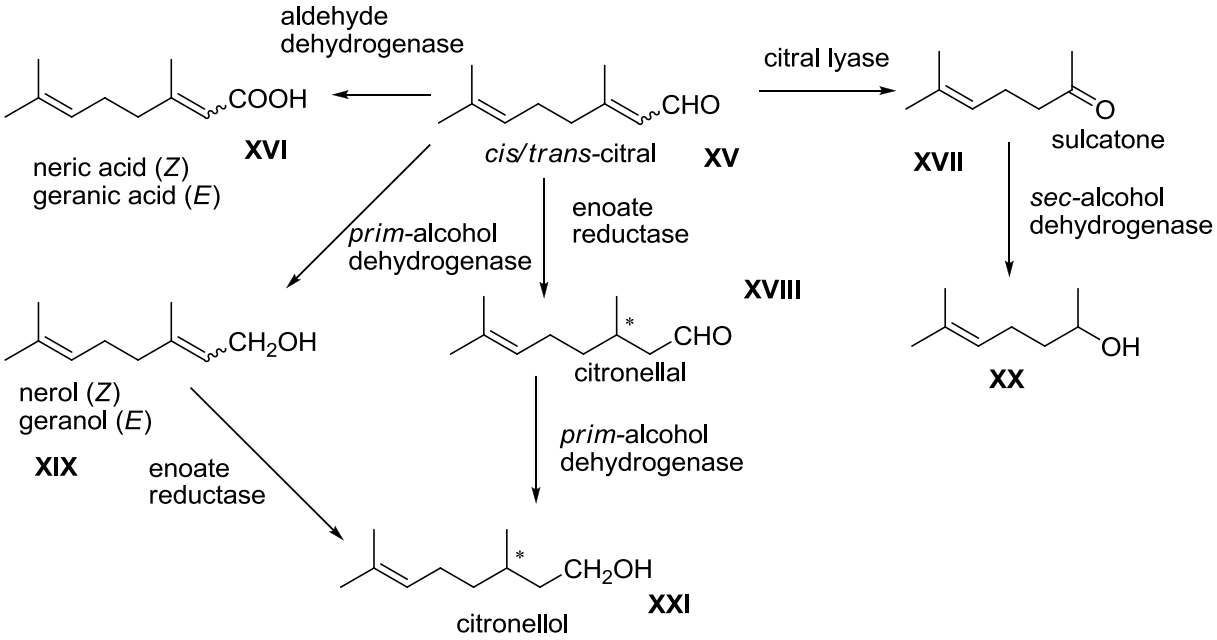


Figure 1.7 Multiple microbial biotransformation reactions of (cis/trans) citral

Whole cell biotransformations (having internal cofactor recycling system) are limited due to poor product yields mainly caused by side reactions such as carbonyl group reduction, ester hydrolysis, acyloin reactions, and acetyl cleavage.[41] In addition, due to the presence of more than one OYE-like enzyme, low ee values are sometimes obtained with different enantiopreferences.[42] To overcome these problems, purified enzymes are used, which require the presence of either large quantities of NAD(P)H or a suitable NAD(P)H recycling system. A range of cofactor recycling systems have been used in the biotransformations of purified OYEs to overcome the expensive large-scale usage of NAD(P)H. In cofactor recycling systems, only a comparatively small quantity of NAD(P)+ is required and the reductive substrate for the enzyme is often cheap. Such as, glucose-6-phosphate dehydrogenase (G6PDH) catalyzes the oxidation of $_D$-glucose-6-phosphate to $_D$-glucono-1,5-lactone-6-phosphate with the simultaneous reduction of NADP+.[43] For OYEs this could be seen as a favored cofactor recycling system, as NADPH is prefered over NADH. However, the presence of large quantities of $_D$-glucose-6-phosphate and $_D$-glucono-1,5-lactone-6-phosphate may decrease the pH of the reaction which affects rate of

[41] a) Fuganti, C.; Grasselli, P.; Servi, S.; Hoegberg, H. E. *J. Chem. Soc., Perkin Trans. 1* **1988,** *12*, 3061–3065. b) Leuenberger, H. G. W.; Boguth, W.; Barner, R.; Schmid, M.; Zell, R. *Helv. Chim. Acta* **1979,** *62*, 455–463.
[42] Hirata, T.; Shimoda, K.; Gondai, T. *Chem. Lett.* **2000,** *29*, 850–851.
[43] Fryszkowska, A.; Toogood, H. S., Sakuma, M.; Gardiner, J. M.; Stephens, G. M.; Scrutton, N. S. *Adv. Synth. Catal.* **2009**, *351*, 2976–2990.

both the OYE-catalyzed reactions and the cofactor recycling enzyme. Alternatively, NAD+ cofactor recycling systems are used in OYE biotransformations such as glucose dehydrogenase[44] (GDH) and formate dehydrogenase[37] (FDH) which oxidize glucose and formate, respectively. Reduction of a variety of α,β-unsaturated carbonyl compounds by OYEs in the presence of NAD(P)H or various cofactor recycling systems has suggested that the source of reducing counterparts can affect the enantiopurity, switch the enantiopreference of the product, influence yields or even increase the probability of catalyzing side reactions.[45] For example, the reduction of ketoisophorone catalyzed with PETN reductase experienced an improvement of ee by 36% when changing from NADPH to the NADP+/G6PDH recycling system. Similarly, the ee of the OPR3-catalyzed reduction of ketoisophorone was enhanced from 33–55% to 99% when switching from NAD(P)H or NAD+/FDH to the NADP+/G6PDH recycling system.[46] In contrast, OPR1-catalyzed reduction of citral caused a remarkable loss in yield from >99% to 20%, when switched from NAD(P)H to either of the three recycling systems. In the case of NAD+/FDH recycling system, the established reaction was the reduction of the carbonyl group to give the subsequent allylic alcohol (>95% yield), which was not noticed considerably in the reactions with NAD(P)H or the other two recycling systems. Reduction of citral with OYE1 in the presence of NAD(P)H and the NADP+/G6PDH recycling system produced the (*S*)-product with low ee values. While the reaction with the NAD+/GDH recycling system yielded the (*R*)-enantiomer with a considerably high ee value. Above examples showed the importance of changing cofactor recycling system when investigating the quantity and quality of the products in scale-up biotransformations. In some biotransformations artificial electron donors, such as reduced methyl viologen, can be used an alternative to NAD(P)H.[47] They are less expensive as compare to NAD(P)H and enzymatic reductions can be carried out in electrochemical cells. However, methyl viologen (paraquat) is extremely toxic, which makes it not acceptable for use in pharmaceutical syntheses. In addition, due to the direct reduction of variety of α,β-unsaturated

---

[44] Mueller, N. J.; Stueckler, C.; Hauer, B.; Baudendistel, N.; Housden, H.; Bruce, N. C.; Faber, K. *Adv. Synth. Catal.* **2010,** *352*, 387–394.
[45] Fryszkowska, A.; Toogood, H. S.; Sakuma, M.; Gardiner, J. M.; Stephens, G. M.; Scrutton, N. S. *Adv. Synth. Catal.* **2009**, *351*, 2976–2990.
[46] a) Hall, M.; Stueckler, C.; Kroutil, W.; Macheroux, P.; Faber, K. *Angew. Chem.* **2007,** *119*, 4008–4011. b) *Angew. Chem. Int. Ed.* **2007,** *46*, 3934–3937.
[47] a) Bader, J.; Simon, H. *Microbiol.* **1980,** *127*, 279–287. b) Simon, H.; Bader, J.; Gunther, H., Neumann, S., Thanos, J. *Angew. Chem.* **1985**, *97*, 541–555. c) *Angew. Chem. Int. Ed. Engl.* **1985,** *24*, 539–553.

aldehydes to form racemic products, the use of methyl viologen for the biotransformations is limited.

There are a number of OYEs which catalyze the disproportionation reaction in which activated alkenes can act as both the reducing and oxidizing substrates.[48] For example, oxidized OYE1 catalyzes the aromatisation of 3-oxo-decalin-4-ene-10-carboxaldehyde (ODEC) to 3-hydroxy-6,7,8,9-tetrahydronaphthalene by stereospecific trans-dehydrogenation with the subsequent reduction of FMN. The second reaction proceeds as a typical OYE-like reduction of a second molecule of ODEC. In an equivalent reaction, EBP1 disproportionates 2-cyclohexenone to phenol and cyclohexanone in the absence of NADPH.[49] The presence of the phenol was indicated by the characteristic broad, low intensity, long wavelength charge-transfer absorbance band seen in the binding of phenol to the enzyme. Recent studies have shown that OYEs YqjM, OYE2, and (to a minor extent) OYE3 and *Zymomonas* NAD(P)H-dependent 2-cyclohexen-1-one (NCR)-reductase are competent of disproportionating 2-cyclohexenone.[50] Using either 3-methylcyclohexen-1-one or cyclohexane-1,4-dione as the sacrificial hydrogen donors asymmetric bioreductions of a number of activated alkenes were achieved. This simplified approach has advantages over both nicotinamide-dependent and other nicotinamide-independent systems; however, careful selection of the co-substrate is required to make certain the oxidative substrate does not participate in the reductive reaction resulting in a loss of the preferred product.

Using free FMN, EDTA, and white light under aerobic conditions as the source of reducing equivalents has bee recently described in the asymmetric bioreduction of ketoisophorone to (*R*)-levodione by YqjM.[51] The reaction continued rapidly with quantitative yields, but somewhat less enantioselectivity as compare to reductions in the presence of GDH/NAD+ (88 vs 92% ee). This loss in the enantioselectivity of product was due to a background reaction in which reduced flavin, directly reduced the substrate to produce a racemic product in the absence of YqjM.

---

[48] a) Stueckler, C.; Reiter, T. C.; Baudendistel, N.; Faber, K. *Tetrahedron,* **2010,** *66*, 663–667. b) Vaz, A. D.; Chakraborty, S.; Massey, V. *Biochemistry*, **1995,** *34*, 4246–4256.
[49] Buckman, J.; Miller, S. M. *Biochemistry*, **1998,** *37*, 14326–14336.
[50] Stueckler, C.; Reiter, T. C.; Baudendistel, N.; Faber, K. *Tetrahedron,* **2010,** *66*, 663–667.
[51] Taglieber, A.; Schulz, F.; Hollmann, F.; Rusek, M.; Reetz, M. T. *ChemBio- Chem*, **2008,** *9*, 565–572.

1.8 Old Yellow Enzyme (OYE)

Old yellow enzymes OYEs are very important in biotransformations, giving the enzyme a significant place in the history of enzymology. Warburg & Christian[52] isolated the OYE from brewers' bottom yeast during efforts to explicate the nature of biological oxidations. Glucose 6-phosphate was oxidized by methylene blue in the presence of two components of erythrocytes, an enzyme, and a small heat-stable `Coferment'. Following this, Warburg identified a yellow enzyme that allowed the system to form a complete respiratory chain reacting with molecular oxygen. Following the isolation of a second, `new', yellow enzyme from yeast by Haas[53], Warburg's enzyme was called `Old Yellow Enzyme', a name that has continued to this day. In 1935 Theorell purified the OYE; it consists of a colorless apoprotein and a yellow dye, both necessary for enzyme activity. The purification of enzyme assisted to identify the vital role of this protein in enzyme catalysis. The yellow dye was analogous in nature to vitamin B_2 (riboflavin). Thus OYE provided the first biochemical role for a vitamin. Theorell[54] confirmed that the yellow cofactor was riboflavin-5'-phosphate, now also termed flavin mono-nucleotide (FMN). Since then, OYE has been characterized in great detail by Massey, and a considerable amount is now known about the mechanism of the enzyme. NADPH is the physiological reductant of OYE. Methylene blue, Fe^{3+}, quinones, cytochrome c[55] and ferricyanide are capable of reoxidizing OYE. Molecular oxygen can help in the reoxidation to yield hydrogen peroxide and superoxide; however, this reaction is very appealing, as the rate observed is significantly lower than that observed with oxidase enzymes, and is lower than obtained with substrates such as quinones. Many different potential ligands have been screened in an attempt to identify possible physiological substrates.[56]

OYE from *Saccharomyces carlsbergensis* was the first flavin-containing enzyme to be identified[57] and is the important example of this rapidly growing family. Now new members are

---

[52] Warburg, O.; Christian, W. *Naturwissenschaften,* **1932**, *20,* 688-696.
[53] Haas, E. *Biochem,* **1938**, *298,* 378-390.
[54] Theorell, H. **1955.** Nobel Prize Lecture (http://nobelprize.org/nobel_prizes/medicine/laureates/1955/theorell-lecture.pdf).
[55] Massey, V.; Strickland, S.; Mayhew, S. G.; Howell, L. G.; Engel, P. C.; Matthews, R. G.; Schuman, M.; Sullivan, P. A**.** *Biochem Biophys Res Commun* **, 1969**. *36,* 891-897.
[56] a) Schopfer, L. M.; Massey, V. Old Yellow Enzyme. In *A Study of Enzymes*, **1991.** 247-269. Edited by S. A. Kuby. Cleveland, OH: CRC Press. **b)** Vaz, A. N.; Chakraborty, S.; Massey, V. *Biochemistry*, **1995,** *34,* 4246-4256.
[57] Warburg, O.; Christian, W. *Biochem. Z.* **1933,** *263,* 228–229.

mainly being discovered through genome sequencing projects. Some members of the OYE family are related somewhat distantly to OYE, which includes the oestrogen-binding protein of *Candida albicans*[58], the bile-acid-inducible flavoenzymes BaiH and BaiC[59], and trimethylamine dehydrogenase.[60] More narrowly correlated enzymes have been characterized in other yeasts, Gram-positive and Gram-negative bacteria, monocotyledonous and dicotyledonous plants, *Caenorhabditis elegans* and *Trypanosoma cruzi*[61]. Bacterial homologues that have been described so far include PETN (pentaerythritol tetranitrate) reductase[62], GTN (glycerol trinitrate) reductase[63], MR (morphinone reductase)[64], 2-cyclohexenone reductase[65], the xenobiotic reductases A and B from *Pseudomonas* sp.[66] and NEM (*N*-ethylmaleimide) reductase[67]. More recently, YqjM from *Bacillus subtilis*, was studied[68], and this was the first OYE homologue from a Gram-positive bacterium.

The old yellow enzyme (OYE) family is capable of catalyzing the reduction of many commercially valuable substrates. These oxidoreductases contain flavin mononucleotide (FMN), and NAD(P)H dependent are known to catalyze the bioreduction of α,β-unsaturated ketones, aldehydes, carboxylic acids, carboxylic esters, nitroalkenes, and derivatives, giving products with a diverse biotechnological and pharmaceutical applications.[69] These enzymes can also

---

[58] Madani, N. D.; Malloy, P. J.; Rodriguez-Pombo, P.; Krishnan, A. V.;Feldman, D. *Proc. Natl. Acad. Sci. U.S.A.* **1994,** *91*, 922–926.
[59] Terez, B.; Hanan, L. M.; Carlo, P.; Neil, C. B.; Nigel, S. S.; Peter, C.; Moody, E. *The Journal of Biological Chemistry.* **2002,***277*, 30976-30983.
[60] Lim, L.W.; Shamala, N.; Mathews, F.S.; Steenkamp, D.J.; Hamlin, R.; Xuong, N.H. *J. Biol. Chem.***1986**, *261*, 15140-15146.
[61] Stott, K.; Saito, K.; Thiele, D. J.; Massey, V. *J. Biol. Chem*. **1993,** *268*, 6097–6106.
[62] French, C. E.; Nicklin, S.; Bruce, N. C. *J. Bacteriol.* **1996,** *178*, 6623–6627.
[63] Snape, J. R.; Walkley, N. A.; Morby, A. P.; Nicklin, S.; White, G. F. *J. Bacteriol.* **1997,** *179*, 7796–7802.
[64] French, C. E.; Bruce, N. C. *Biochem. J.* **1995,** *312*, 671–678.
[65] Rohde, B. H.; Schmid, R.; Ullrich, M. S. *J. Bacteriol.* **1999,** *181*, 814–822.
[66] Blehert, D. S.; Fox, B. G.; Chambliss, G. H. *J. Bacteriol.* **1999,** *181*, 6254–6263.
[67] Miura, K.; Tomioka, Y.; Suzuki, H.; Yonezawa, M.; Hishinuma, T.; Mizugaki, M. *Biol. Pharm. Bull.* **1997,** *20*, 110–112.
[68] Fitzpatrick, T. B.; Amrhein, N.; Macheroux, P. *J. Biol. Chem.* **2003** *278*, 19891–19897.
[69] a) Toogood, H. S.; Fryszkowska, A.; Hare, V.; Fisher, K.; Roujeinikova, A.; Leys, D.; Gardiner, J. M.; Stephens, G. M.; Scrutton, N. S. *Adv. Synth. Catal.* **2008**, *350*, 2789 –2803. b) Chaparro-Riggers, J. F. Rogers, T. A.; Vazquez-Figueroa, E.; Polizzi, K. M.; Bommarius, A. S. *Adv. Synth. Catal.* **2007**, 349, 1521 –1531. c) Swiderska, M. A.; Stewart, J. D. *Org. Lett*. **2006**, *8*, 6131 – 6133. d) Hall, M.; Stueckler, C.; Kroutil, W.; Macheroux, P.; Faber, K. *Angew. Chem*. **2007**, *119*, 4008 –4011; *Angew. Chem. Int. Ed*. **2007**, *46*, 3934 –3937.

reduce nitrate esters, nitroglycerin, nitroaromatic explosives and cyclic triazines, and have been used in the phytoremediation of explosive-contaminated soils.[70]

The OYE family of enzymes has been examined comprehensively for its biocatalytic potential in improving well-known synthetic processes such as reduction of β-nitroacrylates by OYE1, which was used in the synthesis of chiral β^2-amino acids.[28] In addition, recognized OYEs from a *Citrobacter freundii* and *Zymomonas mobilis* were revealed to produce *(R)*- and *(S)*-citronellal, the latter of which is an essential intermediate in the production of l-menthol in the Takasago process.[71] OYEs, such as YqjM, usually catalyze the reduction of ketoisophorone to obtain the *(R)*-enantiomeric product; a necessary intermediate in the synthesis of carotinoid.[72] Morphinone reductase (MR) catalyzes the first step in the degradation of morphine and codeine, making it potentially valuable in the manufacture of semi-synthetic opiate drugs.[73]

The dawn of automated protein sequence alignments, combined with the more recent development into whole genome sequencing, has led to an apparent increase in the identification of possible OYE family members across kingdoms. Some of these genes code for uncharacterized proteins, while others were originally isolated and characterized as different types of enzymes. 12-Oxophytodienoate reductases OPR1-3 orginates from the *S. lycopersicum* (tomato plant). Enzymes such as estrogenbinding protein EBP1 (*Candida albicans*),[74] xenobiotic reductase (XenA; *Pseudomonas putida*),[75] *cis*-enoyl-CoA reductase (later renamed N-ethylmaleimide reductase; *Escherichia coli*),[76] and recently a thermostable chromate reductase (*Thermus scotoductus* SA-01)[77] were recognized as OYE like proteins after biochemical, sequence, and/or structural studies. Here, we designate enzymes as OYE homologues if they show significant amino acid sequence and/or structural homology to known

---

[70] Khan, H.; Harris, R. J.; Barna, T.; Craig, D. H.; Bruce, N. C.; Munro, A. W.; Moody, P. C.; Scrutton, N. S. *J. Biol. Chem.* **2002,** *277*, 21906 – 21912.
[71] a) Muller, A.; Hauer, B.; Rosche, B. *J. Mol. Catal. B,* **2006,** *38*, 126 –130. b) Muller, A.; Hauer, B.; Rosche, B. *Biotechnol. Bioeng*. **2007,** *98*, 22 –29
[72] Leuenberger, H. G. W.; Boguth, W.; Widmer, E.; Zell, R. *Helv. Chim. Acta* **1976,** *59*, 1832 –1849
[73] Craig, D. H.; Moody, P. C.; Bruce, N. C.; Scrutton, N. S. *Biochemistry* **1998,** *37*, 7598–7607
[74] Buckman, J.; Miller, S. M. *Biochemistry.* **1998,** *6*, 37, 14326-36.
[75] Blehert, D. S.; Fox, B. G.; Chambliss, G. H. *J. Bacteriol*. **1999,** *181*, 6254–6263.
[76] a) Miura, K.; Tomioka, Y.; Suzuki, H.; Yonezawa, M.; Hishinuma, T.; Mizugaki, M. *Biol. Pharm. Bull.* **1997,** *20*, 110–112. b) Mizugaki, M.; Unuma, T.; Yamanaka, H. *Chem. Pharm. Bull.* **1979,** *27*, 2334 – 2337. Vaz, A. D.;Chakraborty, S.; Massey, V. *Biochemistry* **1995,** *34*, 4246 –4256.
[77] a) Opperman, D. J.: Piater, L. A.; Heerden, E. *J. Bacteriol.* **2008,** *190*, 3076 –3082. b) Opperman, D. J.; Sewell, B. T.; Litthauer, D.; Isupov, M. N.; Littlechild, J. A.; van Heerden, E. *Biochem. Biophys. Res. Commun*. **2010,** *393*, 426 –431.

OYE members (e.g., OYE1) and also catalyze the NAD(P)H-dependent reduction of activated carbon carbon double bond substrates. A summary of substrate profiles of isolated OYE-like family members is given in Table1.1.

Organism	Ene-Reductase	Substrate Profile
S. pastorianus	OYE1[78,79,23,49,61,69,93]	Substituted and nonsubstituted α,β-unsaturated aldehydes, ketones, imides, nitroalkenes, carboxylic acids, and esters, cyclic and acyclic enones, nitrate esters, nitroaromatics (TNT), terpenoids, N-ethyl maleimide and codeinone.
S. cerevisiae	OYE2, OYE3, Lot6P[80,81,82,8384]	Substituted and nonsubstituted α,β -unsaturated cyclic and acyclic aldehydes, ketones, imides, carboxylic acids, esters,terpenoids, CH, ketoisophorone and thioanisole.
S. lycopersicum	Tomato, LeOPR-1, LeOPR3[81,35,37]	α,β-unsaturated aldehydes, ketones, maleimides, nitroalkenes, dicarboxylates, dimethyl and 12-oxophytodienoic acid (OPDA)and citral.
E. cloacae	PETN reductase[24,38,21,79,85,86]	β,β- and α,β-disubstituted nitroalkenes, substituted cyclic α,β-unsaturated cyclic and acyclic aldehydes and ketones, terpenoids, aromatic and aliphatic explosives, codeinone.
B. subtilis	YqjM [87, 37,51,69]	α,β-unsaturated aldehydes, ketones, maleimides and nitroalkenes, dicarboxylic acids, dimethyl esters and explosives.
T. pseude Thanolicus	TOYE[81]	α,β-unsaturated aldehydes, ketones and N-substituted maleimides.
T. scotoductus SA-01	CrS[77]	CH, 3-methyl cyclohexen-1-one, ketoisophorone, cinnamaldehyde, and terpenoids
E. coli	NEM reductase[79,35,86]	α,β-unsaturated aldehydes, ketones and, explosives and codeinone.
P. putida M10	MR[79,35,88]	α,β-unsaturated aldehydes, ketones and nitroalkenes explosives and codeinone.

---

[78] Meah, Y.;Massey, V. *Proc. Natl. Acad. Sci. USA*, **2000**, *97*, 10733 – 10738.
[79] Williams, R. E.; Rathbone, D. A.; Scrutton, N. S.; Bruce, N. C. *Appl. Environ. Microbiol.* **2004**, *70*, 3566 –3574.
[80] Wada, M.; Yoshizumi, A.; Noda, Y.; Kataoka, M.; Shimizu, S.; Takagi, H.; Nakamori, S. *Appl. Environ. Microbiol.* **2003**, *69*, 933 –937.
[81] a) Muller, R.; Sturmer, B.; Hauer, B.; Rosche. *Angew. Chem*. **2007,** *119*, 3380–3382. b) *Angew. Chem. Int. Ed.* **2007,** *46*, 3316 –3318. c) Adalbjçrnsson, B. V.; Toogood, H. S.; Fryszkowska, A.; Pudney, C. R.; Jowitt, T. A.; Leys, D.; Scrutton, N. S. *ChemBioChem.* **2010,** *11*, 197–207.
[82] Niino, Y. S.; Chakraborty, S.; Brown, B. J.; Massey, V. *J. Biol. Chem*. **1995**, *270*, 1983 –1991.
[83] Mueller, N. J.; Stueckler, C.; Hall, M.; Macheroux, P.; Faber, K. *Org. Biomol. Chem.* **2009**, *7*, 1115– 1119
[84] Strassner, J.; Furholz, A.; Macheroux, P.; Amrhein, N.; Schaller, A.; *J. Biol. Chem.* **1999,** *274*, 35067–35073.
[85] Khan, H.; Barna, T.; Harris, R. J.; Bruce, N. C.; Barsukov, I.; Munro, A. W.; Moody, P. C.; Scrutton, N. S. *J. Biol. Chem*. **2004**, *279*, 30563 – 30572.
[86] a) French, C. E.; Nicklin, S.; Bruce, N. C. *J. Bacteriol.* **1996,** *178*, 6623–6627. b) Miseviciene, L.; Anusevicius, Z.; Sarlauskas, J.; Harris, R. J.; Scrutton, N. S.; Cenas, N. *Acta Biochim. Pol.* **2007**, *54*, 379 –385. c) Nivinskas, H.; Sarlauskas, J.; Anusevicius, Z.; Toogood, H. S.; Scrutton, N. S.; Cenas, N.; *FEBS J.* **2008**, *275*, 6192– 6203.d) Dillewijn, P. V.; Wittich, R. M.; Caballero, A.; Ramos, J. L. *Appl. Environ. Microbiol.* **2008**, *74*, 6820 – 6823.
[87] Hall, M.; Stueckler, C.; Ehammer, H.; Pointner, E.; Oberdorfer, G.; Gruber, K.; Hauer, B.; Stuermer, R.; Kroutil, W.; Macheroux, P.; Faber, K. *Adv. Synth. Catal.* **2008,** *350*, 411–418.
[88] a) Messiha, H. L.; Bruce, N. C.; Sattelle, B. M.; Sutcliffe, M. J.; Munro, A. W.; Scrutton, N. S. *J. Biol. Chem*. **2005**, *280*, 27103– 27110. b) Bougioukou, D. J.; Kille, S.; Taglieber, A.; Reetz, M. T. *Adv. Synth. Catal.* **2009**, *351*, 3287–3305.

Arabidopsis thaliana	OPR1-2, AtOPR3[89]	9S-13S-, 9R-13R-OPDA,CH and OPDA.
C. albicans st. 22	EBP1[35,49]	α,β-unsaturated aldehydes, ketones, nitriles, and carboxylic acids
Gluconobacter oxydans 621H	Gox2107/ 0502/2684[90]	α,β-unsaturated ketones, terpenoids.
K. lactis ATCC 8787	KYE1 (ER)[69]	Substituted and nonsubstituted α,β-unsaturated aldehydes, ketones, maleimides, maleic acid derivatives, estersand terpenoids.
P. putida II-B	XenA[69,66]	Substituted and nonsubstituted α,β-unsaturated aldehydes, ketones, maleimides, maleic acid derivatives, esters and terpenoids
S. oneidensis	SYE1, SYE3, SYE4[91]	α,β-unsaturated aldehydes, N-ethylmaleimide, CH and nitroglycerin.N ethylmaleimide, CH, acrolein and nitroglycerin.
Yersinia bercovieri	Yers-ER[69]	Substituted and nonsubstituted α,β-unsaturated aldehydes, ketones, maleimides, maleic acid derivatives, esters and terpenoids
commercially available ERs	ERED101-114[92]	α,β-unsaturated nitriles and 2,3-disubstituted cyclopenten-3-ones.

Table 1.1 Substrate profiles of OYE-like family members

1.9 *Shewanella* Yellow Enzyme (SYE)

Recently, old yellow enzyme (OYE) from *Saccharomyces carlsbergensis* was re-discovered for the chemo- and stereoselective conversion of enones and nitroolefins into saturated compounds.[93] Homologs of this enzyme from *Shewanella oneidensis* (SYEs) were shown to be catalytically active when recombinantly expressed as GST fusion proteins. *Shewanella oneidensis* is an important model organism in bioremediation studies because it is characterized by unique respiratory capabilities, such as the possibility to reduce heavy metals[94]. The bacterium *S. oneidensis* is exceptional in encoding proteins with high sequence similarity to

---

[89] a) Schaller, F.; Weiler, E. W. *J. Biol. Chem*. **1997**, *272*, 28066 –28072. b) Costa, C. L.; Arruda, P.; Benedetti, C. E. *Plant Mol. Biol.* **2000**, *44*, 61– 71.
[90] a) Schweiger, P.; Gross, H.; Wesener, S.; Deppenmeier, U. *Appl. Microbiol. Biotechnol.* **2008**, *80*, 995– 1006. b) Yin, B.; Yang, X.; Wei, G.; Ma, Y.; Wei, D. *Mol. Biotechnol*. **2008**, *38*, 241 – 245.
[91] a) Brige, A.; van den Hemel, D.; Carpentier, W.; de Smet, L.; van Beeumen, J.J. *Biochem. J.* **2006**, *394*, 335-344. b) van den Hemel, D., Brige, A.; Savvides, S. N.; Beeumen, J. V. *J. Biol.Chem*. **2006**, *281*, 28152–28161.
[92] Kosjek, B.; Fleitz, F. J.; Dormer, P. G.; Kuethe, J. T.; Devine, P. N. *Tetrahedron:Asymmetry*, **2008**, *19*, 1403-1406.
[93]a) Swiderska, M.A.; Stewart, J.D. *J. Mol. Catal. B: Enzym.* **2006**, *42*, 52-54. b) Swiderska, M.A.; Stewart, J.D. *Org. Lett.* **2006**, *8*, 6131-6133.
[94]Heidelberg, J. F.; Paulsen, I. T.; Nelson, K. E.; Gaidos, E. J.; Nelson, W. C.; Read, T. D.; Eisen, J. A.; Seshadri, R.; Ward, N.; Methe, B. et al. *Nature Biotech*. **2002,** *20*, 1118–1123.

OYE, yet the mutual identities between these enzymes (38–52%) are significantly lower compared with the identities beween the different OYE isoforms in yeasts and plants (69–91%).

Van Beeumen et al.[91] studied the characterization and expression analysis of *Shewanella* Yellow Enzyme. Probing the *Shewanella oneidensis* MR-1 genome for OYE-family members led to the identification of four homologues (NP718044, NP718043, NP719682 and NP718946), which were termed SYE1–4. The four proteins show high sequence similarities to a wide variety of members belonging to the OYE family of α,β-barrel flavoprotein oxidoreductases. The highest percentages of identity and similarity were found (from SYE1 to SYE4) with *Vibrio parahaemolyticus* NEM reductase 2 (79% identity and 88% similarity), *Photobacterium profundum* SS9 NEM reductase (71% identity and 85% similarity), *Vibrio cholerae* NEM reductase (72% identity and 82% similarity) and *P. profundum* SS9 NADH-dependent flavin oxidoreductase (78% identity and 87% similarity)[91]. In *vivo* analysis showed that only SYE4 is induced under conditions of eminent oxidative stress, while in *vitro* characterization established prominent differences in ligand binding, catalytic efficiency, and substrate specificity between SYE4 and the other SYE homologues.

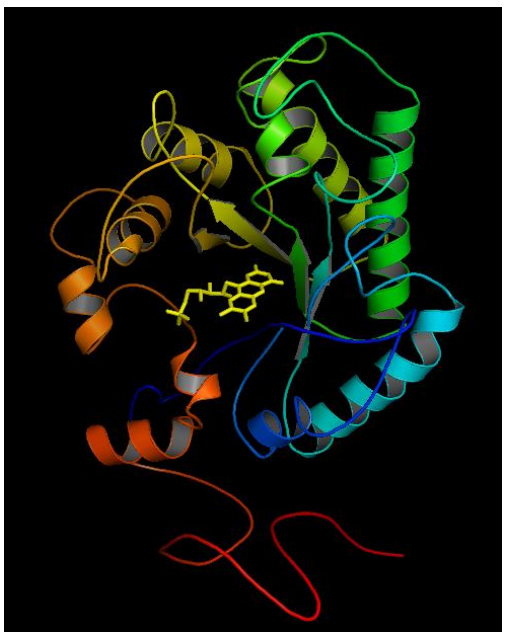


Figure1.8 Homology approximation of SYE-4 based on 1Z41. Estimated by Deep View/Swiss-Pdb Viewer 3.7 and viewed in PyMOL[95]

[95] The PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC.

In general, the residues that make side-chain interactions with FMN are strictly conserved within the OYE family, whereas the residues that contact FMN by their main chain are not, although their Cα positions are structurally well conserved, which is essential to establish the FMN hydrogen-bonding network. This general rule also applies to the SYEs meaning that all requirements are fulfilled for a functional FMN-binding site.

1.10 12-Oxophytodienoate Reductase OPR1 and OPR3 (Tomato Enzymes)

12-Oxophytodienoate reductases (OPRs) are a small group of flavin-dependent oxidoreductases in plants related to old yellow enzyme (OYE). OPR1 and OPR3 are interesting candidates for asymmetric catalysis as they are able to asymmetrically reduce various activated alkenes with high, in part complementary, enantiomeric excess.

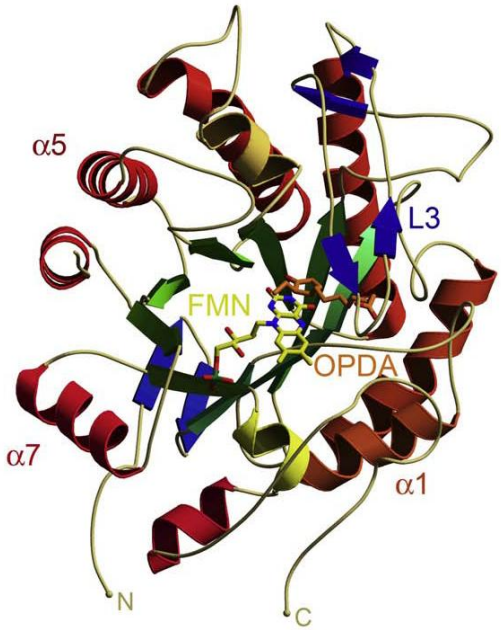


Figure 1.9 Ribbon diagram of the OPR1 structure in complex with (*9R,13R*)-OPDA

Like all members of the OYE family, OPR1 and OPR3 from *S. lycopersicum* (tomato) exhibit the frequently observed $(\alpha/\beta)_8$ barrel fold, in which the cylindrical eight-stranded parallel β-sheet is surrounded by eight α-helices. The FMN cofactor is bound noncovalently at the C terminal end of the β-barrel, where loops L1–L8 (loops are numbered according to the preceding β-

strand, e.g., L8 follows β8) set up the active-site cavity above the FMN and the substrate specificity regions.[96]

1.11 YqjM Enzyme

YqjM enzyme from *Bacillus subtilis* shares many biochemical properties with the homologous yeast Old Yellow Enzyme (OYE); the enzyme binds FMN strongly but noncovalently, preferentially uses NADPH as a source of reducing equivalent, and forms charge transfer complexes with phenolic compounds such as *p*-hydroxybenzaldehyde. Like yeast OYE and other members of the family, YqjM catalyzes the reduction of the olefins bearing a carbonyl or nitro group as activating substituent. Thus, enals, enones, maleidimides, and nitroolefins were reduced with excellent stereoselctivities to the corresponding saturated products at the expense of cofactor (NADH or NADPH).[87] The important differences between YqjM and the other members of the OYE family is that YqjM forms a tetramer, whereas all other members so far characterized in this regard form either monomers or dimers in solution.

The similarity of YqjM to other members of the OYE family derives from amino acid sequence alignments and shows an average of 33% identity and 50% similarity to OYE1 from *Saccharomyces carlsbergensis*, OYE1-2 from *Saccharomyces cerevisiae*, the three isozymes from *Arabidopsis thaliana*, *N*-ethylmaleimide reductase from *E. coli*, and pentaerythritol tetranitrate reductase from *Enterobacter cloacae*, with highest identity to *P. putida* XenA (40%), a xenobiotic oxidoreductase. Due to this high degree of similarity, it can be safely predicted that YqjM also folds into an $(\beta\alpha)_8$-barrel structure as found for other members of the family.[68]

---

[96] Breithaupt, C.; Kurzbauer, R.; Schaller, F.; Stintzi, A.; Schaller, A, Huber, R.; Macheroux, P.; Clausen,T. *J Mol. Biol.* **2009,** *392*, 1266–1277

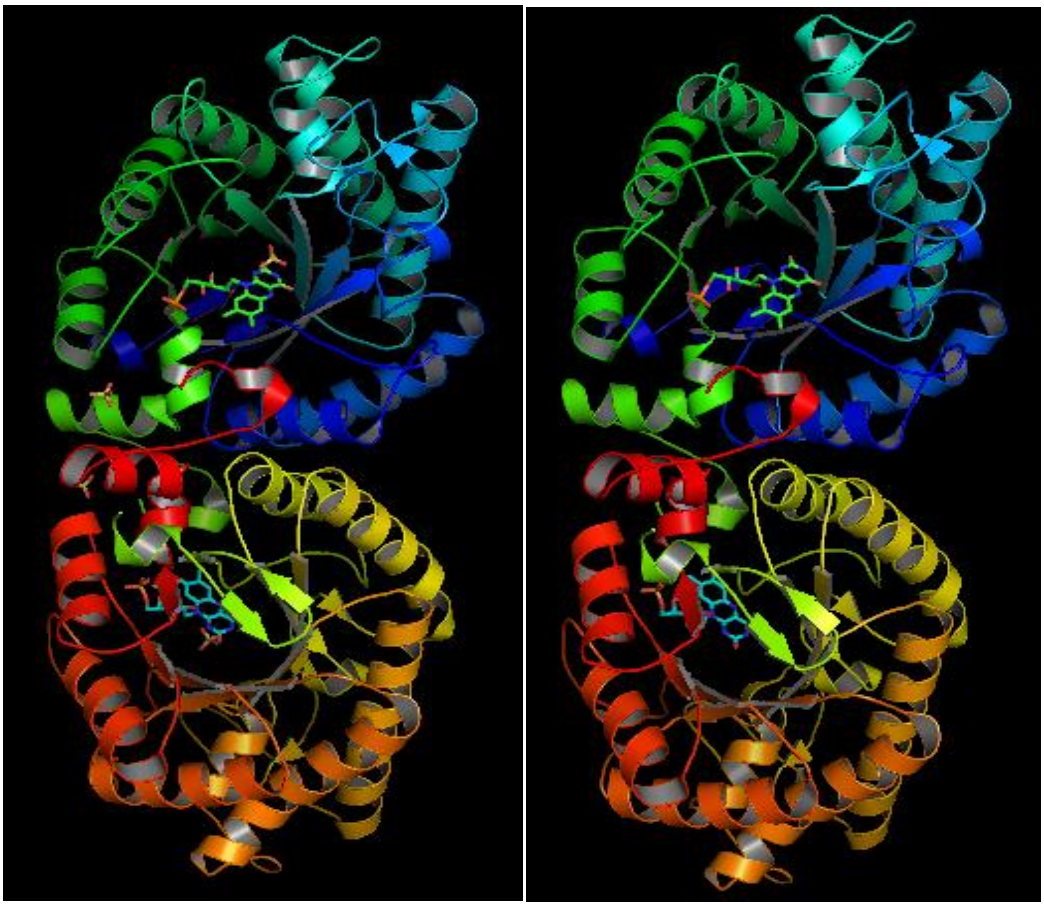


Figure 1.10 Homology approximation of YqjM (left oxidized, right reduced) based on 1Z41. Estimated by Deep View/Swiss-Pdb Viewer 3.7 and viewed in PyMOL[95]

1.12 Bioreduction of Different Substrates with Ene-Reductases

1.12.1 Bioreduction of α,β-Unsaturated Carbonyl Compounds

Aldehydes are a commercially important group of compounds due to their applications in the manufacture of dispersants and detergents. Naturally occurring aldehydes are found in essential oils such as cinnamaldehyde and vanillin and play an important role in the perfume industry. Bioreduction of cinnamaldehyde is a conventional reaction catalyzed by the OYE family, as high reactivity has been found with more than nine OYEs.[97]

---

[97] a) Chaparro-Riggers, J. F.; Rogers, T. A.; Vazquez-Figueroa, E.; Polizzi, K. M.; Bommarius, A. S. *Adv. Synth. Catal.* **2007**, *349*, 1521–1531. b) Fryszkowska, A.; Toogood, H. S.; Sakuma, M.; Gardiner, J. M.; Stephens, G. M. N.; Scrutton, S. *Adv. Synth. Catal.* **2009,** *351*, 2976 –2990. c) Vaz, A. D.; Chakraborty, S.; Massey, V.; *Biochemistry*. **1995,** *34*, 4246–4256.

Biotransformations of α,β-unsaturated enals using whole cell Baker's yeast show an over-reduction of the saturated aldehydes to form the corresponding primary alcohol. This is likely catalyzed by constitutive *prim*-alcohol dehydrogenases, as seen with *C. parapsilosis*.[98] Biotransformation of a variety of acyclic enals in presence of R1 and R2 substituents was conducted efficiently by OYEs provided only one of the groups was bulky and substituents on α-C should not be large and may be electron withdrawing (Figure 1.11).[99] Many acyclic α,β-unsaturated alkyl aldehydes were reduced by a variety of OYEs, often with high reactivity and/or stereospecificity. Another possible 'diagnostic' OYE-like reaction is the reduction of the short chain 2-methyl pentenal (**XXIV**) (Figure 1.11), which produces (*S*)-product (**XXV**) exploiting OYE1-3, OPR1, NCR, YqjM, PETN reductase and TOYE with often-poor ee values.[81] The (*R*) enantiomeric product was produced in the reaction with OPR3, although with poor enantiomeric excess of only 19%.[81] Yield of (*S*)-2-methylpentanal (**XXV**) was not quantitative with PETN reductase due to the presence of unidentified side products, most probably formed by non-enzymatic substrate and/or product decomposition, and product racemization.

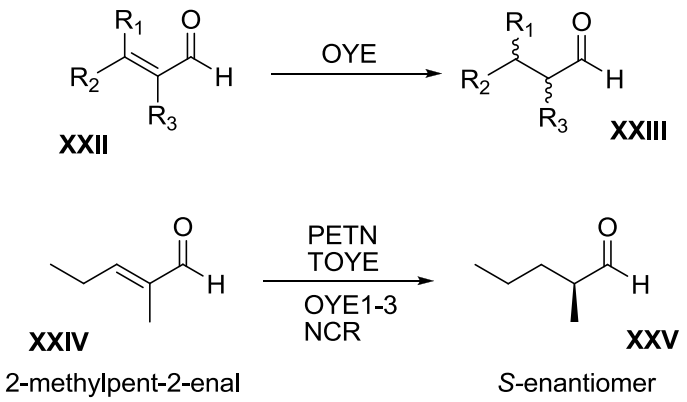


Figure 1.11 Asymmetric bioreduction of α,β -unsaturated alkenes with aldehyde activating groups by OYEs.

Ketones are an important class of compounds produced industrially at huge scales due to their use as synthons or solvents in many processes such as polymerizations and pharmaceutical drug

[98] Hall, M.; Hauer, B.; Stuermer, R.; Kroutil, W.; Faber, K. *Tetrahedron: Asymmetry.* **2006,** *17*, 3058– 3062.
[99] a) Fardelone, L. C.; Augusto, J.; Rodrigues, R.; Moran, P. J. S. *J. Mol. Catal. B.* **2004**, *29*, 41–45. b) Fronza, G. C. Fuganti, S. Serra, *Eur. J. Org. Chem.* **2009,** 6160 –6171.

synthesis.[100] Some of the industrially most important ketones are acetone, methylethylketone, ketoisophorone (**XXX**),[101] and cyclohexanone. OYEs catalyzed reduction of 2-cyclohexen-1-one to cyclohexanone is a standard reaction of this family of enzymes and can be an industrially useful process. The most important bioreduction by Baker's yeast is the large-scale production of (*R*)-levodione (**XXXI**) from the diketone ketoisophorone (**XXX**). Reaction conditions were cautiously optimized to minimize the activity of the competing *sec*-alcohol dehydrogenase activity to yield >80% of the desired product.

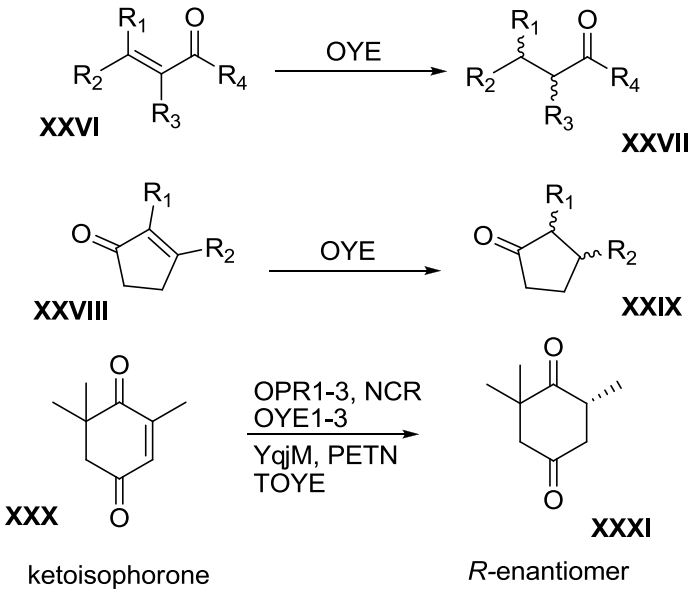


Figure 1.12 Asymmetric bioreduction of α,β -unsaturated alkenes with ketone activating groups by OYEs.

Ketoisophorone (**XXX**) is a highly accepted substrate for isolated OYEs, with at least 11 family members known to produce the (*R*)-product (**XXXI**) (Figure 1.12).[102] As this biotransformation is liable to water-mediated product racemization, the ee values are often quite poor. Although with PETN reductase a dramatic improvement in product enantiomeric excess was found (99% ee).

---

[100] Kirk, R. E.; Othmer, D. F. *Encyclopedia of Chemical Technology*, *14*, John Wiley & Sons, NY, **2005**.
[101] Leuenberger, H. G. W.; Boguth, W.; Widmer, E.; Zell, R.; *Helv. Chim. Acta* **1976,** *59*, 1832–1849.
[102] a) Kataoka, M.; Kotaka, A.; Thiwthong, R.; Wada, M.; Nakamori, S.; Shimizu, S. *J. Biotechnol.* **2004,** *114*, 1–9. b) Kataoka, M.; Kotaka, A.; Hasegawa, A.; Wada, M.; Yoshizumi, A.; Nakamori, S.; Shimizu, S. *Biosci. Biotechnol. Biochem.* **2002,** *66*, 2651–2657.

Bioreduction of a variety of substituted 2-cyclopenten-1-one (**XXVIII**) and cyclohexen-1-one substrates by isolated OYEs allows both recognizing the substrate specificity of these enzymes as well as forecasting the likely ligand binding conformation. Unsubstituted 2-cyclohexen-1-one appears to be a near common substrate for OYEs, whereas alkyl substitution at the α- and β-carbons can have a large influence on reactivity, substrate binding, and product enantioselectivity (Figure 1.12). Old yellow enzymes have significant similarities in the enantiopreference of products; however, yields, product optical purities, and reaction rates diverge between OYEs and are often dependent on the cofactor source[28].

Generally, the reduction of both 3-methyl substituted cyclopentenone and cyclohexenone proceeds to the corresponding (*S*)-products in high optical purity although often in poor yields. A recent study of the substrate specificity of a large number of site-saturated mutants of YqjM with 3-alkyl-substituted 2-cyclohexenones and 2-cyclopentenones showed that only small numbers of carefully positioned specific mutations were required to produce biocatalysts capable of improved substrate tolerance combined with different enantioselectivities.[28,103]

Acyclic α,β-unsaturated enones are also known substrates of a variety of isolated OYEs.[104] For example, the short chain compound methylvinylketone is a substrate for OYE1, EBP1, KYE1, XenA, and Yers-ER. Reduction of the long chain alkyl enones mesityl oxide and (*cis* or *trans*)-4-hexen-3-one was also considerable, as well as the 1,1-substituted alkene, 2-ethyl acrolein.[105]

---

[103] Bougioukou, D. J.; Kille, S.; Taglieber, A.; Reetz, M. T. *Adv. Synth. Catal.* **2009**, *351*, 3287–3305.
[104] Chaparro-Riggers, J. F.; Rogers, T. A.; Vazquez-Figueroa, E.; Polizzi, K. M.; Bommarius, A. S. *Adv. Synth. Catal.* **2007,** *349*, 1521–1531.
[105] a) Vaz, A. D.; Chakraborty, S.; Massey, V. *Biochemistry.* **1995,** *34*, 4246 –4256. b) Kataoka, M.; Kotaka, A.; Hasegawa, A.; Wada, M.; Yoshizumi, A.; Nakamori, S.; Shimizu, S.; *Biosci. Biotechnol. Biochem*. **2002,** *66*, 2651–2657.

1.12.2 Bioreduction of Maleimides

Maleimides are also an important class of organic compounds used as building blocks in organic synthesis. The double bond of maleimides reacts readily with hydroxy, amine, or thiol functionalities to generate stable C-C, C-N, and C-S bonds, respectively[28].

α,β-Unsaturated carboxylic acid derivatives, maleimides and acid anhydride are known to be good substrates for both isolated OYEs and whole cell biotransformations. Cultured suspensions of plant cells (*N. tabacum* and *Cathranthus roseus*) reduce the double bond of *N*-substituted maleimides to generate the corresponding succinimides. For example, biotransformations of *N*-phenyl-2-methylmaleimide (**XXXII**) by whole cells of *N. tabacum* generated (*R*)-*N*-phenyl-2-methylsuccinimide (**XXXIII**) with high product enantioselectivity (99% ee) and yields.[106] In addition, whole cell biotransformation of *Synechococcus* sp. produced (*R*)-2-methylsuccinimide with high enantiomeric purity.[107]

The bioreduction of *N*-substituted maleimides by isolated OYEs mostly produces (*R*)-products in high yields and enantiopurities (Figure 1.13).[108] Only purified MR gave low conversion but excellent ee values for the reduction of 2-methylmaleimide and *N*-phenyl-2-methylmaleimide.[109] Reduction of *N*-ethylmaleimide was also seen with OYE1[48], LeOPR, KYE1, XenA, and Yers-ER, although the stereoselectivity of the reaction was not determined[28]. Additional substrates such as maleic anydride and maleic acid were also reduced with low specificity.[84]

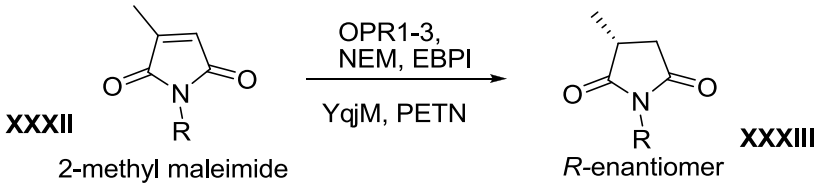


Figure 1.13 Asymmetric bioreduction of α,β-unsaturated maleimides

---

[106] Hirata, T.; Takarada, A.; Matsushima, A.; Konodo, Y.; Hamada, H.; *Tetrahedron: Asymmetry*. **2004**, *15*, 15–16.
[107] Stuermer, R.; Hauer, B.; Hall, M.; Faber, K. *Curr. Opin. Chem. Biol*. **2007,** *11*, 203–213.
[108] a) Hall, M.; Stueckler, C.; Kroutil, W.; Macheroux, P.; Faber, K. *Angew. Chem.* **2007,** *119*, 4008–4011. b) *Angew. Chem. Int. Ed.* **2007,** 46, 3934 –3937.
[109] Mueller, N. J.; Stueckler, C.; Hauer, B.; Baudendistel, N.; Housden, H.; Bruce, N. C.; Faber, K. *Adv. Synth. Catal*. **2010,** *352*, 387–394.

1.12.3 Bioreduction of Carboxylic Acids and Esters

Carboxylic acids and esters are employed in the production of polymers, pharmaceuticals, solvents, chemicals and food additives. Carboxylic acids can be used to produce esters, amides, and thioesters. The advantage of biocatalytic reduction of carboxylic acid containing alkenes over chemical methods may be as it does not require protection and deprotection steps of the reactive group.

The bioreduction of (*E*)- and (*Z*)-2-methylmaleic acid (**XXXIV**) and their corresponding methyl esters by majority of OYEs proved a clear selectivity for the (*Z*)-isomer (Figure 1.14).[37] The (*Z*)-diacid and diesters were reduced to the corresponding (*R*)-enantiomer (**XXXV**) with high optical purity, only exception was NEM reductase which gave 71–79% ee. Whereas the (*E*)-diacid was only reduced by EBP1 to form the (*S*)-product (**XXXVI**) with 46% enantiomeric excess. All products of (*E*)- and (*Z*)-diester reduction yielded the (*R*)-enantiomer (**XXXVIII**) except for the reaction with YqjM, which produced the (*S*)-product (**XXXIX**). Substrates more difficult to reduce were *exo*-methylene succinic acid and its dimethyl ester.

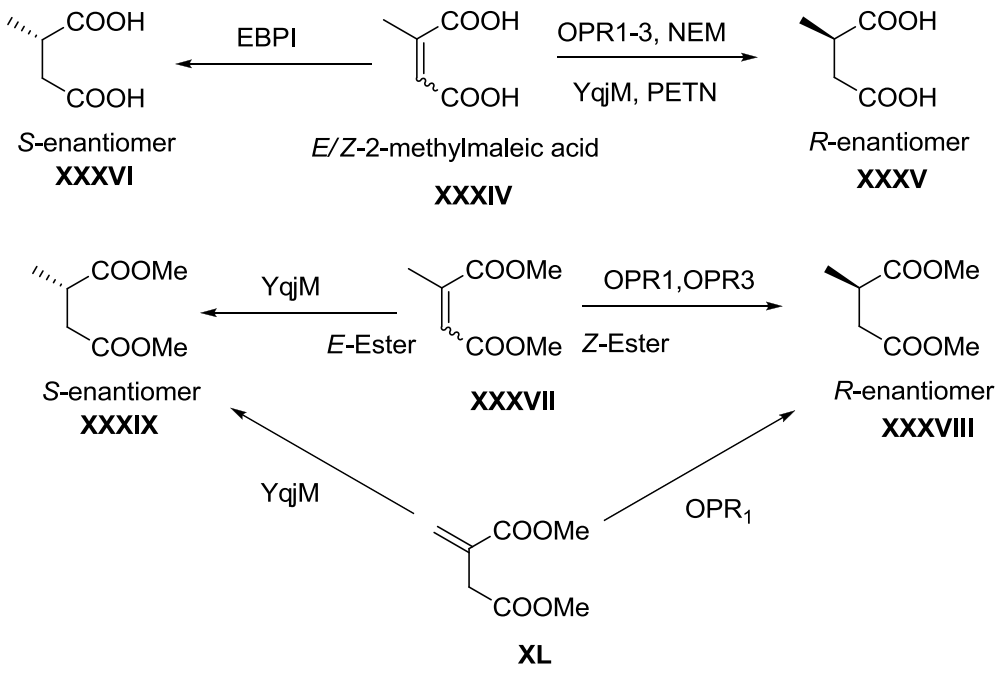


Figure 1.14 Asymmetric bioreduction of α,β-unsaturated alkenes with carboxylic acid and ester activating groups by OYEs.

Significant reduction was only achieved with dimethyl 2-methylenesuccinate (**XL**) by OPR1 and YqjM, which produced the (*R*)- and (*S*)-products, respectively, at high enantiopurity (Figure 1.14).[28,37]

1.12.4 Bioreduction of Terpenoids

The terpenoids, sometimes called isoprenoids, are a large and diverse class of naturally occurring cyclic and acyclic molecules derived from five-carbon isoprene units assembled and modified in highly modular ways. Plant terpenoids are used extensively for their aromatic qualities. They play a role in traditional herbal remedies and are under investigation for antibacterial, antineoplastic, and other pharmaceutical functions. The terpenoids are found in eucalyptus oil and the essential oil of ginger.[110] Well-known terpenoids used industrially include carvone (*R* and *S*), citral (geranial and neral), and camphor.

In terpenoid synthesis Baker's yeast has been used by catalyzing the asymmetric reduction of 3-phenylthiomethyl-2-butenolide to (*R*)-3-phenylthiomethyl-2-butanolide in high enantio-selectivity.[111] A reductase isolated from *N. tabacum* was found to reduce the C=C bond of (*5S*)-carvone (**XLI**) to (*2S,5S*)-dihydrocarvone (**XLV**). Recently, bioreduction with 16 strains of yeast from six genera (*Candida, Cryptococcus, Hanseniaspora, Kluyveromyces*, *Pichia* and *Saccharomyces*) showed that (*5S*)-(+)-carvone (**XLI**) yielded a variety of products such as (*2S,5S*)- and (*2R,5S*)-dihydrocarvone (**XLV** & **XLIV**), plus (*1S,2S,5S*)-, (*1S,2R,5S*)- and (*1R,2S,5S*)-dihydrocarveol.[112] The product yields varied (0.1–30%) dependent on the strain and the physiological state of the organism (growing, resting, or lyophilized cells). Whole cell bioreduction of (*5R*)-carvone (**XLII**) with *Astasia longa* gave both (*2R,5R*)-dihydrocarvone (**XLIII**) and (*1R,2S,5R*)-neoisodihydrocarveol, due to reduction of the C=C bond and carbonyl group, respectively. In contrast, reactions with (5*S*)-carvone (**XLI**) produced (2S,5S)-dihydro-carvone (**XLV**), (*2R,5S*)-isodihydrocarvone (**XLIV**), and (*1R,2S,5S*)-neoisodihydrocarveol.[113]

---

[110] Eich, E. Solanaceae and convolvulaceae: *Secondary Metabolites Biosynthesis, Chemotaxonomy, Biological and Economic Significance.* Springer, Berlin, **2008**.
[111] Takabe, K.; Hiyoshi, H.; Sawada, H.; Tanaka, M.; Miyazaki, A.; Yamada, T.; Katagiri, T.; Yoda, H. *Tetrahedron: Asymmetry.* **1992,** *3*, 1399–1400.
[112] Goretti, M.; Ponzoni, C.; Caselli, E.; Marchigiani, E.; Cramarossa, M. R.; Turchetti, B.; Buzzini, P.; Forti, L.; *Enzyme Microb. Technol.* **2009**, *45*, 463–468.
[113] Shimoda, K.; Hirata, T. *J. Mol. Catal.* B. **2000**, *8*, 255–264.

The bioreduction of (*S/R*)-carvone by OYE1, PETN reductase, and TOYE yielded the same diastereoisomeric products (*2R,5S*)-dihydrocarvone (**XLIV**) and (*2R,5R*)-dihydrocarvone (**XLIII**), respectively (Figure 1.15).[114] These results suggest a common binding mode of both substrates, with the presence of the large substitutent group not affecting the stereochemical outcome. In contrast, reductases I and II from *A. longa* stereospecifically reduced (*5R*)- and (*5S*)-carvone to (*2R,5R*)-dihydrocarvone (**XLIII**) and (*2R,5S*)-dihydrocarvone (**XLIV**), respectively. Other OYEs (Gox1-3, CrS, KYE1, XenA, and Yers-ER) reduced (*S/R*)-carvone, but the stereochemistry of the products was not assigned.[115]

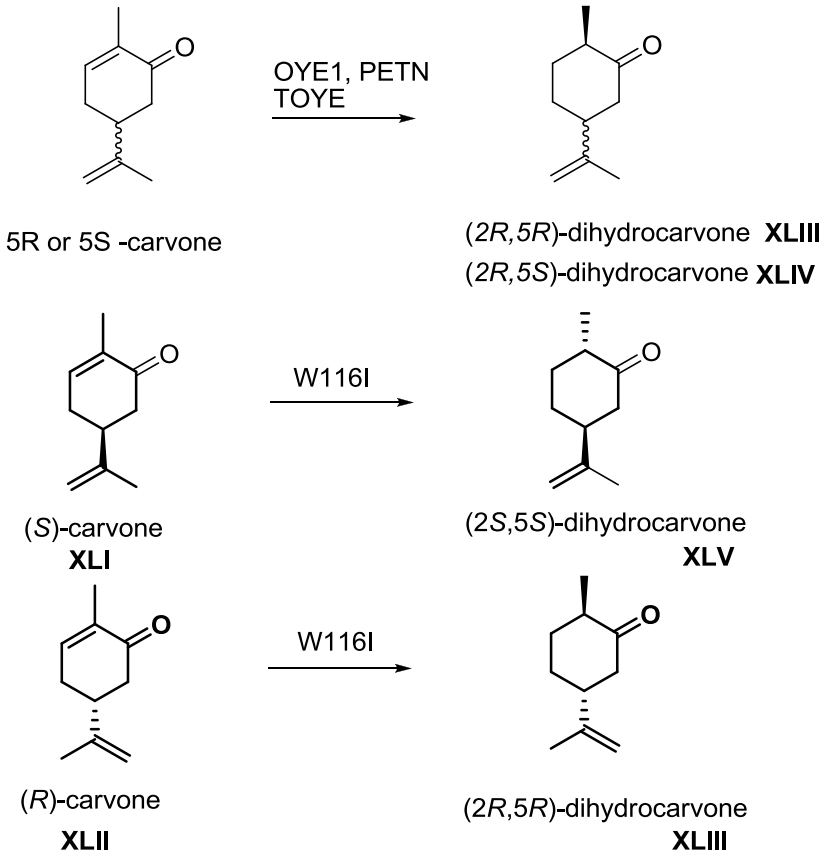


Figure 1.15 Asymmetric bioreduction of α,β-unsaturated terpenoids by OYEs.

---

[114] Padhi, S. K.; Bougioukou, D. J.; Stewart, J. D. *J. Am. Chem. Soc.* **2009,** *131*, 3271 –3280.
[115] Opperman, D. J.; Sewell, B. T.; Litthauer, D.; Isupov, M. N.; Littlechild, J. A.; Heerden, E. *Biochem. Biophys. Res. Commun*. **2010,** *393*, 426–431.

Many OYEs catalyze the bioreduction of citral (neral and geranial isomers) to (*S*)-citronellal in excellent yields and enantioselectivity.[116] PETN reductase yielded only 57% of the anticipated (*S*)-enantiomeric product with 87% ee due to the formation of unknown side products. Additionally, the opposite (*R*)-enantiomeric product was obtained with OYE2 and EBP1 catalyzed bioreduction at low ee values.[117] Other OYEs were found to react with citral isomer(s) under steady state conditions.[118]

Site-saturation mutagenesis was used to produce all possible replacements for Trp 116 of *Saccharomyces pastorianus* (*Saccharomyces carlsbergensis*) old yellow enzyme (OYE). This yielded reversed stereochemical outcomes compared to that of the wild-type OYE. For example, W116I OYE reduced (*R*)- and (*S*)-carvone to enantiomeric products, rather than the diastereomers produced by the wild-type OYE.[119] (*S*)-carvone (**XLI**) reduction by W116I OYE occurred via the same way as for the wild type (net *trans*-addition of H_2), providing that different substrate binding orientations were responsible for the divergent products. (Figure 1.15)

1.12.5 Bioreduction of α,β-Unsaturated Nitroalkenes

Nitroalkanes are readily converted into the corresponding amines, carboxylic acids, aldehydes, oximes, hydroxylamines, or denitrated compounds. Due to this reason, nitroalkanes are industrially important synthons.[120] Synthesis of chiral nitroalkanes by the asymmetric reduction of nitroalkenes is an attractive method due to the creation of up to two new stereogenic centers (Figure 1.17). Baker's yeast reduces a variety of substituted α,β-unsaturated 1-nitro-1-alkenes to the corresponding 1-nitroalkanes at good enantiopurities (83–98% ee).[121] Classic substrates included aryl, *p*-halide substituted aryl, and linear alkyl chain substituted nitroalkenes. β,β-Disubstituted nitroalkenes were reduced to the (*R*)-enantiomer with high enantiopurity,

---

[116] a) Knowles, W. S.; *Acc. Chem. Res.* **1983**, *16*, 106–112. b) Tuttle, J. B.; Ouellet, G. G.; MacMillan, D. W. C. *J. Am. Chem. Soc*. **2006,** *128*, 12662–12663. c) Adalbjçrnsson, B. V.; Toogood, H. S.; Fryszkowska, A.; Pudney, C. R.; Jowitt, T. A.; Leys, D.; Scrutton, N. S. *ChemBioChem.* **2010,** *11*, 197–207.
[117] Hall, M.; Stueckler, C.; Hauer, B.; Stuermer, R.; Friedrich, T.; Breuer, M.; Kroutil, W.; Faber, K. *Eur. J. Org. Chem*. **2008,** 1511–1516.
[118] Opperman, D. J.; Sewell, B. T.; Litthauer, D.; Isupov, M. N.; Littlechild, J. A.; van Heerden, E. *Biochem. Biophys. Res. Commun.* **2010**, *393*, 426–431.
[119] Padhi, K. S.; Bougioukou, D. J.; Stewart, J .D. *J. Am. Chem.Soc*, **2009**, *131*, 3271-3280.
[120] Ono. N. *The Nitro Group in Organic Synthesis.* Wiley-VCH**,** Weinheim, **2001**.
[121] Ohta, H.; Kobayashi, N.; Ozaki, K. *J. Org. Chem.* **1989,** *54*, 1802–1804.

whereas α,β-disubstituted nitroalkenes tend to be converted into products with poor enantiopurity.[122]

Whole cell biotransformations using a variety of yeasts and bacteria have been successful in the asymmetric reduction of a variety of α,β-unsaturated nitroalkenes. Anaerobic organisms (*Eubacterium spp.),* yeasts (*Candida, Torulopsis,* and *Rhodotorula*), and bacteria (*Klebsiella, B. subtilis, Rhodococcus, Nocardia and E. coli*) are shown to be active in bioreduction of nitroalkenes.[123] Aerobic bioreduction of (*E*)-α- or β-alkyl-β-arylnitroalkenes with a number of isolated OYEs and whole cell biotransformation with *Clostridium sporogenes* (anaerobic reactions) showed unconsistancy in both enantiopreference and optical purity of the products.[124] The MR gave (*S*)-enantiomeric products at low to medium ee, whereas NCR reductase produced (*S*)-products with high ee. (*R*)-Enantiomeric product was formed with high ee value employing OPR1 reductse, whereas OPR3 and YqjM produced the (*S*)-product with medium to high enantioselectivity. While NEM reductase, OYE1-2, and EBP1 furnished the (*R*)-enantiomeric products at low to medium ee, whereas OYE3 and *C. sporogenes* generated optically pure (*R*)-products. PETN reductase showed enantiopreference to produce the (*R*)-products under alternative biotransformation conditions (i.e., aerobic reactions in a different buffer) with medium ee values from the bioreduction of α- or β-alkyl-β-arylnitroalkene (Figure 1.16). [125]

The reduction of (*Z*)-3-phenyl-2-nitro-2-butene (**XLVIII**) by nitroalkene reductases (YNAR-1 and II) from Baker's yeast gave the diastereoisomeric products (*2R,3R*)- and (*2S,3R*)-3-phenyl-2-nitrobutane (**XLIX**), although each with high enantioselectivity (97% ee).[28]

---

[122] Kawai, Y.; Inaba, Y.; Hayashi, M.; Tokitoh, N. *Tetrahedron Lett.* **2001**, *42*, 3367–3368.
[123] Mori, A.; Ishiyama, I.; Akita, H.; Suzuki, K.; Mitsuoka, T.; Oishi, T. *Chem. Pharm. Bull.* **1990**, *38*, 3449-3451.
[124] a) Kataoka, M.; Kotaka, A.; Thiwthong, R.; Wada, M.; Nakamori, S.; Shimizu, S. *J. Biotechnol.* **2004**, *114*, 1–9. b) Fryszkowska, A.; Fisher, K.; Gardiner, J. M.; Stephens, G. M. *J. Org. Chem.* **2008,** *73*, 4295–4298.
[125] Mueller, N. J.; Stueckler, C.; Hauer, B.; Baudendistel, N.; Housden, H.; Bruce, N. C.; Faber, K. *Adv. Synth. Catal*. **2010,** *352*, 387–394.

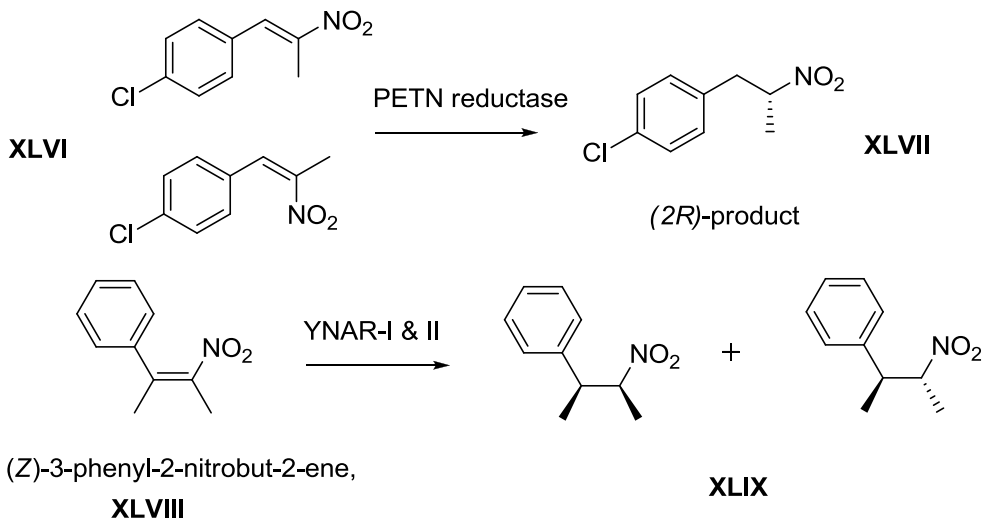


Figure 1.16 Asymmetric bioreduction of α,β-unsaturated nitroalkenes by OYEs.

1.13 Monooxygenase-Mediated Baeyer-Villiger Oxidations

The Baeyer-Villiger oxidation[126] of ketones represents a powerful methodology for breaking carbon-carbon bonds in an oxygen-insertion process. Widely used oxidants for Baeyer-Villiger reactions are *m*-chloroperoxybenzoic acid, trifluoroperoxyacetic acid and hydrogen peroxide.

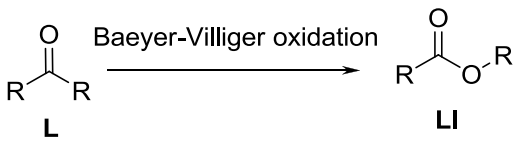


Figure 1.17 Baeyer–Villiger oxidation

The regiochemistry of the reaction is governed by predictable conformational, steric, and electronic effects,[127] and the rearrangement process of the tetrahedral peroxo Criegee intermediate proceeds with strict retention of configuration.[128] These factors are key prerequisites for performing the Baeyer–Villiger oxidation in an enantioselective manner.

The conversion of cyclic ketones in to optically pure lactones, in particular, allows access to highly flexible compounds as platforms for the subsequent synthesis of bioactive compounds and natural products. Consequently, enantioselective Baeyer–Villiger oxidations have become a

---

[126] Baeyer, A.; Villiger, V. *Chem. Ber.* **1899**, *32*, 3625–3633.
[127] Noyori, R.; Sato, T.; Kobayashi, H. *Bull. Chem. Soc. Jpn.* **1983**, *56*, 2661–2679.
[128] Mislow, K.; Brenner, J. *J. Am. Chem. Soc.* **1953**, *75*, 2318–2322.

highly active field in asymmetric chemistry in recent years.[129] Currently, two major strategies are being developed with implementation of the "green-chemistry" concept aimed at sustainable, environmentally benign, and atom-efficient processes. Metal-based, *de novo* designed chiral catalysts have been continuously improved and are becoming promising candidates for industrial-scale applications.[130] By taking advantage of the vast catalytic repertoire of enzymes in nature, biocatalysis offers alternative entities for stereoselective oxidation processes with molecular oxygen utilized as the oxidant.[131] An increasing number of flavin-containing Baeyer–Villiger monooxygenases (BVMOs) have been identified during the past decade, and several such proteins display a remarkably broad acceptance profile for nonnatural substrates. Baeyer-Villiger monooxygenases are enzymes that catalyze the insertion of an oxygen atom in a ketone, next to the carbonyl carbon atom. These enzymes typically contain FAD or FMN as a cofactor and catalyze highly regio- and stereoselective oxygenations at the expense of NAD(P)H and molecular oxygen.

1.14 Mechanism of BVMOs

The generally accepted mechanism for the enzymatic Baeyer-Villiger oxidation is based on results obtained with cyclohexanone monooxygenase (CHMO) isolated from *Acinetobacter calcoaceticus* NCIMB 9871.[132] The enzyme possesses flavin adenine dinucleotid (FAD) as a prosthetic group and was found to be NADPH- and oxygen-dependent. Walsh and co-workers proposed several steps for the oxidation and determined a number of rate constants.[133]

The biocatalytic process is initiated by reduction of the tightly bound FAD by NADPH and subsequent rapid oxidation by molecular oxygen to produce the flavin 4a-peroxide anion (**LIII**) (Figure 1.19). This intermediate constitutes the oxygenating species in the subsequent Baeyer-

---

[129] Mihovilovic, M. D.; Rudroff, F.; Grötzl, B. *Curr.Org.Chem.* **2004**, *8*, 1057–1069.
[130] Bolm C. in *Peroxide Chemistry* (Ed.: W. Adam), Wiley-VCH, Weinheim, **2000**, 494–510; Strukul, G. *Angew. Chem.* **1998**, *110*, 1256–1267. *Angew. Chem. Int. Ed.* **1998**, *37*, 1198–1209.
[131] a) Li, Z.; Van Beilen, J. B.; Duetz, W. A.; Schmid, A.; De Raadt, A.; Griengl, H.; Witholt, B. *Curr. Opin. Chem. Biol.* **2002**, *6*, 136–144. b) Burton, S. G. *Trends Biotechnol.* **2003**, *21*, 543–549.
[132] Donoghue, N. A.; Norris, D. B.; Trudgill, P. W. *Eur. J. Biochem.* **1976**, *63*, 175–92.
[133] Ryerson, C. C.; Ballou, D. P.; Walsh, C. *Biochemistry.* **1982**, *21*, 2644–55.

Villiger reaction. The anion is formed initially and, in the absence of the substrate, exists in equilibrium with the 4a-hydroperoxide (**LIV**) as a stabilized form.[134]

The peroxide acts as a nucleophile, attacking the substrate carbonyl group to give the "classical" tetrahedral Criegee intermediate (**LV**). Rearrangement of the species results in formation of the product lactone and 4a-hydroxyflavin (**LVI**). The catalytic cycle is closed by elimination of water to form FAD and release of product and cofactor.

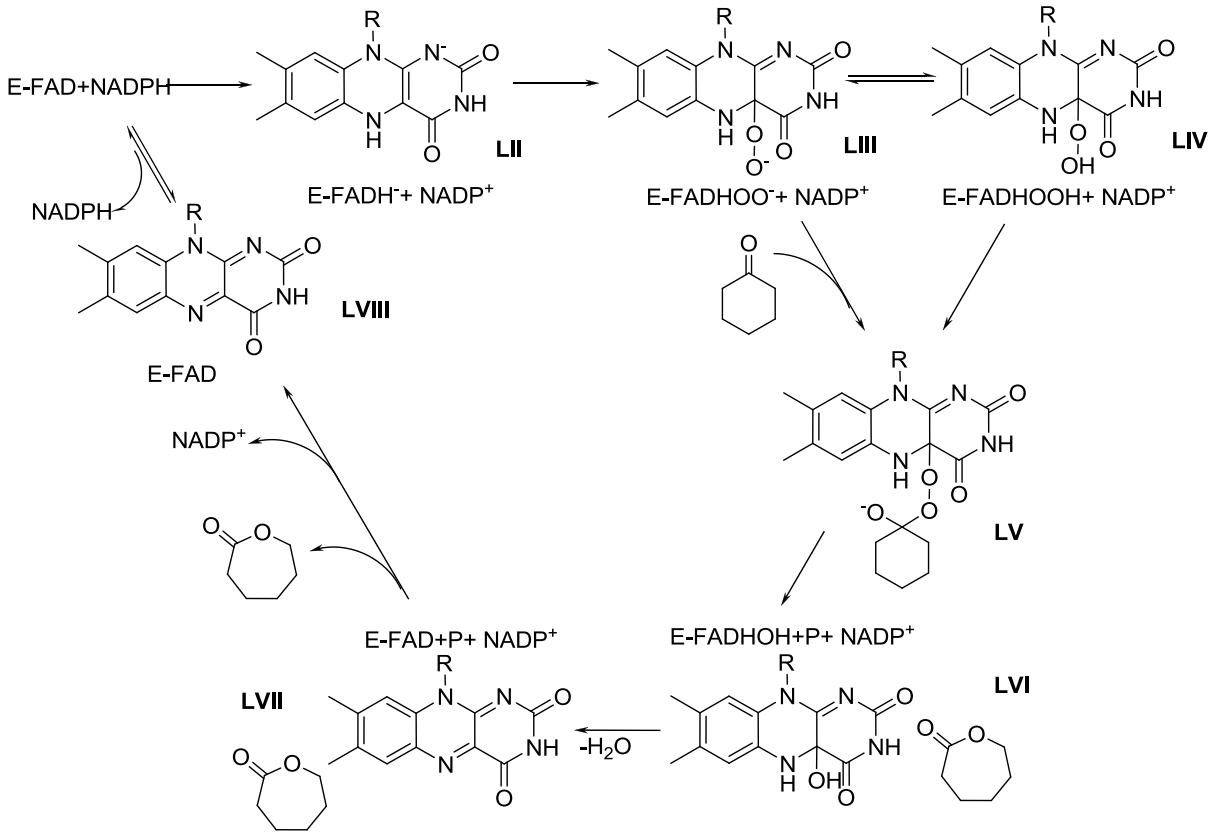


Figure 1.18 Mechanism of BVMOs

The rearrangement is governed by the same stereoelectronic effects as in the non-enzymatic Baeyer-Villiger oxidation.[135,136] It has been demonstrated that two preconditions must be satisfied for successful alkyl migration: 1) the migrating C—C bond has to be in an antiperiplanar position with respect to the peroxy bond, and 2) electron release from the hydroxy

---

[134] Sheng, D.; Ballou, D. P.; Massey, V. *Biochemistry.* **2001**, *40*, 11156-11167.
[135] Chandrasekhar, S.; Roy, C. D. *Tetrahedron Lett.* **1987**, *28*, 6371-72.
[136] Deslongchamps, P. *Organic Chemistry Series*, Vol. 1, Pergamon, Oxford, **1983**.

oxygen atom to the originally attached migrating entity is essential for the alkyl shift and requires a lone pair in the *anti* position at the oxygen atom (Figure 1.18).[137]

This mechanism for CHMO and the directing effects involved generally serve as a model for other BVMOs with some differences; both NADH and NADPH are found as cofactors for monooxygenases, and the prosthetic group FAD can be replaced by FMN with no essential changes in the mechanism.

BVMOs, containing interesting and different properties with respect to chemo-, regio-, and stereoselectivity became available for synthetic applications.[138,139,140] Substrate acceptance profiles and stereopreferences of cyclohexanone (CH) and cyclopentanone (CP) monooxygenases originating from *Acinetobacter* ($CHMO_{Acineto}$), *Arthrobacter* ($CHMO_{Arthro}$), *Brachymonas* ($CHMO_{Brachy}$), *Brevibacterium* ($CHMO_{Brevi1}$, $CHMO_{Brevi2}$), *Rhodococcus* ($CHMO_{Rhodo1}$, $CHMO_{Rhodo2}$), and *Comamonas*($CPMO_{Coma}$) species was compared in whole-cell mediated Baeyer–Villiger oxidations with recombinant *E. coli* as the host organism.[141]

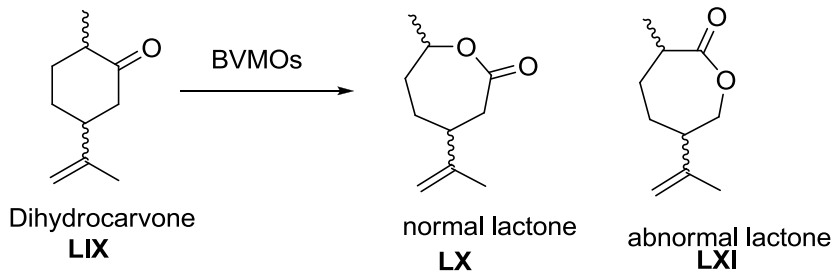


Figure 1.19 Baeyer–Villiger oxidation of carvones with BVMOs

[137] Noyori, R.; Sato, T.; Kobayashi, H. *Bull. Chem. Soc. Jpn.* **1983**, *56*, 2661-79.
[138] Kamerbeek, N.M.; Janssen, D.B.; van Berkel, W.J.H.; Fraaije, M.W. *Adv. Synth. Catal.* **2003**, *345*, 667-678.
[139] Mihovilovic, M.D.; Müller, B.; Stanetty, P. *Eur. J. Org. Chem.* **2002**, 3711-3730.
[140] Mihovilovic, M.D.; *Curr. Org. Chem.* **2006**, *10*, 1265-1287.
[141] Cernuchova, P.; Mihovilovic, M.D. *Org. Biomol. Chem.* **2007**, *5*, 1715–1719.

1.15 Aims of PhD Thesis

- Develop the substrate profile for the *Shewanella* Yellow Enzymes (SYE-3 and SYE-4).
- Synthesis of Baylis Hillman and Aza Baylis Hillman products for the bioreduction studies.
- Bioreduction studies of Baylis Hillman products with different Ene-reductases (SYE-3, SYE-4, OPR1, OPR3, YqjM).
- Bioreduction studies of Aza Baylis Hillman products with different Ene-reductases (SYE-3, SYE-4, OPR1, OPR3, YqjM) to the synthesis of β-amino acids.
- Bioreduction studies of carvones with different Ene-reductases (SYE-3, SYE-4, OPR1, OPR3, YqjM, W116I).
- Biooxygenation studies of dihydrocarvones with different BVMOs ($CHMO_{Acineto}$, $CHMO_{Brevi1}$, $CHMO_{Brevi2}$ and $CPMO_{Coma}$).
- One-pot redox reactions of carvones using different combinations of Ene-reductases (SYE-3, SYE-4, OPR1, OPR3, YqjM, W116I) and BVMOs ($CHMO_{Acineto}$, $CHMO_{Brevi1}$, $CHMO_{Brevi2}$ and $CPMO_{Coma}$).

Results and Discussion

2.1 SubstrateProfiling for *Shewanella* Yellow Enzyme

2.1.1 Synthesis of Substrates and Reference Materials for Substrate Screening with SYE Proteins

2.1.1.1 Synthesis of α,β-Unsaturated Cyclic Ketones

2-Methylcyclopent-2-enone (**1**), 3-methylcyclopent-2-enone (**2**), 3-methylcyclohex-2-enone (**3**), 1-cyclopentenylethanone (**4**) and 1-cyclohexenylethanone (**5**) were commercially available unsaturated ketones and used for the bioreduction.

For the synthesis of compounds (**8** & **9)** the methodology was simple condensation of cyclopenatanone (**6**) and cyclohexanone (**7**) with benzaldehyde in the presence of sodium hydroxide[142] (Figure 2.1).

2.1.1.2 Synthesis of Saturated Cyclic Ketones

These unsaturated cyclic ketones were reduced by catalytic hydrogenation with Pd/C to obtain reference material for GC analysis. The catalytic reaction was straight forward with high yields (Figure 2.1).

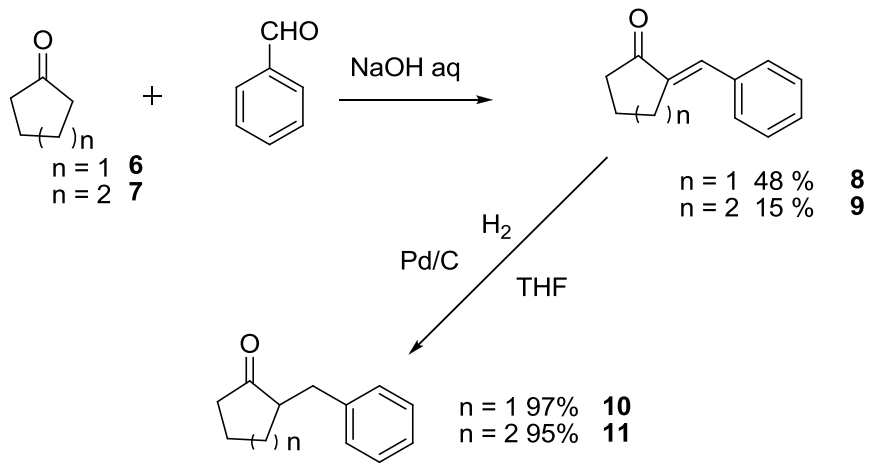


Figure 2.1 Synthesis of α,β-unsaturated cyclic ketones and saturated ketones

[142]Vatsadze, S. Z.; Sviridenkova, N. V.; Manaenkova, M. A.; Semashko, V. S.; Zyk, N. V. *Russian Chemical Bulletin, International Edition*, **2005**, *54*, 9, 2224-2225.

2.1.1.3 Synthesis of Unsaturated Diesters

Different types of diesters were synthesized by esterification of 2-methylmaleic acid (**12**) and 2-methylenesuccinic acid (**15**).[117] A solution of 2-methylmaleic acid (**12**) in BF_3/MeOH (14%) was stirred at 100 °C for 1h to obtain dimethyl 2-methylmaleate (**13**). Similarly treatment of 2-methylmaleic acid (**12**) with BF_3/EtOH (14%) gave diethyl 2-methylmaleate (**14**). Dimethyl and diethyl esters of 2-methylenesuccinic acid (**16** & **17**) were prepared using the same protocol in good yields (Figures 2.2 & 2.3).

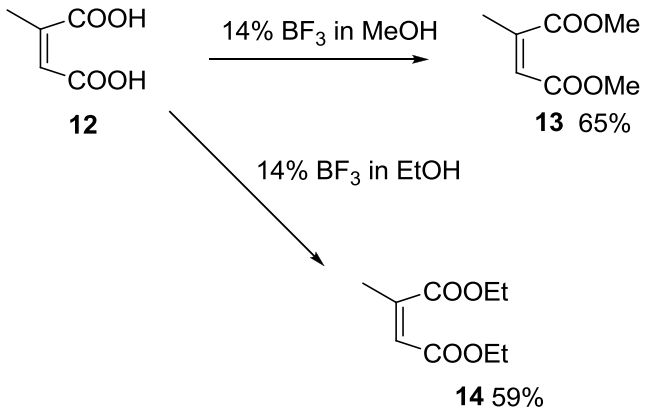


Figure 2.2 Synthesis of unsaturated diesters from 2-methylmaleic acid

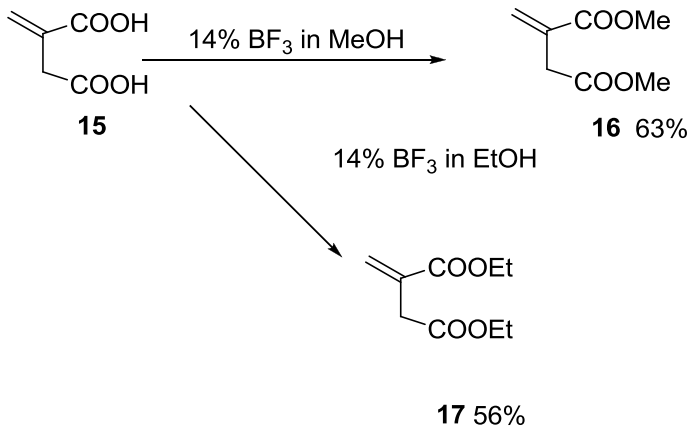


Figure 2.3 Synthesis of unsaturated diesters from 2-methylenesuccinic acid

2.1.1.4 Synthesis of Saturated Diesters

Saturated diesters were synthesized for analytical reference purposes during bioreduction screenings. 2-Methylmaleic acid (**12**) was first reduced to racemic 2-methylsuccinic acid (**18**) by catalytic reduction with 5% Pd/C using THF/EtOH (1:1) as solvent. Then esterification of

2-methylsuccinic acid (**18**) was conducted using the same method as above to obtain dimethyl 2-methylsuccinate (**19**) and diethyl 2-methylsuccinate (**20**) (Figure 2.4).

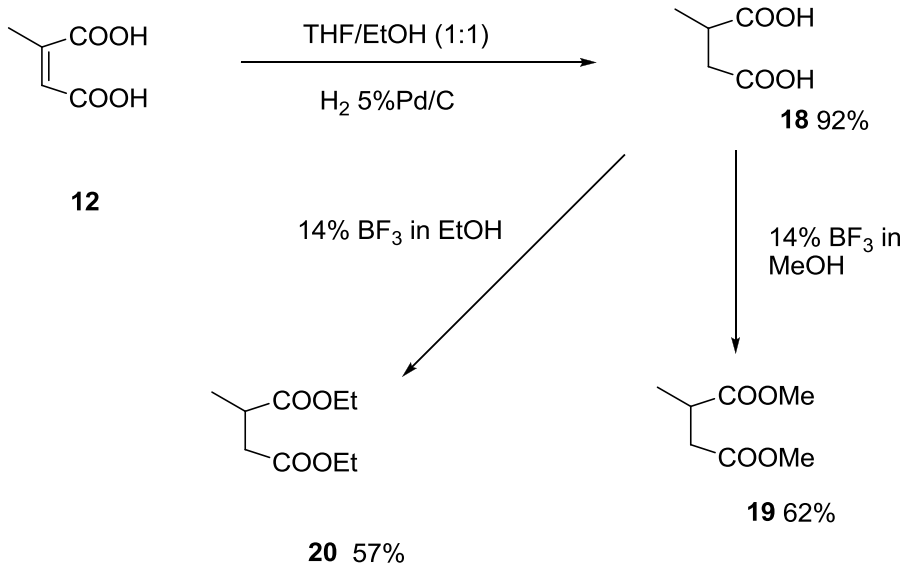


Figure 2.4 Synthesis of saturated diesters

2.1.1.5 Synthesis of *N*-Substituted Imides

Different methods were investigated for the synthesis of imides.[143] First method was the reaction of maleic anhydride (**21**) and amine in the presence of glacial acetic acid but in this case yields were only moderate. Second attempt was to irradiate the mixture of maleic anhydride (**21**) and amine within a microwave reactor instead of conventional heating.[144] Due to low power of irradiation (150W), short time (5-30min) and low temperature (100^{o}C) the yields were again not convincing.

---

[143] Dragutin, F.; Radivoje, V.; Ana Erceg, K.; Grozdana, B.; Vlasta, P.; Damir, K.; Kreimir, M.; Kristina, W.; Draen, V. *Croat. Chem. Acta,* **2003,** *76,* 1, 69–74.

[144] Harsha, N.; Borah, R. C.; Boruah, Jagir, S. S. *J. Chem. Research (S),* **1998**, 272-273.

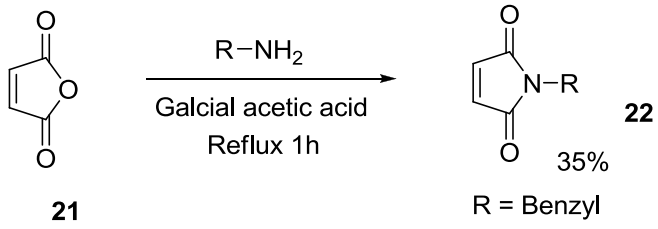


Figure 2.5 Synthesis of 1-benzyl-1H-pyrrole-2,5-dione (**22**) under reflux conditions

To optimize the reaction conditions, a mixture of maleic anhydride (**21**) and benzyl amine in glacial acetic acid was subjected to microwave irradiation with power of 150W and temperature 200^{o}C for 1h. This time reasonable yields were observed under these conditions (Figure 2.7). This method is novel for the synthesis of substituted imides using microwave.

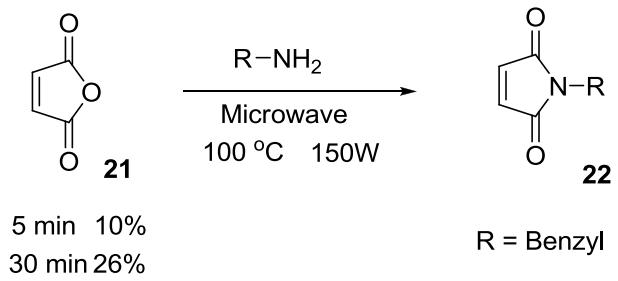


Figure 2.6 Synthesis of 1-benzyl-1H-pyrrole-2,5-dione (**22**) under microwave conditions

Different maleimides were synthesized by the reaction of 2-methyl maleic anhydride with a variety of amines under optimized conditions. Reference products for biotransformations were also prepared by the same method using 2-methyl succinic anhydride and amines (Figure 2.8).

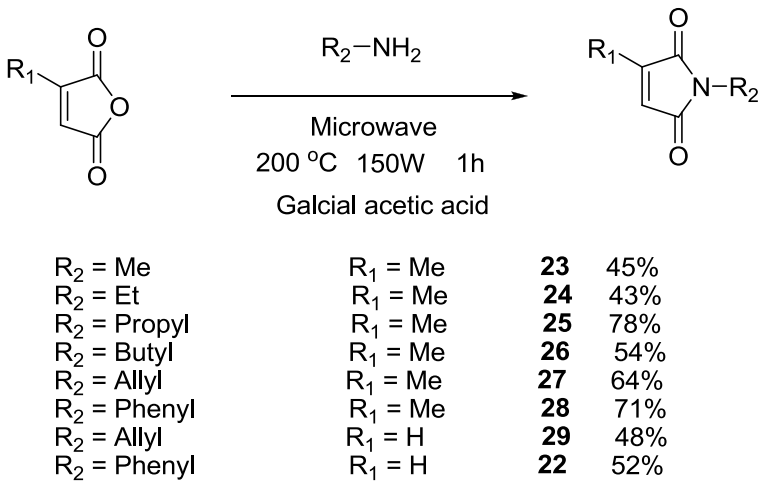
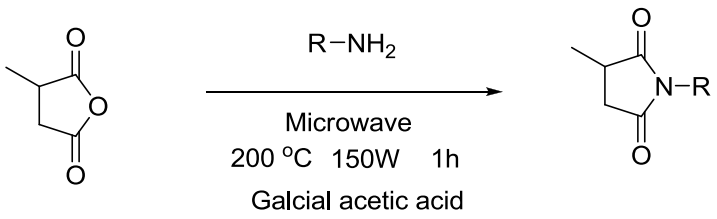


Figure 2.7 Synthesis of unsaturated substituted imides



R = Benzyl	**30**	38%
R= Allyl	**31**	42%
R = Methyl	**32**	83%
R = Ethyl	**33**	77%
R = Propyl	**34**	75%
R = Butyl	**35**	80%

Figure 2.8 Synthesis of saturated substituted imides

2.1.2 Bioreductions with SYE-3 and SYE-4

Bioreductions of the above synthesized substrates were performed with *E.coli* BL21 (DE3) recombinant whole cells harboring the expression plasmid for SYE-3 and SYE-4 and with crude cell protein extracts. In whole cell bioreductions cells were grown on the LB agar media plates supplemented with chloroamphenicol at 37°C. Precultures were inoculated with a single colony from $LB_{Chlamph}$ media plates. An Erlenmeyer flask containing sterile TB medium supplemented with chloroamphenicol was incubated with 2% vol of overnight culture up to O.D. of 1 within 3h at 28°C. Production of enzyme was induced by IPTG; substrates were added after 1h. Samples were analyzed after 3h, 6h and 24h using chiral GC. (Table 2.1–2.6)

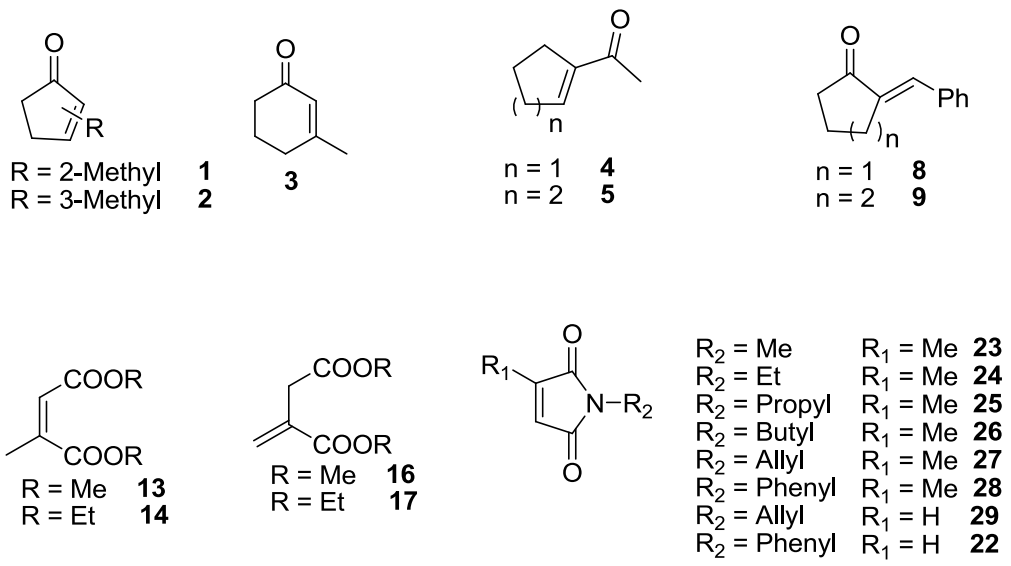


Figure 2.9 Substrates for bioreduction

2.1.2.1. Whole Cell Bioreductions of α,β-Unsaturated Cyclic Ketones with *Shewanella* Yellow Enzyme SYE-3

The bioreductions of α,β-unsaturated ketones (**1-3** & **8-9**) with cells expressing SYE-3 were conducted in Erlenmeyer shaking flask containing sterile $TB_{Chlamph}$ media, the screening volume (34µg/mL substrate) was incubated with 2% vol of overnight culture *E.coli* BL21(DE3) having expression plasmid for SYE-3 grown on $LB_{Chlamph}$ media up to O.D. of 1 within 3h at 28°C. Production of enzyme was induced by IPTG (0.5mM). Substrates were added after 1h of IPTG addition. Samples were collected after 3h, 6h and 24h for GC analysis. SYE-3 expressing cells bioreduction gave only poor results with all cyclic ketones. Only traces of products were observed in case of compound (**8**)

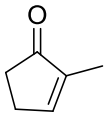
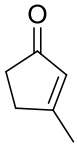
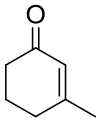
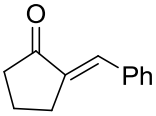
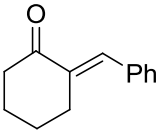
Substrate/Time	3h	6h	24h
 **1**	nc[a]	nc	nc
 **2**	nc	nc	nc
 **3**	nc	nc	nc
 **8**	1.2%[b] 99ee[c]	1.3% 99ee	1% 99ee
 **9**	nc	nc	nc

Table: 2.1 Bioreduction screening results of α,β-unsaturated cyclic ketones with SYE-3 expressing cells. a) no conversion, b) % conversion, c) enantiomeric excess.

2.1.2.2 Whole Cell Bioreductions of Unsaturated Esters with *Shewanella* Yellow Enzyme SYE-3

Similarly, the bioreduction of unsaturated diesters (**13**-**14** & **16**-**17**) was performed using the same SYE-3 expressing cells biotransformation protocol. Dimethyl 2-methylmaleate (**13**) and diethyl 2-methylmaleate (**14**) gave traces of product but in case of dimethyl-2-methylenesuccinate (**16**) and diethyl-2-methylenesuccinate (**16**) no product was observed. (Table 2.2)

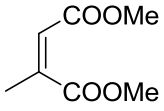
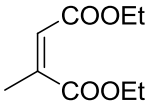
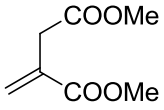
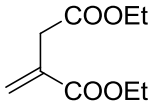
Substrate/Time	3h	6h	24h	Absolute Configuration
 **13**	1%[a] 29.7ee[b]	2.5% 63.5ee	2.7% 64.2ee	*R*
 **14**	1.6% 99.9ee	2.2% 99.9ee	2.5% 99.9ee	*R*
 **16**	nc[c]	nc	nc	nd[d]
 **17**	nc	nc	nc	nd

Table: 2.2 Bioreduction screening results of unsaturated diesters with SYE-3 expressing cells. a) % conversion, b) enantiomeric excess, c) no conversion, d) not determined.

2.1.2.3 Whole Cell Bioreductions of *N*-Substituted Imides with *Shewanella* Yellow Enzyme SYE-3

Bioreductions of *N*-substituted imides with SYE-3 expressing cells showed reasonable results as compared to unsaturated ketones and unsaturated diesters (Table 2.1 & 2.2). The *N*-methyl substituted imide (**23**) was converted fully into reduced product after 15h with SYE-3 expressing cells. With increasing size of substituent at nitrogen (**23**-**28**) the conversion decreases to trace amounts in case of *N*-phenyl substituted imide (**28**). The imides (**29** & **22**) bearing no methyl substituent at 3 position were converted fully to reduced products within 24h even when having large substituents on nitrogen atom. The bioreduction of *N*-substituted imides gave reduced product with high enantiopurity.

So in general SYE-3 tolerates only very small substitutens on imine substrates to give reasonable conversions.

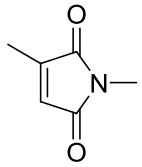
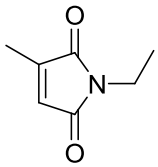
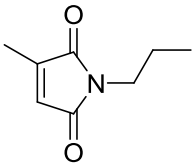
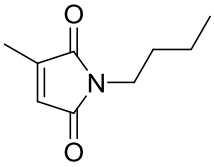
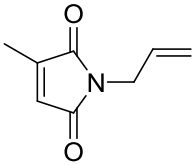
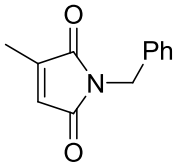
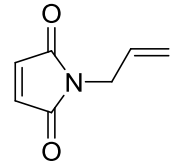
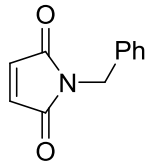
Substrate/Time	3h	6h	24h	Absolute Configuration
 **23**	--	100%[a] 99ee(15h)[b]	--	*R*
 **24**	--	27.6% 99ee(15h)	31.8% 99ee	*R*
 **25**	--	5.6% 99ee(15h)	6.1% 99ee	*R*
 **26**	--	4.5% 99ee(15h)	4.3% 99ee	*R*
 **27**	--	4.7 % 96ee	5% 97ee	*R*
 **28**	--	0.7 % 99ee	0.8% 99ee	*R*
 **29**	--	21 %	99%	na[c]
 **22**	--	30%	98%	na

Table: 2.3 Bioreduction screening results of *N*-substituted imides with SYE-3 expressing cells. a) % conversion, b) enantiomeric excess, c) not applicable.

2.1.2.4 Whole Cell Bioreductions of α,β-Unsaturated Cyclic Ketones with *Shewanella* Yellow enzyme SYE-4

The bioreduction of the above synthesized substrates (Figure 2.9) with SYE-4 expressing cells were also conducted using the same whole cell biotransformation protocol as in case of SYE-3 expressing cells.

In case of SYE-4 expressing cells, bioreduction of 2-methylcyclopent-2-enone (**1**) gave almost full conversion after 24h, but a significant decrease in enantiomeric excess was observed over time. Most likely this can be attributed to a certain acidity of the α-proton in the bioreduction product. Only 2.8% conversion was observed with compound (**3**), while all other ketones (**2**, **8** and **9**) did not give any conversion to reduced products.

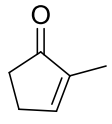
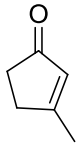
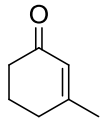
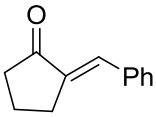
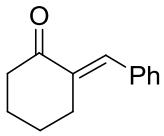
Substrate/Time	3h	6h	24h
 **1**	4.5%[a] 92.9 ee[b]	22.3% 81.1ee	97% 9.6 ee
 **2**	nc[c]	nc	nc
 **3**	nc	nc	2.8% 99.9ee
 **8**	nc	nc	nc
 **9**	nc	nc	nc

Table: 2.4 Bioreduction screening results of unsaturated cyclic ketones with SYE-4 expressing cells. a) % conversion, b) enantiomeric excess, c) no conversion.

2.1.2.5 Whole Cell Bioreductions of Unsaturated Diesters with *Shewanella* Yellow Enzyme SYE-4

Bioreduction of diesters (**13** & **14**) having both ester groups directly attached to the double system by SYE-4 expressing cells gave full conversion to reduced products with high

enantiomeric purity after 24h (Table 2.5). Dimethyl diester (**16**) bearing a terminal double bond gave 33.5% conversion. In contrast, diethyl ester (**17**) was not accepted by SYE-4 expressing cells.

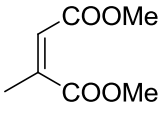
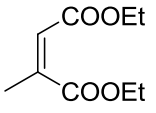
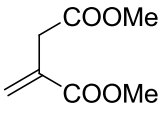
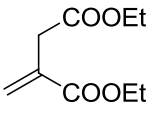
Substrate/Time	3h	6h	24h	Absolute Configuration
 **13**	3.4%[a] 99.9ee[b]	10.8% 99.9ee	100% 99.9ee	*R*
 **14**	14.8% 99.9ee	39.3% 99.9ee	100% 99.9ee	*R*
 **16**	0.3% 99.9ee	--	33.5% 99.9ee	*R*
 **17**	nc[c]	nc	nc	na[d]

Table: 2.5 Bioreduction screening results of unsaturated diesters with SYE-4 expressing cells. a) % conversion, b) enantiomeric excess, c) no conversion, d) not applicable

2.1.2.6 Whole Cell Bioreductions of *N*-Substituted Imides with *Shewanella* Yellow Enzyme SYE-4

N-Substituted imides (**23** & **24**) bearing *N*-methyl and *N*-ethyl substituent at nitrogen gave full conversion with high stereoselectivity within 15h. Imides **25** to **28** showed no significant conversion with SYE-4 expressing cells as the size of substituent increases. Imides **29** & **22** gave full conversion as there was no methyl substituent with the double bond. The enantiomeric excess values in all reduced products displayed high enantioselectivity for the bioreduction.

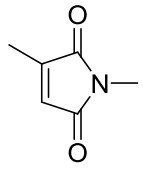
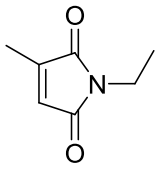
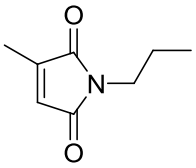
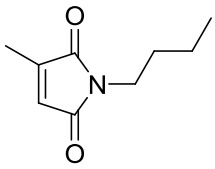
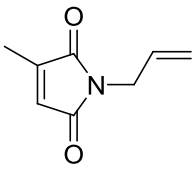
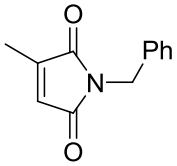
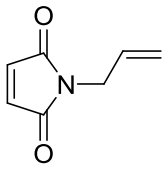
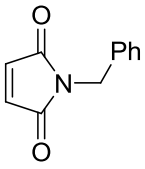
Substrate/Time	3h	6h	24h	Absolute Configuration
 **23**	--	100%[a] 99ee(15h)[b]	--	*R*
 **24**	--	100% 99ee(15h)	--	*R*
 **25**	--	18.2% 99ee(15h)	18.3% 99ee	*R*
 **26**	--	4.1% 99ee(15h)	6.8% 99ee	*R*
 **27**	--	1 % 96ee	2.4% 97ee	*R*
 **28**	--	nc[c]	nc	na[d]
 **29**	--	62 %	100%	na
 **22**	--	99.9%	100%	na

Table: 2.6 Bioreduction screening results of substituted imides with SYE-4 expressing cells.
a) % conversion, b) enantiomeric excess, c) no conversion, d) not applicable

2.1.2.7 Isolation of Crude Cell Extract Proteins

In some substrates there may be problems for penetrating through the cell wall during the whole cell biotransformation, so crude cell extract (CCE) proteins were isolated from SYE-3 & SYE-4 expressing cells. Cells grown in $TB_{Chlamph}$ media were induced by IPTG solution for 15h. After collecting cell pellets by centrifugation, cells were suspended in PBS and ruptured by sonication using pulse for 10 seconds after every 1 min. After sonication, suspension was centrifuged to get cell lysate, which was stored at -20°C. Bradford assay was conducted to calculate the concentration of protein in cell lysate.

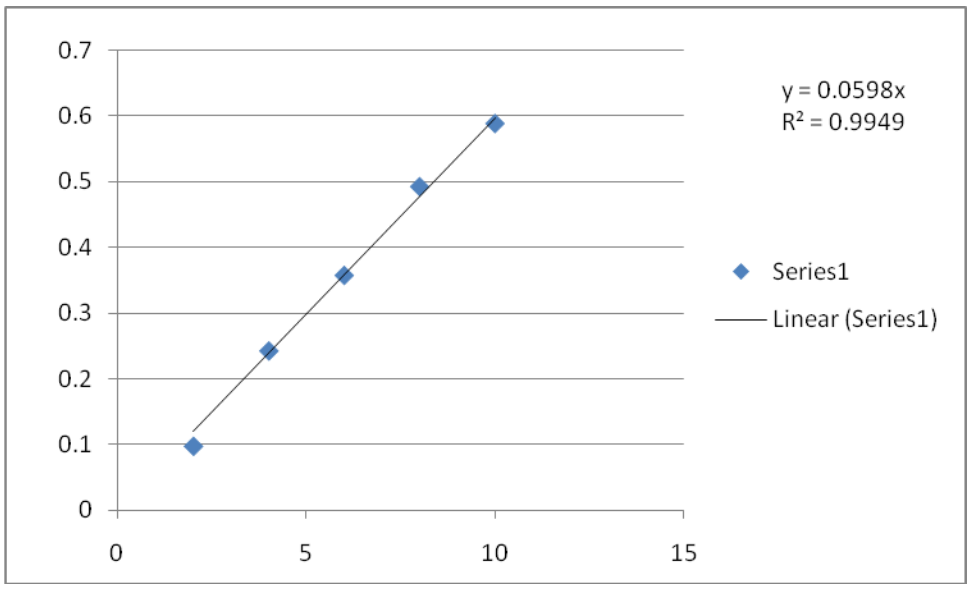


Figure 2.10 Standard curve of Bradford assay for the protein concentration

Protein concentrations from BL21 (DE3) Gold cells

SYE-3 = 24.2 mg/mL

SYE-4 = 15.3mg/mL

In another attempt protease inhibitor (benzyl sulfonyl fluoride 1µL/mL) was added to improve the concentration of proteins in cell extract. Glycerol was also added as additional nutrition in the TB media. So as a result increase in concentration was observed with these modifications.

SYE-3 = 33.4 mg/mL

SYE-4 = 52.2mg/mL

Biotransformations of above substrates (Figure 2.9) were performed with these CCEs using the cofactor recycling system. It was reported that SYE-3 is NADH dependent and SYE-4 is NADPH dependent for cofactor recycling.[91]

Bioreduction screening experiments with CCE were performed in sterile multiwell plates in the presence of Tris HCl (pH 8), NAD^+ (SYE-3) or $NADP^+$ (SYE-4), glucose-6-phosphate, glucose-6-phosphate dehydrogenease and substrate at 28°C. Analysis was conducted by chiral GC and results are shown in Table 2.7-12. Results of above substrates (Figure 2.9) clearly indicate the improvement in conversion and reaction time as compare to whole cell biotransformations.

2.1.2.8 Bioreductions of α,β-Unsaturated Cyclic Ketones with *Shewanella* Yellow Enzyme SYE-3 CCE

Bioreduction of α,β-unsaturated cyclic ketone (**1-3**) with SYE-3 CCE gave almost no conversion but **8** & **9** showed little conversion, again with decreasing enantiomeric excess over time.

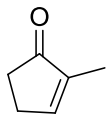
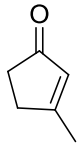
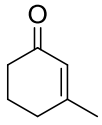
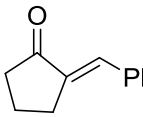
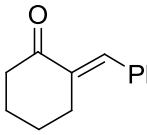
Substrate/Time	1h	3h	6h	Overnight
 **1**	nc[a]	nc	nc	--
 **2**	nc	nc	nc	--
 **3**	nc	nc	nc	--
 **8**	0.6%[b] 65ee[c]	2% 44ee	2% 41ee	--
 **9**	0.9% 99ee	5.5% 73.3ee	6.5% 65ee	--

Table: 2.7 Bioreduction screening results of unsaturated ketones with SYE-3 CCE. a) no conversion, b) % conversion, c) enantiomeric excess.

2.1.2.9 Bioreduction of Unsaturated Diesters with *Shewanella* Yellow Enzyme SYE-3 CCE

As compared to whole cell bioreductions, SYE-3 CCE gave improved results. Diethyl ester (**14**) was fully converted to reduced product after only 3h with 99% ee. However the dimethyl ester (**13**) was converted to reduced product with only 14.6% with decrease in enantiomeric excess after overnight reaction. But in case of whole cell biotransformations none of these diesters gave any significant product peak in GC. (Table 2.8)

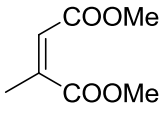
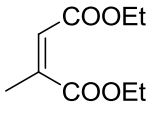
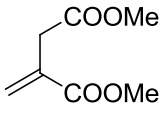
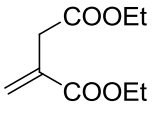
Substrate/Time	1h	3h	6h	Overnight	Absolute Configuration
 **13**	3.7%[a] 12ee[b]	6.8% 37.3ee	9.1% 51.6ee	14.6% 25ee	*R*
 **14**	65% 99.9ee	100% 99.9ee	--	--	*R*
 **16**	nc[c]	nc	nc	--	na[d]
 **17**	nc	nc	nc	--	na

Table: 2.8 Bioreduction screening results of unsaturated diesters with SYE-3 CCE. a) % conversion, b) enantiomeric excess, c) no conversion, d) not applicable

2.1.2.10 Bioreductions of *N*-Substituted Imides with *Shewanella* Yellow Enzyme SYE-3 CCE

Conversions of all imides (**22-29**) with SYE-3 CCE showed significant improvement as compare to whole cell biotransformations. Enantioselectivity was also a prominent factor in this conversion. *N*-Mmethyl substituted imide (**23**) was fully reduced to the product after 1h with 99% ee. The increase of substituent size at nitrogen atom decreased the rate of reaction, as in (**24**) complete conversion was observed after overnight reaction. Imids (**25** & **26**) also gave very good conversion to reduced products after overnight reaction. However imides with phenyl and allyl substituents (**27** & **28**) at nitrogen were converted to product at low rates. The imides without C-methyl substituent (**22** & **29**) were also fully converted to products (Table 2.9).

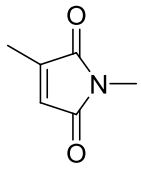
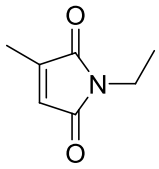
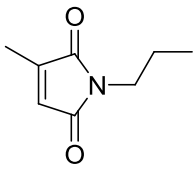
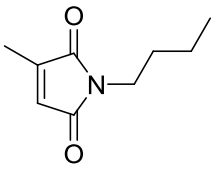
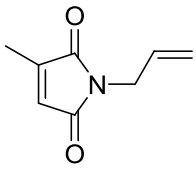
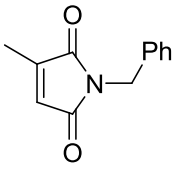
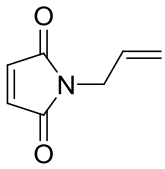
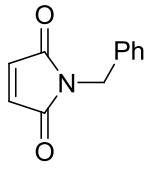
Substrate/Time	1h	3h	6h	Overnight	Absolute Configuration
 **23**	100%[a] 99.9ee[b]	--	--	--	*R*
 **24**	16% 99.9ee	67% 99.9ee	71% 99.9ee	99% 99ee	*R*
 **25**	9.3% 99.9ee	27% 99.9ee	44.4% 99.9ee	96.4% 99.7ee	*R*
 **26**	5.3% 99ee	28.1% 99ee	64% 99ee	85% 99ee	*R*
 **27**	6% 99ee	19% 99ee	20% 99ee	--	*R*
 **28**	6.2% 99ee	26% 99ee	31% 99ee	--	*R*
 **29**	99% na[c]	--	--	--	na
 **22**	99% na	--	--	--	na

Table: 2.9 Bioreduction screening results of substituted imides with SYE-3 CCE. a) % conversion, b) enantiomeric excess, c) not applicable.

2.1.2.11 Bioreductions of α,β-Unsaturated Cyclic Ketones with *Shewanella* Yellow Enzyme SYE-4 CCE

The results from biotransformation of the various substrates with SYE-4 CCE are summarized in the Tables 2.10-2.12. The bioreduction of 2-methylcyclopent-2-enone (**1**) resulted in the decrease of enantiomeric excess to 52% with the passage of time. The overall conversion of the substrate was 92% after 6h time. Ketones (**2** & **3**) gave no conversion even after 6h time; this may be attributed to the position of the substituent on these cyclic ketones.

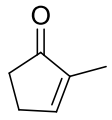
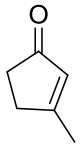
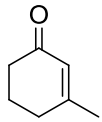
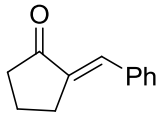
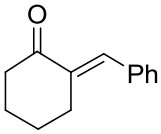
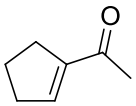
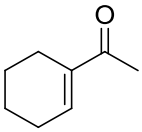
Substrate/Time	1h	3h	6h
 **1**	67.6%[a] 79.3ee[b]	77.4% 68.6ee	92.2% 52.8ee
 **2**	nc[c]	nc	nc
 **3**	nc	nc	nc
 **8**	3.73% 5ee	1% 1.4ee	3.7% 1.4ee
 **9**	64.4% 60ee	83.7% 51.1ee	92.2% 36.3ee
 **4**	--	60% (12h)	71% (24h)
 **5**	--	5% (12h)	9% (24h)

Table: 2.10 Bioreduction screening results of α,β-unsaturated cyclic ketones with SYE-4 CCE. a) % conversion, b) enantiomeric excess, c) no conversion.

Substrate (**1**) having substituent at position 2 was reactive towards enzymes whereas cyclic ketones (**2** & **3**) bearing substituents at position 3 were not accepted by the SYE-4 protein.

Similar results were obtained using substrates (**8** & **9**)[142] providing saturated products with only moderate ee values. In case of **9** ee value was found to be 36.3%, after 92.2% conversion within 6h biotransformation time. Compounds **1**, **8** and **9** possess structural similarities, so they behave in a similar manner for the enzymes. Similar problems were reported by the Faber group using different EREDs with these substrates.[93,145,146]. Furthermore, cyclic ketones (**4** & **5**) were reduced to saturated ketones with SYE-4 CCE in reasonable to good yields (Table 2.10).

2.1.2.12 Bioreductions of Unsaturated Diesters with *Shewanella* Yellow Enzyme SYE-4 CCE

Substrates with diester functionality gave good to acceptable results with high enantioselectivity. Compounds **13** & **14**, having both the ester group directly linked with double bond, were fully converted to yield enantiomerically pure. In contrast, substrates **16** & **17** displayed little or almost no conversion, probably due to the attachment of a methylene group to the double bond. So *exo*-methylene analogs **16** & **17** proved to be difficult substrates[37] (Table 2.11).

Although α,β-unsaturated esters were suspected to be good substrates for EREDs,[109] it turned out that ester hydrolysis occurred first when expressing cells were used, which rendered the corresponding α,β-unsaturated carboxylic acids as the actual substrates. This drawback was successfully circumvented by using isolated enzymes instead of whole cell systems. By SYE-4 CCE undesired ester hydrolysis was entirely eliminated.

---

[145] Hall, M., Stueckler, C., Kroutil, W., Macheroux, P., Faber, K., *Angew. Chem.* **2007**, *119*, 4008–4011.
[146] Hall, M.; Stuekler, C.; Hauer, B.; Stuermer, R.; Friedrich, T.; Breuer, M.; Kroutil, W.; Faber, K. *Eur. J. Org. Chem.* **2008**, 1511-1516.

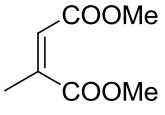
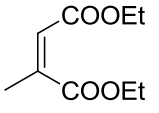
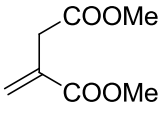
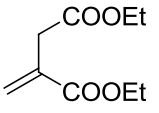
Substrate/Time	1h	3h	6h	Absolute Configuration
 **13**	70%[a] 99.9ee[b]	76.4% 99.9ee	99% 98ee	*R*
 **14**	65% 99.9ee	99% 99.9ee	99% 99.9ee	*R*
 **16**	5.4% 99.9ee	13.3% 99.9ee	19.3% 89.6ee	*R*
 **17**	nc[c]	nc	nc	na[d]

Table: 2.11 Bioreduction screening results of unsaturated diesters with SYE-4 CCE. a) % conversion, b) enantiomeric excess, c) no conversion, d) not applicable.

2.1.2.13 Bioreductions of *N*-Substituted Imides with *Shewanella* Yellow Enzyme SYE-4 CCE

The compounds with imide functionality (**22-29**) were investigated to extend the substrate tolerance of SYE-4 protein on complex cyclic carboxylic acid derivatives.

Such α-methylmaleimides turned out to be excellent substrates for SYE-4 CCE, with almost 100% conversion and high enantioselectivity within 1-3h of biotransformation (Table 2.12). These imides were also reported as substrates for OPR1, OPR3, YqjM and other OYE within previous publications[146] but we investigated in detail the substituents tolerance in terms of stereoslectivity and conversion. We observed that large variety of substuents was tolerated with high stereroselectivity (only*(R)*-configuration).

The tolerance for the functional groups proved to be remarkably broad. Compounds **27** & **29** bearing two cumulated C=C double bonds, only α,β-bond was reduced selectivity, whereas the non activated double bond remained intact.

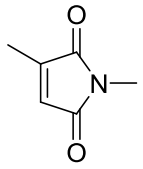
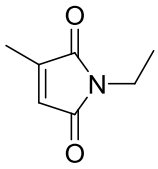
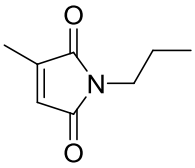
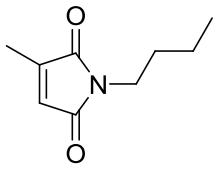
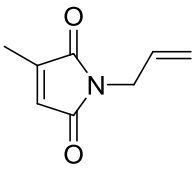
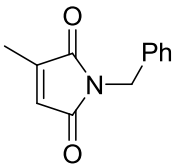
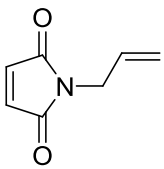
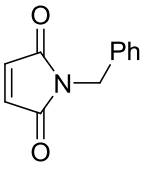
Substrate/Time	1h	3h	6h	Absolute Configuration
 **23**	99%[a] 99.9ee[b]	99% 99.9ee	--	*R*
 **24**	86% 99.9ee	99% 99.9ee	--	*R*
 **25**	96.2% 99.9ee	99.6% 99.9ee	--	*R*
 **26**	97.6% 99ee	99% 99.9ee	--	*R*
 **27**	99% 99.7ee	99% 99ee	--	*R*
 **28**	98.4% 99.9ee	99% 99.9ee	--	*R*
 **29**	99% na[c]	--	--	*na*
 **22**	98% na	--	--	*na*

Table: 2.12 Bioreduction screening results of substituted imides with SYE-4 CCE
a) % conversion, b) enantiomeric excess, c) not applicable.

2.1.2.14 pH Studies for SYE-4 CCE

In order to investigate the loss in enantiomeric excess with time in biotransformations of cyclic ketones (**1**, **8** and **9**) screening with SYE-4 cell extract proteins was conducted at three different pH values acidic (4.5), neutral (7) and basic (9) (Table 2.13). The optimal pH used by VAN Beeumen and coworkers[91] for the SYE-4 proteins studied by was 7.3. Result in the table indicated that there was acceleration at pH 9 as compare to neutral and acidic pH but no significant difference in loss of enantiomeric excess on different pH values.

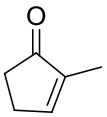
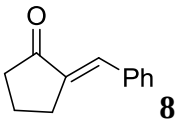
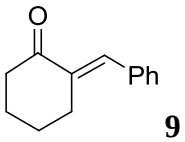
Time	pH	 **1**	 **8**	 **9**
	4.5	21%[a] (77.7ee)[b]	7% (27ee)	91% (62.3ee)
1h	7	18% (80ee)	5.5% (26ee)	77% (62ee)
	9	18% (81ee)	4% (22ee)	80% (61ee)
	4.5	25% (74ee)	13% (18ee)	95% (60ee)
3h	7	25% (70ee)	6.7% (28ee)	93% (60ee)
	9	66% (77ee)	13% (20ee)	93% (60ee)
	4.5	90% (60ee)	49% (34ee)	Decom.
6h	7	25% (64ee)	18% (32ee)	Decom.
	9	99% (56ee)	--	95% (57ee)

Table: 2.13 pH studies for SYE-4 CCE. a) % conversion, b) enantiomeric excess.

2.1.2.15 Determination of Absolute Configuration

Scale up reactions were conducted for diesters and imides which were fully converted to products with high enantiomeric excess using SYE-4 CCE. Absolute configurations of bioreduction products were determined by comparing the sign of optical rotation of reference compounds from the literature with products isolated from biotransformations. The absolute configuration of bioreduction products of dimethyl 2-methylsuccinate (**13**) and diethyl

2-methylsuccinate (**14**) was determined and both dimethyl 2-methylsuccinate (**19**) and diethyl 2-methylsuccinate (**20**) were identified as *R*-enantiomers.

The product from 1-benzyl-3-methyl-1H-pyrrole-2,5-dione (**28**) was chosen as representative case study for the absolute configuration determination and all other imides were considered similar to that. The absolute configuration of 1-benzyl-3-methylpyrrolidine-2,5-dione (**30**) was also found to be *R*-enantiomer.

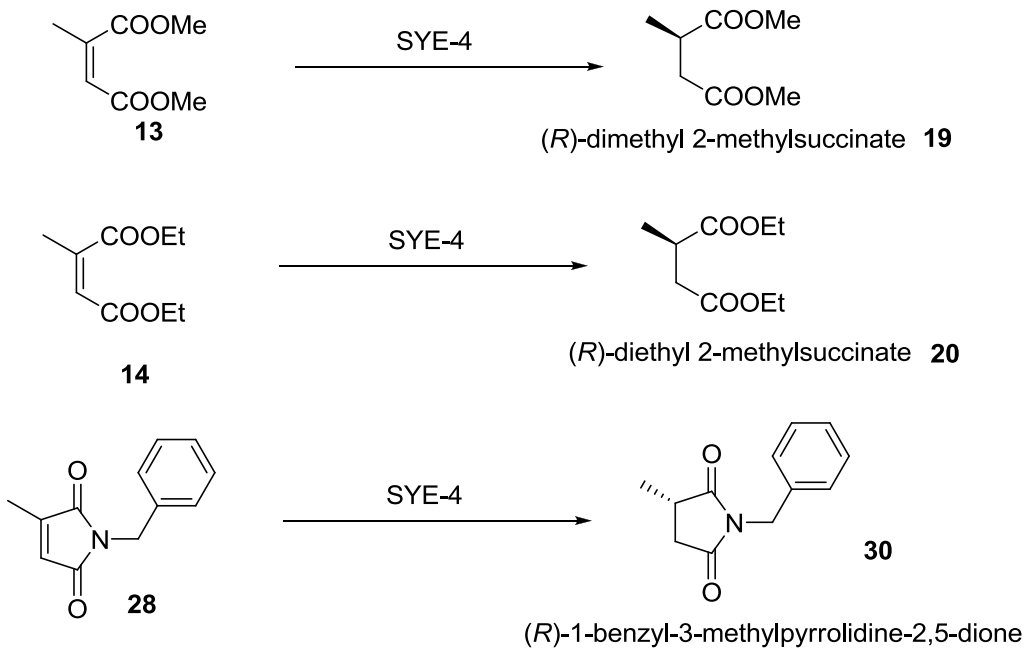


Figure 2.11 Scale up bioreduction with SYE-4 CCE

2.2 Bioreduction of Baylis Hillman Products

2.2.1 Synthesis of Baylis Hillman Products

The Baylis Hillman reaction represents the coupling of an activated alkene derivative with an aldehyde and is catalyzed by a tertiary amine (for example: DABCO = 1,4-diaza-bicyclo[2.2.2]octane). Applying general Baylis Hillman conditions adducts was synthesized by using aldehyde (aromatic and aliphatic), acrylate (methyl acrylate and acrylamide), and DABCO in good to moderate yields. The reaction of benzaldehyde with methyl acrylate in the presence of DABCO gave product (**31**) after several days stirring (Figure 2.12).

The Baylis Hillman adduct methyl 2-(hydroxy-(phenyl)-methyl) acrylate (**31**) is a very important model substrate for the subsequent bioreduction studies.

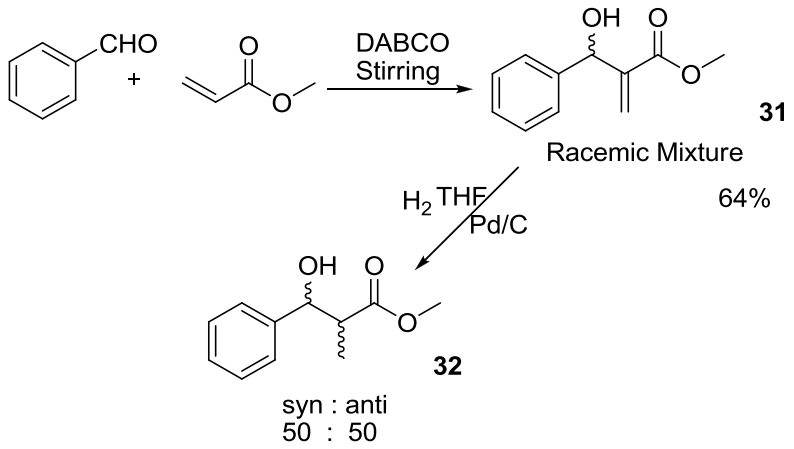


Figure 2.12 Synthesis of Baylis Hillman products and reduction

Baylis Hillman reactions in the absence of a chiral catalyst end up with racemic mixtures. These substrates having a double bond attached with electron withdrawing groups fulfill the requirement for the bioreduction through ene-reductases. Catalytic reduction of double bond without the presence of chiral catalyst gave racemic mixtures of 50% *syn* and 50% *anti* diastereoisomers (as shown in Figure 2.13).

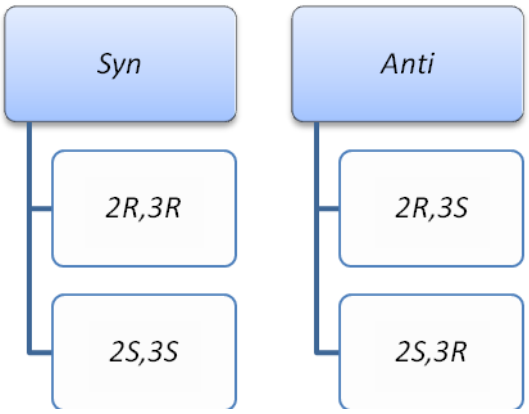


Figure 2.13 Possible diastereoisomers after reduction of Baylis Hillman products

Theoretically, in the presence of a chiral catalyst reduction products will be enantiomerically pure *syn* and *anti* diastereoisomers in 50:50 mixture upon complete consumption of racemic starting material. Ene-reductases offer the prospect of highly appealing chiral biocatalysts for these types of substrates bearing a chiral centre.

2.2.1.1 Synthesis of Baylis Hillman Products with Aliphatic Substituent

To cover the whole range of structure diversity Baylis Hillman products were synthesized using different aliphatic aldehydes under normal Baylis Hillman condition. The synthesis was carried out using general Baylis Hillman procedure[147] by mixing aldehyde in methyl acrylate with catalytic amount of DABCO and stirring at room temperature for several days. The yields of these reactions were moderate to good (Figure 2.14).

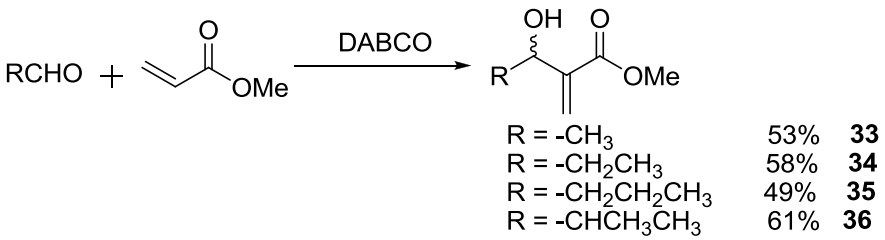


Figure 2.14 Synthesis of Baylis Hillman products with aliphatic substituent

[147] Holz, J.; Schäffner, B.; Zayas, O.; Spannenberg, A.; Bcrnera, A. *Adv. Synth. Catal.* **2008**, *350*, 2533–2543.

2.2.1.2 Synthesis of Baylis Hillman Products with Aromatic Substituent

Using aromatic aldehydes, methyl acrylate and DABCO under basic Baylis Hillman conditions a variety of products was synthesized with moderate to good yield (Figure 2.15).

Figure 2.15 Synthesis of Baylis Hillman products with aromatic substituent

2.2.1.3 Synthesis of Baylis Hillman Products with Heterocyclic Substituent

To extend the substituent diversity in the substrates for bioreduction the synthesis of Baylis Hillman compounds with heterocyclic substituents was carried out, following the procedure of Hu et al.[148], using aldehyde, methyl acrylate, and DABCO dissolved in a mixture of dioxane and water (Figure 2.16).

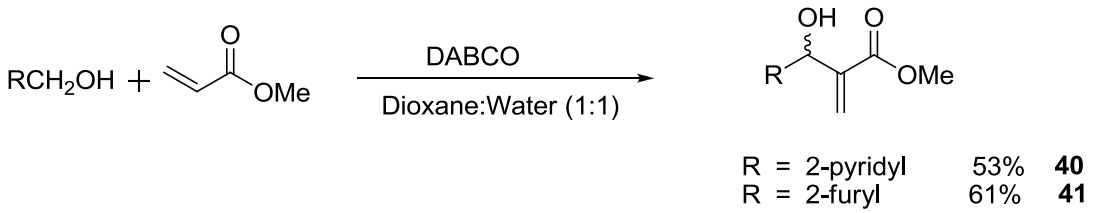


Figure 2.16 Synthesis of Baylis Hillman products with heterocyclic substituent

[148] Yu, C.; Liu, B.; Hu, L. *J. Org. Chem*. **2001**, *66*, 5413–5418.

2.2.1.4 Synthesis of Baylis Hillman Products with Acrylonitrile and Acryl Amide

Other modification of the Baylis Hillman reaction for the bioreduction studies exploited the use of acrylonitrile and acryl amide instead of methyl acrylate. Compound (**42**) was synthesized by the reaction of benzaldehyde with acrylonitrile in the presence of DABCO after 3 days stirring. Similarly, the reaction of benzaldehyde with acrylamide in the presence of dioxane and water mixture as a solvent gave product (**43**) in 43% yield. However, Baylis Hillman reaction of *p*-chlorobenzaldehyde with acrylamide under these conditions was not possible, so *t*-BuOH and water mixture was used as solvent instead of dioxane and water. Catalytic amounts of phenol were also added to enhance the reaction. The origin of the autocatalysis was the hydroxyl group of the Baylis-Hillman product, which promoted the reaction through hydrogen bonding. This observation led to the addition of hydrogen bond donors to further enhance the rate of the reaction. Although the exact role of phenol as additive is as yet unclear, it was proposed that phenol can act both as a Brønsted acid alkoxide scavenger and as a H-bonding (or Brønsted acid) catalyst in its own right.[149] As a result 51% yield of isolated product (**44**) was obtained after chromatography (Figure 2.17).

These reaction conditions did not work for the the synthesis of (**45**) from furfural and acrylamide. Hence, base catalysis was investigated for this reaction. Aggarwal et al.[150] reported quinuclidine in the presence of methanol as a better catalyst than DABCO for the Baylis Hillman reaction of acrylamide with different aldehydes. So reaction of furfural with acrylamide in the presence of quinuclidine and methanol gave the desired product (**45**) in 61% purified yield (Figure 2.17).

---

[149] Cornelia, F.; Eimear, M. F.; Stephen, J. C. *J. Org. Chem.* **2004,** *69*, 6496-6499.
[150] Aggarwal, V. K.; Emme, I.; Fulford, S. Y. *J. Org. Chem*, **2003,** *68*, 3, 692-700.

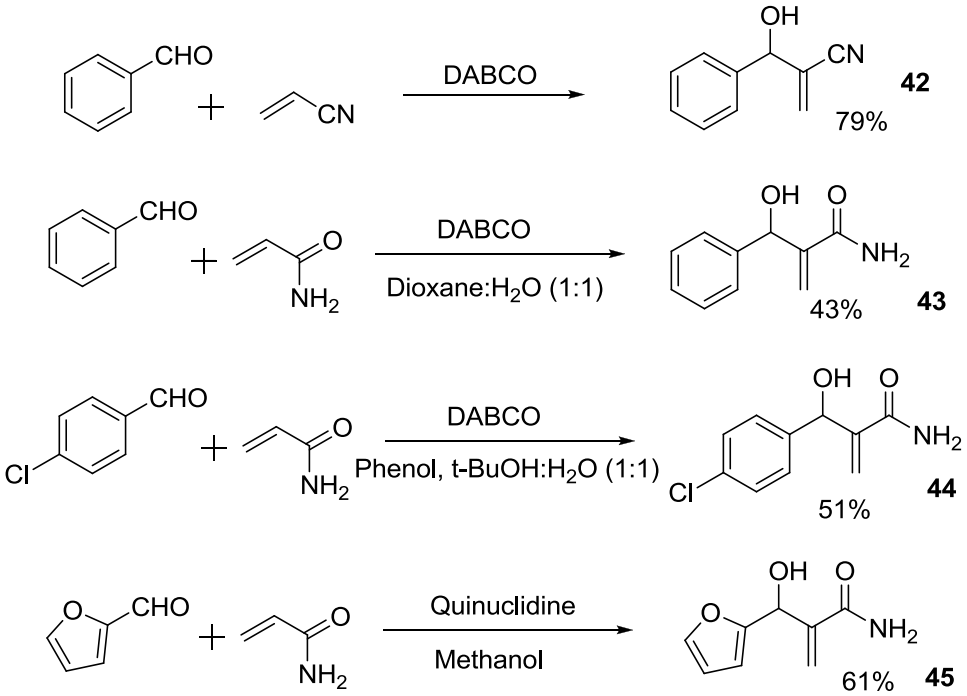


Figure 2.17 Synthesis of Baylis Hillman products with acryl amide and acrylonitrile

2.2.1.5 Synthesis of Derivatives of Baylis Hillman Products

Baylis Hillman products with different modification were synthesized through different routes for ultimate application in bioreductions. The Baylis Hillman ester (**46**) synthesized from normal protocol using DABCO and relative aldehyde. The ester (**46**) was hydrolyzed to the acid (**47**) with potassium hydroxide in methanol with good yield. (Figure 2.18)

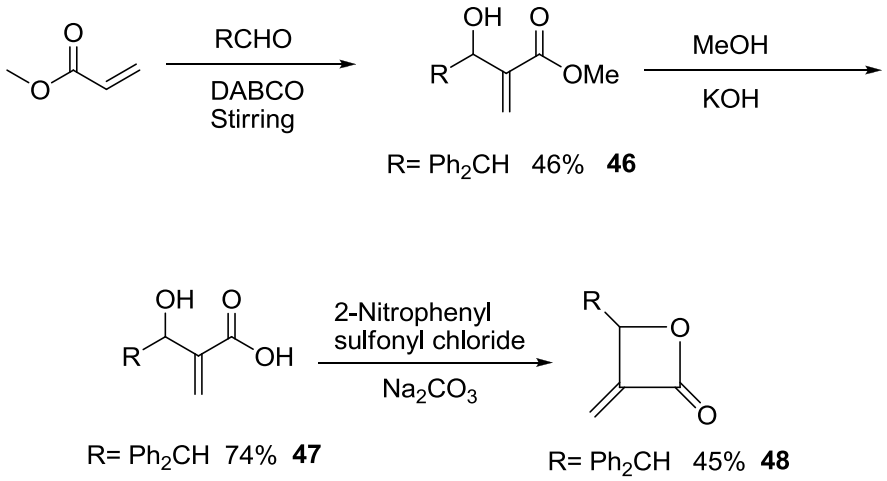


Figure 2.18 Synthesis of lactone (**48**)

2-Nitrophenyl sulfonyl chloride, a common promoter of lactonization of β-hydroxy acid (**47**), in the presence of sodium carbonate provided (**48**) in reasonable yield.[151]

Synthesis of lactam ring compound for bioreduction studies from the Baylis Hillman products was investigated. Formation of the corresponding amid from acid (**47**) with *p*-anisidine as nitrogen source and dicyclohexylcarbodiimide (DCC) as coupling agent was investigated. The hydroxy amide (**49**) was converted to the mesyl amide (**50**) in good yield with mesyl chloride and triethylamine. The cyclization of the mesyl amide (**50**) to lactam (**51**) was not successful following a literature protocol [152] (Figure 2.19).

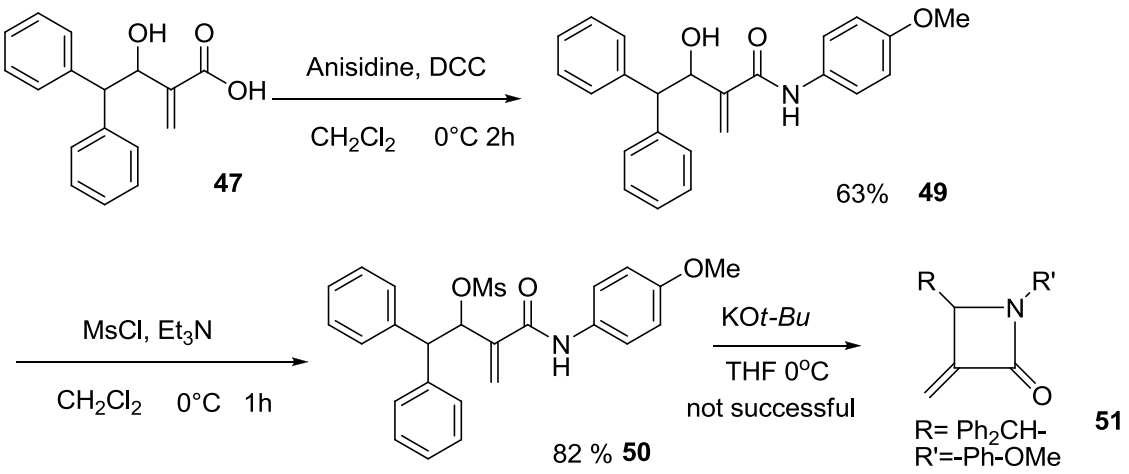


Figure 2.19 Synthesis of lactam (**51**)

2.2.2 Synthesis of Aza-Baylis Hillman Products

It was found that the amine (DABCO) catalyzed reaction of acrylates with imine compounds is much faster than that with common aldehydes at room temperature. Keeping in mind this factor hydoxy group of Baylis Hillman adducts was replaced with nitrogen functionality through the synthesis of imines and then treating with methyl acrylate in presence of DABCO for overnight. Imine (**52**) was synthesized by the reaction of tosyl amine and benzaldehyde in the presence of

[151] Isamir, M.; Alexander, E. A.; Joseph, D. E.; Albert, J. N.; Jun, W.; Amy, R. H. *Org. Lett.*, **2003,** *5*, 399-402.
[152] Waldemar, A.; Peter, G.; Hans-Ulrich, H.; Chantu, R. S. *J. Org. Chem.* **2000,** *65*, 4919-4922.

triflouroacetic anhydride using CH_2Cl_2 as solvent, which was then condensed with methyl acrylate to obtain product (**53**) in moderate yield[153].

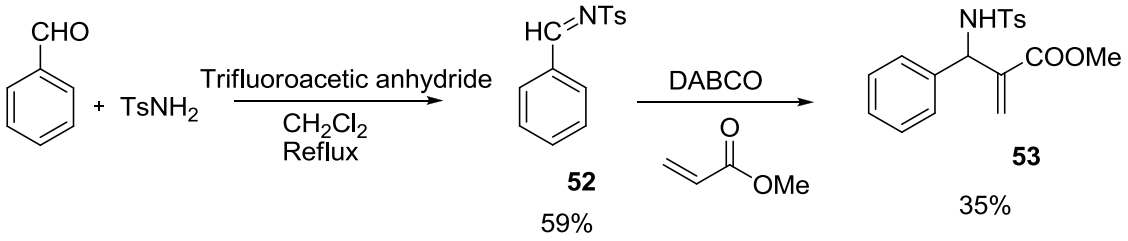


Figure 2.20 Synthesis of carbamate (**53**)

However the synthesis of imine (**54**) was not successful by the above method, which was then synthesized by the reported procedure.[154] Benzaldehyde was converted by using *n*-butyl lithium and hexamethyldisilazane (HMDS) to get silyl imine (**54**).[155] *N*-Moc imine (**55**) was prepared by acylation of the corresponding *N*-silylimine (**54**) in the presence of methyl chloroformate.[156] This *N*-Moc imine (**55**) undergoes aza Baylis Hillman reaction with methyl acrylate in the presence of DABCO to the desired product (**56**) in 59% yield using a reported procedure.[157]

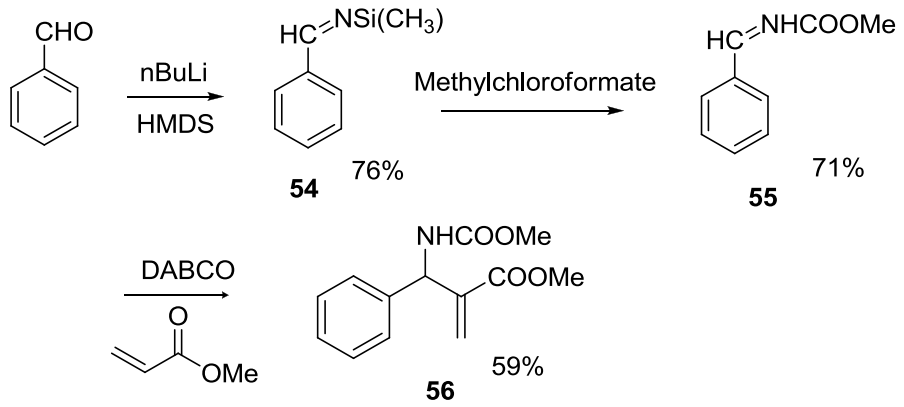


Figure 2.21 Synthesis of carbamate (**56**)

---

[153] Xu, X.; Wang, C.; Zhou, Z.; Tang, X.; He, Z.; Tang, C. *Eur. J. Org. Chem.* **2007**, 4487–4491.
[154] a) Hart, D. J.; Kanai, K-I.; Thomas, D. G.; Y, T-K. *J. Org. Chem*. **1983**, *48*, 289-294. b) Vidal, J.; Damaestoy, S.; Guy, L.; Hannachi, J-C.; Aubry, A.; Collet, A. *Chem. Eur. J.* **1997**, *3*, 1691-1709.
[155] Vidal, J.; Damaestoy, S.; Guy, L.; Hannachi, J-C.; Aubry, A.; Collet, A. *Chem. Eur. J.* **1997**, *3*, 1691-1709.
[156] Kupfer, R.; Meier, S.; Wurthwein, E. U. *Synthesis.* **1984,** 688-690.
[157] Yamamoto, K.; Takagi, M.; Tsiji, J. *Bull.Chem. Soc. Jpn.* **1988**, *61*, 319-321.

The above protocol for the synthesis of compound (**56**) was not successful when substituted benzaldehydes (4-chloro, 4-methyl, and 4-methoxy) were used. The main problem observed is associated to limited stability and problematic purification (distillation) of *N*-silylimine and *N*-Moc imines as intermediates derived from substituted benzaldehydes.

Kim and coworkers[158] introduced nucleophiles (carbamates) at the secondary position of Baylis Hillman adducts regio-selectively using Baylis Hillman allylic bromides. Simple Baylis Hillman products (**31**, **37-38**) were converted into allylic bromide treating with HBr solution in the presence of sulphuric acid using the reported protocol.[159] These allylic bromides were treated with nucleophilic carbamtes (ethyl carbamate and methyl carbamte) in the presence of DABCO base gave aza Baylis Hillman products. By this method different aryl substituted products (**61-67**) were synthesiszed in moderate yields which are not accessible by other methods. Electon withdrawing and electron donating substituents were employed in this synthesis, which gave a variety of substartes for the bioreduction studies.

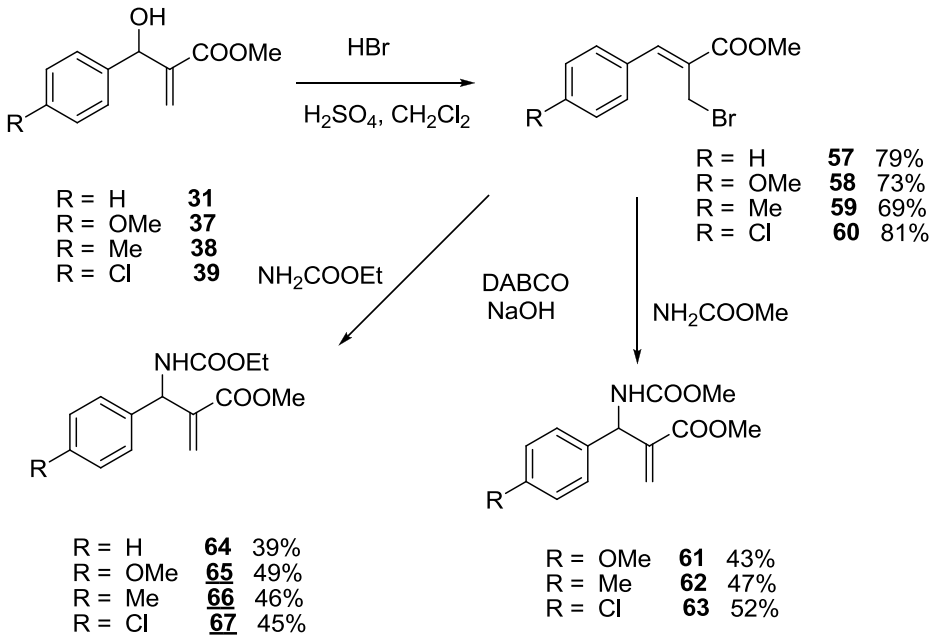


Figure 2.22 Synthesis of Aza Baylis Hillman Products (**61-67**)

[158] Lee, K. Y.; Kim, T. H.; Kim J. N. *Bull. Korean Chem. Soc*. **2004**, *25*, 12
[159] Buchholz, R.; Martin, H.; Hoffmann, R. *Helv. Chim. Acta*. **1991,** 74, 1213-1220

This strategy was extended for the synthesis of aza Baylis Hillman products with heterocylic substituents (**69** & **70**). Allylic bromide synthesis from the Baylis Hillman compound (**41**) was tricky, as it was not stable under acidic condition at room temperature. So, compound (**68**) was prepared at -20°C and utilized for the synthesis of aza Baylis Hillman product (**69** & **70**) with carbamates.

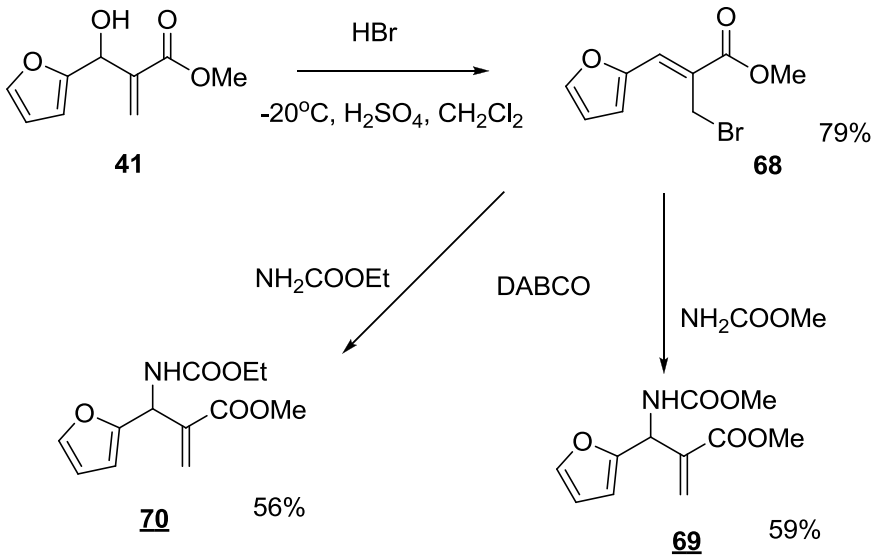


Figure 2.23 Synthesis of Aza Baylis Hillman Products (**69**-**70**)

2.2.3 Bioreduction of Baylis Hillman Adducts

This diverse range of substrates with different modifications was applied to get insight into enzyme selectivity of ERED biotransformations. Bioreductions of these Baylis Hillman products with different Ene-reductases was conducted. Only YqjM enzyme gave reduced products in appreciable amounts; all other enzymes showed no activity with the Baylis Hillman substrates.

2.2.3.1 Bioreduction of Baylis Hillman Product (31)

The bioreduction of compound (**31**) with YqjM enzyme gave 90% conversion after 48h time with enantiomerically pure *syn* (**32**) and *anti* (**32**) products in ratio of 65:35 (results in Table 2.22). The *syn* (**32**) diastereoisomer was found in excess which was confirmed later by spectroscopic data after scale up reaction. The *syn* (**32**) and *anti* (**32**) diastereoisomers were enantiomerically pure according to chiral GC.

Figure 2.24 Bioreduction Baylis Hillman product (**31**)

This *syn*-selectivity did not originate from the enzymatic bioreduction with ene-reductases because we were starting from racemic mixture of Baylis Hillman adducts and enzymic reduction should give only 50:50 mixture of *syn* and *anti* product. The mechanism of bioreduction of ene-reductase shows that only *anti* addition of hydrogen around the double bond is possible. For example *R* enantiomer can only give *syn RR* and *S* enantiomer gives *SR*.

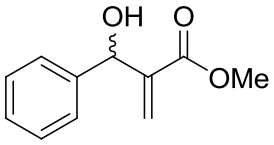
Enzyme	**Time**	 **(31)**
YqjM	24h	70%[a] 65:35[b] (99:98)ee[d]
	48h	90% 65:35 (99:98)ee
OPR1	24h	nc[c]
	48h	nc
OPR3	24h	nc
	48h	nc
SYE-4	24h	nc
	48h	nc

Table 2.14 Bioreduction of Baylis Hillman product (**31**). a) conversion, %, b) diastereomeric ratio, dr, *syn:anti*, c) no conversion, d) enantiomeric excess, (*syn:anti*)

The bioreduction of double bond with ene-reductases can only give one absolute configuration on the new chiral centre. The possibility of *S* enantiomer to *syn RR* diastereoisomer is also possible through the proposed mechanism as shown in Figure 2.25.

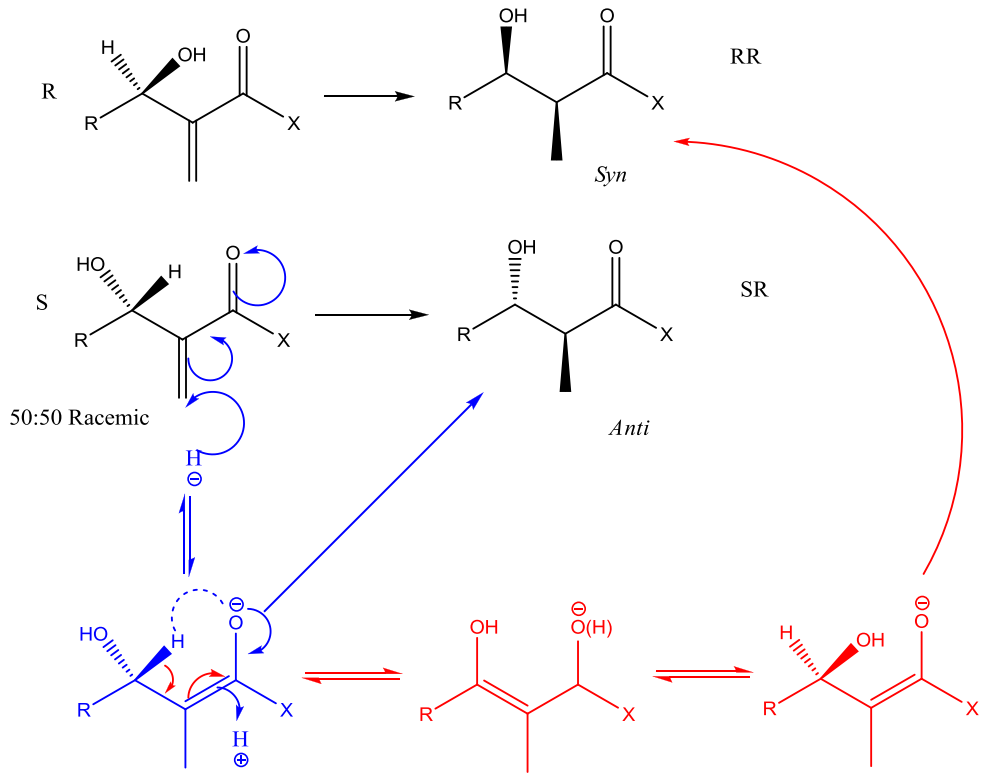


Figure 2.25 Proposed mechanism for *syn*-selectivity

There are two hypotheses to explain this particular behavior in ERED mediated reductions of Baylis Hillman substrates:

According to this proposed mechanism the hydride attacks on the β-carbon followed by a shift of double bond in conjugation with carbonyl group. Then the proton capture from tyrosine residue to give *anti* addition of hydrogen. This is the normal way to obtain *syn* diastereoisomer from *R* and *anti* from *S* enantiomers (labeled as blue in Figure 2.25). If there is a possibility of rearrangement after the attack of hydride ion on β-carbon this may lead to loss of the stereocentre in the *S* enantiomer. Reverse reaction of this rearrangement can give *R* configuration on carbinol carbon upon protonation to ultimately form a *syn* product (labeled as red in Figure 2.25). This probability is very low in case of *R* enantiomer due to position of carbinol proton which does not allow the rearrangement of double bond and loss of stereogenic centre. So *R* enantiomer can only give *syn RR* diastereoisomer. Time screening experiments of compound (**31**) with YqjM enzyme were performed to check the *syn* selectivity originating from the beginning. Samples were analyzed after certain time until 48h, as shown in results Table 2.15. Diastereomeric ratio of *syn* and *anti* remain same from beginning 60:40 to 65:35.

Time	12h	24h	30h	48h
Conversion(%)	10	53	72	93
Diastereomeric ratio (dr) (*syn:anti*)	61:39	63:37	68:32	66:34
Enantiomeric excess ee (*syn:anti*)	99:98	99:97	99:97	98:97

Table 2.15 Time screening experiment for bioreduction of Baylis Hillman product (**31**)

Another possibility to explain *syn* selectivity is that the alcohol is being oxidized to yield a β-keto ester that could undergo facile racemization. In addition, the alkene would present two possible activating groups to the enzyme, and it is not obvious which one (ketone versus ester carbonyls) would pick up the hydrogen bonding and thereby direct the sense of *trans*-addition of hydrogen.

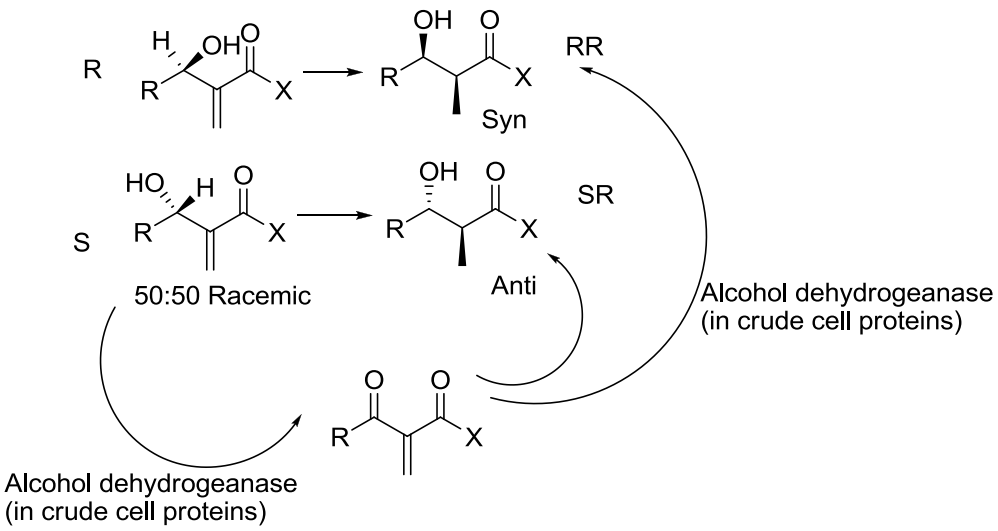
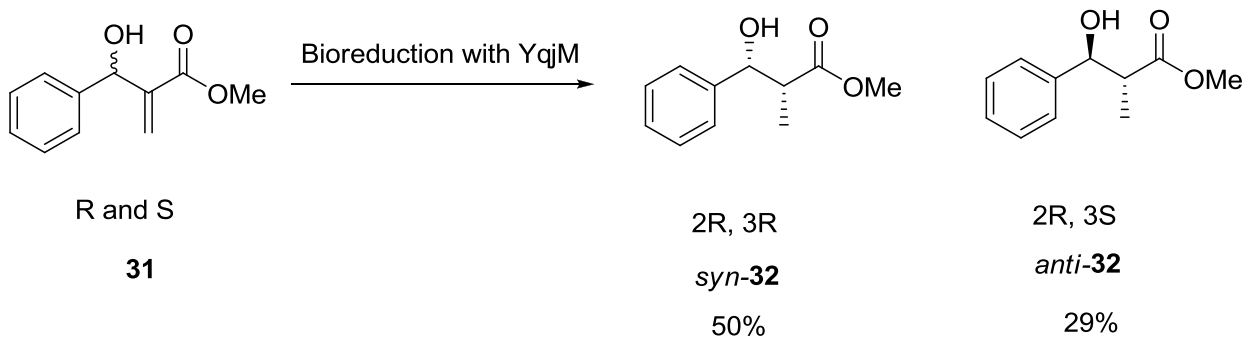


Figure 2.26 Proposed route *syn*-selectivity through racemizaion in the presence of alcohol dehydrogeanase in the crude cell extract.

Servi and coworkers[41] reported such a behavior using bakers' yeast to reduce alkenes that were part of allylic alcohol moieties, which was attributed to a rapid and reversible alcohol-ketone interconversion. However, it has to be mentioned that formation of a keto-intermediate was not

observed based on GC-MS analysis. Since CCE of YqjM was used in the present study, a similar process may be operating based on the redox-activity of a native *E.coli* enzyme. Attempt to purify the YqiM protein was not successful and will be worked out in next project.

Preparative scale bioreduction was performed with YqjM enzyme to confirm the assignment of *syn* and *anti* product and their relative ratios. Bioreductions were carried out on 100mg scale and compound (**31**) gave 50% isolated yield of *syn* diastereoisomer (**32**) and 29% isolated yield of *anti* diastereeoisomer (**32**). Structure was assigned by the ^{1}HNMR peak of carbinol protons: in the *syn* diastereoisomer (**32**) the carbinol proton gave a doublet peak at 5.09 ppm; *anti* diasteroisomer (**32**) displays the carbinol proton peak at 4.72ppm which is according to the literature.[160] The *syn* diastereoisomer (**32**) has *2R,3R* absolute configuration at two chiral centers and *anti* diastereoisomer (**32**) has *2R,3S* based on comparison of optical rotation with reported data.[160]



$[\alpha]_D^{22}$ = +21.8 (c = 1, $CHCl_3$) $[\alpha]_D^{22}$ = -62.5 (c = 0.24, $CHCl_3$)

Figure 2.27 Scale up bioreduction of Baylis Hillman product (**31**) with YqjM enzyme

[160] Gennari, C.; Colombo, L.; Bertolini, G.; Schimperna, G. *J. Org. Chem.* **1987**,52, 2754-2760.

2.2.3.2 Bioreductions of Baylis Hillman Products with Aliphatic Substituents

Bioreductions of compounds (**33-36**) bearing aliphatic substituents were conducted with EREDs and results are compiled in Table 2.16.

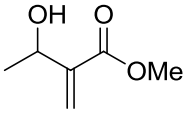
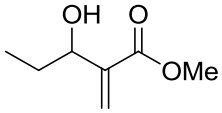
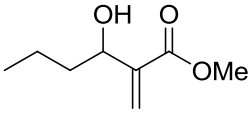
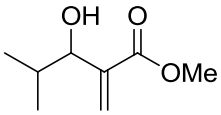
Enzyme	Time	 **(33)**	 **(34)**	 **(35)**	 **(36)**
YqjM					
	24h	71%[a] 54:46[b] (99:99)[c]	85% 73:27 (99:98)	58% 74:26 (99:98)	61% 97:3 (99:98)
	48h	89% 55:45 (99:99)	95% 68:32 (99:97)	80% 73:27 (98:97)	67% 88:12 (98:97)
OPR1					
	24h	nc[d] (na)[e]	2% 92:8 (nd)	4% 99:1 (nd)	4% 99:1 (nd)
	48h	nc (na)	5% 90:10 (nd)	11% 99:1 (nd)	5% 99:1 (nd)
OPR3					
	24h	traces (nd)[f]	3% 99:1 (nd)	2% 86:14 (nd)	nc (na)
	48h	2% 100:0 (nd)	9% 99:1 (nd)	4% 95:5 (nd)	nc (na)
SYE-4					
	24h	nc (na)	nc (na)	nc	nc (na)
	48h	nc (na)	nc (na)	4% 98:2 (nd)	nc (na)

Table 2.16 Bioreduction screening experiment of Baylis Hillman products (aliphatic substituents **33-36**). a) conversion %, b) *diastereomeric ratio, dr, syn:anti*, c) enantiomeric excess, ee, (*syn:anti*), d) no conversion, e) not applicable, f) not determined.

As with the previous model substrate only YqjM enzyme converted these substrates efficiently. After 48h, 89% conversion was observed for substrate (**33**) with 55:45 ratio of *syn* to *anti* diastereoisomers. The *syn* selectivity increased with increase in size and branching of the aliphatic substituent. Compound (**36**) bearing an isopropyl substituent gave 67% conversion of reduced product with 88:12 ratio of *syn* to *anti* diastereoisomers. Compounds (**34**) and (**35**) with ethyl and *n*-propyl substituents, respectively, have intermediate *syn* selectivity. In case of other ene-reductases only traces of products or no conversions were observed. All the reduced products were enantiomerically pure according to chiral GC.

2.2.3.3 Bioreductions of Baylis Hillman Products with Aromatic Substituents

Compounds (**37-39**) bearing aromatic substituent were reduced by EREDs with moderate to good conversion after 48h. High *syn* selectivity was observed with substrate (**38**) bearing a 4-methyl substituent on the phenyl ring and conversions did not exceed 50%. Replacing the group at 4-position of the benzene ring with methoxy (**37**) and chloro (**39**) resulted in decreased *syn* selectivity as compared to 4-methyl group. With OPR1 enzyme compounds (**38**) and (**39**) gave low conversion to only *syn* diastereoisomers. OPR3 and SYE-4 enzyme showed no activity with these substrates.

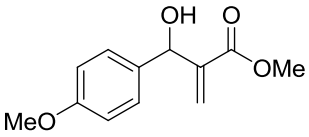
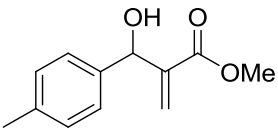
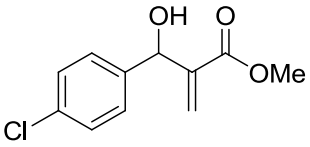
Enzyme	Time	 (37)	 (38)	 (39)
YqjM	24h	15%[a] 85:15[b] (99:99)[c]	27% 90:10 (99:98)	30% 65:35 (99:98)
	48h	83% 78:22 (99:99)	45% 90:10 (98:98)	89% 60:40 (99:98)
OPR1	24h	nc[d] (na)[e]	<1% 100:0 (nd)[f]	7% 99: 1 (nd)
	48h	nc (na)	15% 100:0 (99:0)	30% 99: 1 (99:0)
OPR3	24h	nc (na)	nc (na)	nc (na)
	48h	nc (na)	nc (na)	nc (na)
SYE-4	24h	nc (na)	nc (na)	nc (na)
	48h	nc (na)	nc (na)	nc (na)

Table: 2.17 Bioreduction of Baylis Hillman products with aromatic substituents (**37-39**). a) conversion %, b) *diastereomeric ratio, dr*, *syn:anti*, c) enantiomeric excess, ee, (*syn:anti*), d) no conversion, e) not applicable, f) not determined.

2.2.3.4 Bioreductions of Baylis Hillman Products with Heterocyclic Substituent

β-Hydroxyesters with heterocyclic substituents (**40**) and (**41**) were also investigated for acceptance by the ene-reductase collection. Pyridyl substituted Baylis Hillman adduct (**40**) was not accepted by any ERED, however, compound (**41**) with furyl as heterocyclic substituent gave full conversion with 80:20 *syn:anti* ratio again when using YqjM.

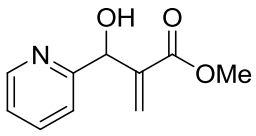
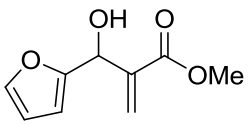
Enzyme	Time	 **(40)**	 **(41)**
YqjM	24h	nc[a] (na)[b]	100%[c] 79:21[d] (99:99)[e]
	48h	nc (na)	100% 80:20 (99:98)
OPR1	24h	nc (na)	45% 99:1 (99:0)
	48h	nc (na)	79% 99:1 (99:0)
OPR3	24h	nc (na)	nc (na)
	48h	nc (na)	nc (na)
SYE-4	24h	nc (na)	nc (na)
	48h	nc (na)	nc (na)

Table 2.18 Bioreduction of Baylis Hillman products with heterocyclic substituent (**40-41**). a) no conversion, b) not applicable, c)conversion, %, d) *diastereomeric ratio, dr*, *syn:anti*, e) enantiomeric excess, ee, (*syn:anti*).

Interesting results in this experiment was the bioreduction of compound (**41**) with OPR1 enzyme with 79% conversion to enantiomerically pure *syn* diastereoisomer, exclusively.

2.2.3.5 Bioreductions of Baylis Hillman Products from Acrylamide and Acrylonitrile

The site for substrate modification within the bioreduction studies was to change the ester functionality to nitrile and amide group. Baylis Hillman product (**42**) synthesized by the reaction of benzaldehyde and acrylonitrile was not accepted by any ene-reductase. The bioreduction of hydroxyl amides (**43-45**) (Baylis Hillman products synthesized by the reaction of acrylamide and different aldehydes) with YqjM enzyme gave good conversion and high stereoselectivity of the reduced products.

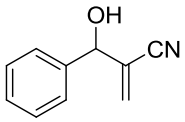
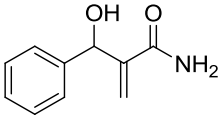
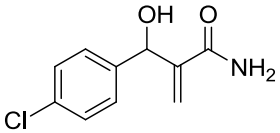
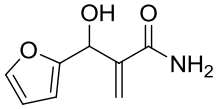
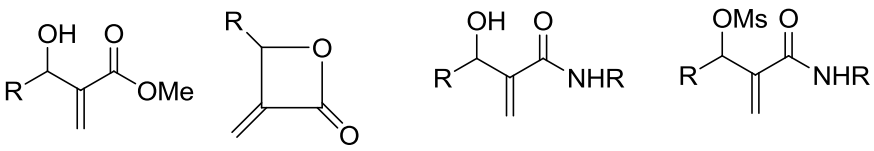
Enzyme	Time	 (42)	 (43)	 (45)	 (46)
YqjM	24h	nc[a] (na)[b]	25%[c] 51:49[d] (99:99)[e]	91% 75:25 (99:98)	92% 44:56 (98:99)
	48h	nc (na)	75% 52:48 (99:98)	92% 74:26 (99:98)	98% 45:55 (97:98)
OPR1	24h	nc (na)	nc (na)	nc (na)	nc (na)
	48h	nc (na)	nc (na)	nc (na)	nc (na)
OPR3	24h	nc (na)	nc (na)	nc (na)	nc (na)
	48h	nc (na)	nc (na)	nc (na)	nc (na)
SYE-4	24h	nc (na)	nc (na)	nc (na)	nc (na)
	48h	nc (na)	nc (na)	nc (na)	nc (na)

Table 2.19 Bioreduction of Baylis Hillman products with acrylamide and acrylonitrile. a) no conversion, b) not applicable, c)conversion, %, d) *diastereomeric ratio*, *dr*, *syn:anti*, e) enantiomeric excess, ee, (*syn:anti*).

In this set of compounds high *syn*-selectivity was observed with 4-cholorobenzyl (**45**) group while phenyl (**43**) and furyl group (**46**) compounds gave almost equal ratio of *syn* and *anti* diastereoisomers. No conversion was observed with these compounds in case of other ene-reductases. All bioreduction products were obtained in excellent enantioselectivity.

2.2.3.6 Bioreduction of Modified Baylis Hillman Products



46 **48** **49** **50**

R= Ph_2CH

Compound (**46**) was used in the synthesis of lactone (**48**) and compound (**49**) and (**50**) were synthesized during the attemped synthesis of lactam (**51**). These compounds were also subjected to bioreduction with all ene-reductases (SYE-4, OPR1, OPR3, YqjM). All these compounds were not accepted by any ene-reductase owing to their bulky substituents.

2.2.4. Bioreduction Aza-Baylis Hillman Products

Another modification was to exchange the hydroxyl group by a protected nitrogen functionality. For this purpose aza-Baylis Hillman reactions were performed for the synthesis of compounds (**53**) and (**56**). Bioreductions of these substrates were conducted with different ene-reductases. N-Tosyl amine substituted Baylis Hillman adduct (**53**) was not accepted by ene-reductases. The reason for this may be the large size of the tosyl group, which is not favourable for the enzymatic reaction. The bioreduction of *N*-substituted Baylis Hillman adducts (**56 & 64**) gave only *anti* diastereoisomers with good conversion with YqjM enzyme. The structural assignments were made on the bases of reported data[161]. The exchange of hydroxyl by *N*-carbamate enhanced the conversion to *anti* diastereoisomers. The stereoselective bioreduction of compounds can lead to the stereoselctive synthesis of *β*-amino acids as a novel bioreductive approach.

At present it is not clear why *anti*-bioreduction products are formed exclusively. Again, this might involve an additional redox process originating from host proteins present in the CCE, but in this particular case activity of an imine-reductase would be required.

---

[161] Takagi, M.; Yamamoto, K. *Tetrahedron.* **1991,** *41*, 42, 8869-8882.

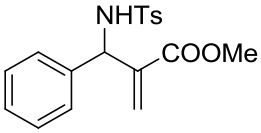
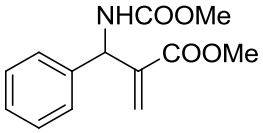
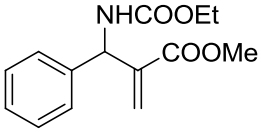
Enzymes	Time	 **(53)**	 **(56)**	 (**64**)
YqjM	24h	nc[a] (na)[b]	67%[c] 1:99[d] (99)[e]	42% 1:99 (99)
	48h	nc (na)	74% 1:99 (98)	92% 4:96 (98)
OPR1	24h	nc (na)	nc (na)	nc (na)
	48h	nc (na)	nc (na)	nc (na)
OPR3	24h	nc (na)	nc (na)	nc (na)
	48h	nc (na)	nc (na)	nc (na)
SYE-4	24h	nc (na)	nc (na)	nc (na)
	48h	nc (na)	nc (na)	nc (na)

Table 2.20 Bioreduction of aza Baylis Hillman products (**53, 56** and **64**). a) no conversion, b) not applicable, c)conversion, %, d) *diastereomeric ratio*, *dr*, *syn:anti*, e) enantiomeric excess, ee, (*anti*).

Different aza Baylis Hillman adducts (**61**-**67**) were synthesized with different substituents to explore the substrate tolerance of EREDs in general and YqjM in particular. Bioreductions of these substrates were performed with all Ene-reductases and results are shown in the Table 2.21 and 2.22.

The bioreduction of these substrates (**61**-**67**) gave similer type of results as in the bioreduction of compounds (**56** & **64**): *N*-sustituted substrates were only accepted by YqjM with good conversion, high *anti* diastereoselectivity and excellent optical purity (based on chiral HPLC).

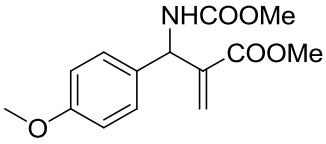
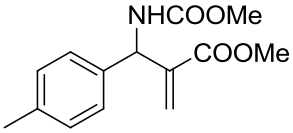
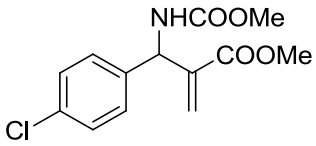
Enzymes	Time	 **(61)**	 **(62)**	 **(63)**
YqjM	24h	53%[a] 1:99[b] (97)[c]	55% 10:90 (98)	83% 10:90 (99)
	48h	75% 2:98 (99)	60% 15:85 (99)	91% 12:88 (98)
OPR1	24h	nc[d] (na)[e]	nc (na)	nc (na)
	48h	nc (na)	nc (na)	nc (na)
OPR3	24h	nc (na)	nc (na)	nc (na)
	48h	nc (na)	nc (na)	nc (na)
SYE-4	24h	nc (na)	nc (na)	nc (na)
	48h	nc (na)	nc (na)	nc (na)

Table 2.21 Bioreduction of aza Baylis Hillman products (**61-63**). a) conversion %, b) *diastereomeric ratio, dr*, *syn:anti*, c) enantiomeric excess, ee, (*anti*), d) no conversion, e) not applicable.

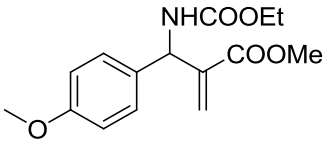
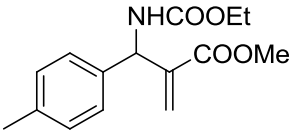
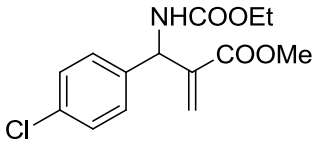
Enzymes	Time	 **(65)**	 **(66)**	 (67)
YqjM	24h	65%[a] 4:96[b] (99)[c]	75% 5:95 (99)	75% 1:99 (99)
	48h	90% 8:92 (99)	76% 5:95 (99)	96% 2:98 (99)
OPR1	24h	nc[d] (na)[e]	nc (na)	nc
	48h	nc (na)	nc (na)	nc (na)
OPR3	24h	nc (na)	nc (na)	nc (na)
	48h	nc (na)	nc (na)	nc (na)
SYE-4	24h	nc (na)	nc (na)	nc (na)
	48h	nc (na)	nc (na)	nc (na)

Table 2.22 Bioreduction of aza Baylis Hillman products (**65**-**67**). a) conversion %, b) *diastereomeric ratio, dr, syn:anti*, c) enantiomeric excess, ee, (*anti*), d) no conversion, e) not applicable.

To elobrate the substrate profile of YqjM enzyme for the aza Baylis Hillman products, compounds (**69** & **70**) with heterocyclic ring were synthesized and submitted to bioreductions (Table 2.23). The furan ring containing aza Baylis Hillman products (**69** & **70**) were accepted very efficiently by YqjM enzyme with almost full conversion and *anti* selectivity. The reaction was very fast and completed in only 24h as compare to other substrates (48h). Similar behaviour was observed in the bioreduction of Baylis Hillman product (**41**) with YqjM enzyme (Table 2.18).

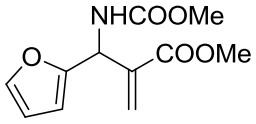
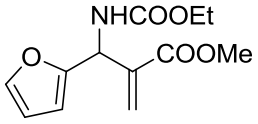
Enzyme	Time	 **(69)**	 **(70)**
YqjM	24h	95%[a] 5:95[b] (99)[c]	97% 8:93 (99)
	48h	--	--
OPR1	24h	nc[d] (na)[e]	nc
	48h	nc (na)	nc
OPR3	24h	nc (na)	nc (na)
	48h	nc (na)	nc (na)
SYE-4	24h	nc (na)	nc (na)
	48h	nc (na)	nc (na)

Table 2.23 Bioreduction of aza Baylis Hillman products (**69**-**70**). a) conversion %, b) *diastereomeric ratio, dr*, *syn:anti*, c) enantiomeric excess, ee, (*anti*), d) no conversion, e) not applicable.

2.2.5 Scale up Bioreduction of Baylis Hillman Products with YqjM Enzyme

The successful screening bioreductions of Baylis Hillman products were performed on larger scale to confirm the structure and absolute configuration of the reduced products. One example from each category of compound was selected and reduced to the product with YqjM enzyme on 100mg scale. The *syn* and *anti* products were isolated from these reactions, structure assignment was confirmed by NMR, and absolute configuration was determined by optical rotation values. On the basis of NMR chemical shift for the carbinol protons we can easily assign the *syn* and *anti* configuration to the compounds. The chemical shift value of carbinol proton is higher for the *syn* diastereoisomer as compared to *anti* diastereoisomer.

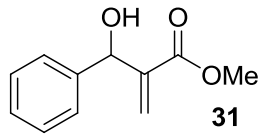
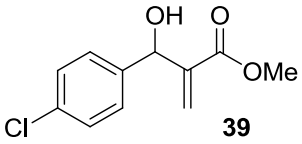
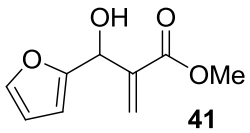
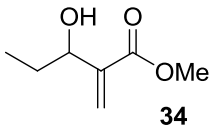
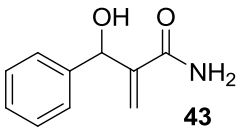
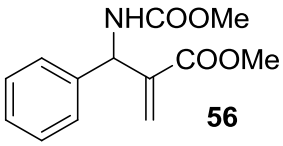
Compounds	Screening Experiment	Preparative scale	Yields	Absolute Configuration
 **31**	90%[a] 65:35[b] (99:98)[c] (48h)[d]	100% 69:31 (99:98) (72h)	*Syn* 50% *Anti* 28%	*Syn 2R, 3R* $[\alpha]_D^{22}$ *=+21.8 (c = 1, $CHCl_3$)* *Anti 2R, 3S* $[\alpha]_D^{22}$ =-62.5 (c = 0.24, $CHCl_3$)
 **39**	89% 60:40 (99:97) (48h)	75% 55:45 (97:96)(72h)	*Syn* 43% *Anti* 37%	*Syn 2R, 3R* *$[\alpha]_D^{22}$ =+12.4 (c = 1.1, $CHCl_3$)* *Anti 2R, 3S* *$[\alpha]_D^{22}$ =+12.4 (c = 1.1, $CHCl_3$)*
 **41**	100% 80:20 (99:98) (48h)	100% 90:10 (98:97) (72h)	*Syn* 79% *Anti* 9%	*Syn 2R, 3R* *$[\alpha]_D^{20}$ =+4..76 (c = 0.55 $CHCl_3$)* *Anti 2R, 3S* *$[\alpha]_D^{20}$ =-1.2 (c = 0.41, $CHCl_3$)*
 **34**	95% 75:25 (99:98) (48h)	100% 93:7 (98:96) (72h)	*Syn* 73% *Anti* 11%	*Syn 2R, 3S* *$[\alpha]_D^{20}$ =-0.92 (c = 0.21, $CHCl_3$)* *Anti 2R, 3R* $[\alpha]_D^{20}$=-10.9(c = 0.92, $CHCl_3$)
 **43**	75% 52:48 (99:99) (48h)	70% 49:51 (98:97) (72h)	*Syn* 23% *Anti* 25%	*Syn 2R, 3R* *$[\alpha]_D^{20}$=+13.33 (c = 1.21, $CHCl_3$)* *Anti 2R, 3S* $[\alpha]_D^{20}$ =-30.26 (c = 1.24, $CHCl_3$)
 **56**	74% 1:99 (99)[e] (48h)	80% 1:99 (97) (72h)	*Anti*	*Anti 2R, 3S* $[\alpha]_D^{20}$ =-37.08 (c = 0.25, EtOH)

Table 2.24 Scale up bioreduction of Baylis Hillman products for characterization. a) conversion %, b) *diastereomeric ratio, dr, syn:anti*, c) enantiomeric excess, ee, (syn:*anti*), d) time, e) enantiomeric excess, ee, (*anti*).

The absolute configuration on each chiral centre was assigned by the comparison of optical rotation value with the reported values in the literature.

2.3 Redox Reactions of Carvones

2.3.1 Bioreduction of Carvones

Bioreductions of carvones were conducted with all ene-reductases: SYE-3, SYE-4 (*Shewanella* Yellow Enzyme), OPR1, OPR3 (12-Oxophytodienoate reductase), YqjM (*Bacillus subtilis*) and mutant W116I. SYE-4, OPR1, OPR3 and YqjM were already used for the bioreduction of Baylis Hillman products and substrate profiling above. Mutant W116I was created and used by Stewart and coworkers[119] for the bioreduction of carvones displaying divergent stereopreferences. Within this study this mutant was used for the bioreduction of carvones and then further in the one-pot redox reactions in combination with BVMOs. Cell lysate for EREDs and BVMO CCE were extracted from *E.coli* BL21 (DE3) cells using the same protocol as described above (Section 2.1.7). At first, bioreduction of enantiomerically pure carvones was studied. Then the reduced products from these biotransformations were subjected to biooxidation screening. Finally, these two steps were performed in one pot starting from enantiomerically pure carvones to different types of lactones. Bioreduction and biooxidation screening experiments with EREDs and BVMOs protein (5mg) in sterile multi-well plates were performed in the presence of Tris HCl (pH 8, 50mM), $NADP^+$ (200μM), glucose-6-phosphate (4mM), glucose-6-phosphate dehydrogenease (1 unit) and substrate (2mM) at 28°C. Conversion of substrates was monitored by GC-MS. After 6h full conversion was observed with both (−)-carvone (**76**) and (+)-carvone (**77**) except mutant W116I which took 24h for reaction completion.

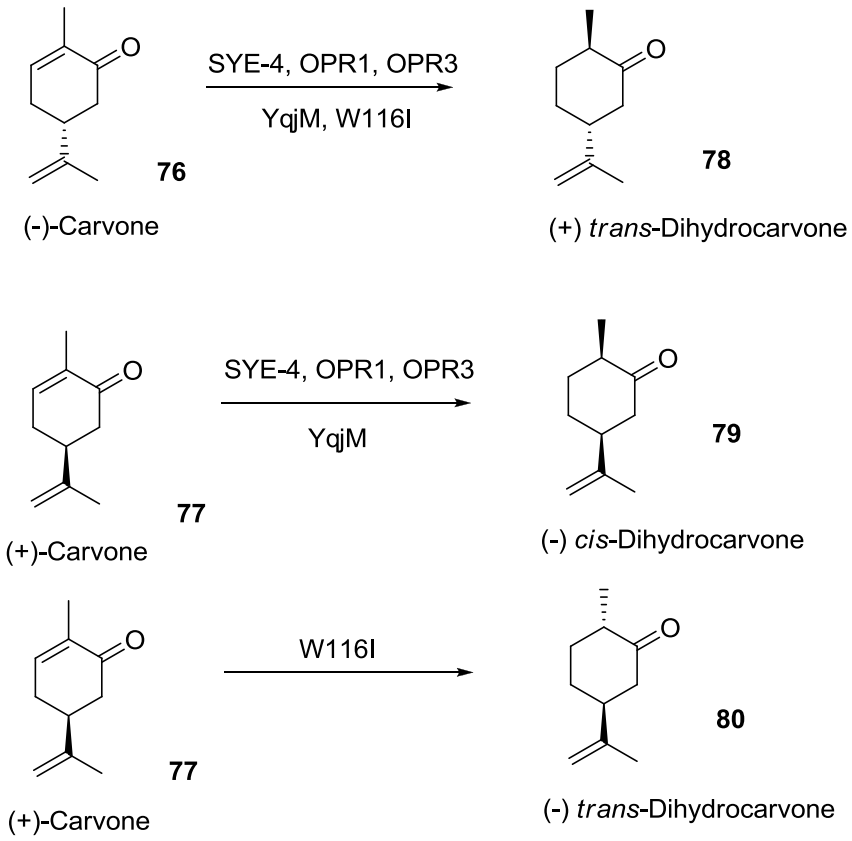


Figure 2.28 Bioreduction of carvones

Enantiomerically pure carvones were selectively reduced to dihydrocarvone with five EREDs: SYE-4 (*Shewanella* Yellow Enzyme), OPR1, OPR3 (12-Oxophytodienoate reductase), YqjM (*Bacillus subtilis*) and mutant W116I. SYE-3 (*Shewanella* Yellow Enzyme) was not able to reduce both enantiomerically pure carvones. (−)-Carvone (**76**) was converted to (+)-*trans*-dihydrocarvone (**78**) with all Ene-reductases SYE-4, OPR1, OPR3, YqjM and W116I, with YqjM and SYE-4 as most selective and efficient biocatalysts. (Table 2.25)

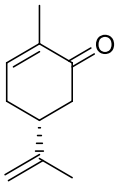
Time	 (−)-Carvone (76)					
	SYE-3	**SYE-4**	**OPR1**	**OPR3**	**YqjM**	**W116I**
1h	nc[a]	100%[b] (3:97)[c]	27% (5:95)	89% (32:68)	100% (4:96)	--
3h	nc	--	97% (8:92)	100% (41:59)	--	35% (4:96)
24h	nc	--	100% (15:85)	--	--	97% (5:95)

Table 2.25 Bioreduction screening experiment results (−)-carvone (**76**) from ene-reductases. a) no conversion, b) conversion %, c) *cis : trans* (dr)

On the other hand (+)-carvone (**77**) was converted to (−)-*cis*-dihydrocarvone (**79**) with SYE4, OPR1, OPR3 and YqjM but mutant W116I reduced (+)-carvone (**77**) to new (-)-*trans*-dihydrocarvone (**80**). This fact was previously explained by the Stewart group[119].

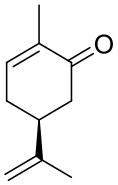
Time	 (+)-Carvone (77)					
	SYE-3	**SYE-4**	**OPR1**	**OPR3**	**YqjM**	**W116I**
1h	nc[a]	100%[b] (95:5)[c]	33% (93:7)	95% (92:8)	93% (91:9)	--
3h	nc	--	100% (85:15)	100% (82:18)	100% (82:18)	22% (9:91)
24h	nc	--	--	--	--	95% (10:90)

Table 2.26 Bioreduction screening experiment results (+)-carvone (**77**) from ene-reductases.

a) no conversion, b) conversion %, c) *cis : trans* (dr)

Scale up bioreductions on 50 mg scale were performed with SYE-4 for characterization of products. (−)-Carvone (**76**) gave 85% isolated yield of (+)-*trans*-dihydrocarvone (**78**) ($[\alpha]_D^{22}$ = +14.2 (c = 0.8, $CHCl_3$)), and (−)-*cis*-dihydrocarvone (**79**) ($[\alpha]_D^{22}$ = −16.9 (c = 1.2, $CHCl_3$)) was obtained in 83% from (+)-carvone (**77**).

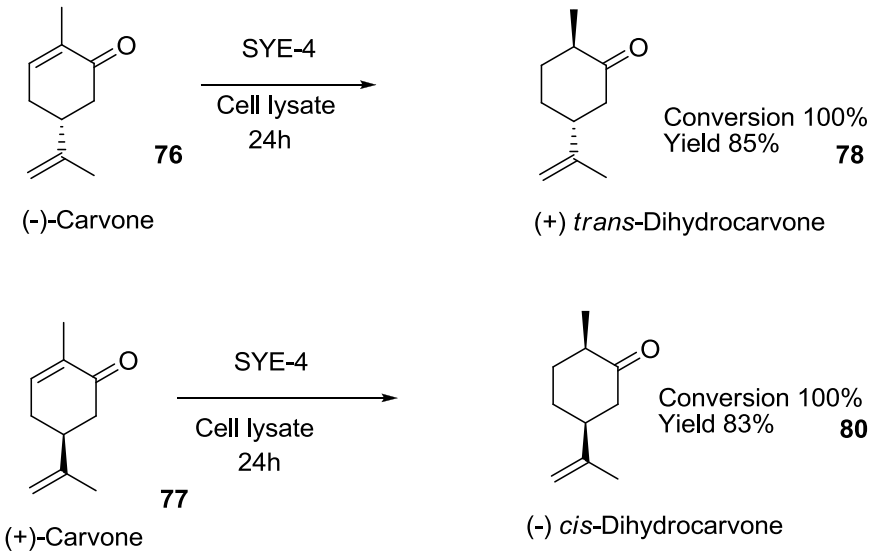


Figure 2.29 Preparative scale bioreduction of carvones with SYE-4 protein

2.3.2 Baeyer–Villiger Biooxidation of Dihydrocarvones

The regiopreference of Baeyer–Villiger biooxidation was first investigated for (+)-*trans*-dihydrocarvone (**78**), (−)-*cis*-dihydrocarvone (**79**), and (−)-*trans*-dihydrocarvone (**80**) with $CHMO_{Acineto}$, $CHMO_{Brevi1}$, $CHMO_{Brevi2}$, and $CPMO_{Coma}$ in sterile multi-well plates. Recently, the existence of two distinct sub-clusters of cycloketone oxidizing BVMOs was proposed based on protein sequence alignment, substrate acceptance, and stereopreference.[162] Baeyer–Villiger oxidations of (+)-*trans*-dihydrocarvone (**78**) catalyzed with "CHMO"-type enzymes ($CHMO_{Acineto}$) led to the formation of "abnormal" lactones (−)-ANL (**81**), with excellent conversion. A somewhat different outcome was observed for the biotransformation of (+)-*trans*-dihydrocarvone (**78**) with $CHMO_{Brevi1}$, where both "normal" and "abnormal" lactones were obtained in approximate ratios of 60:40.

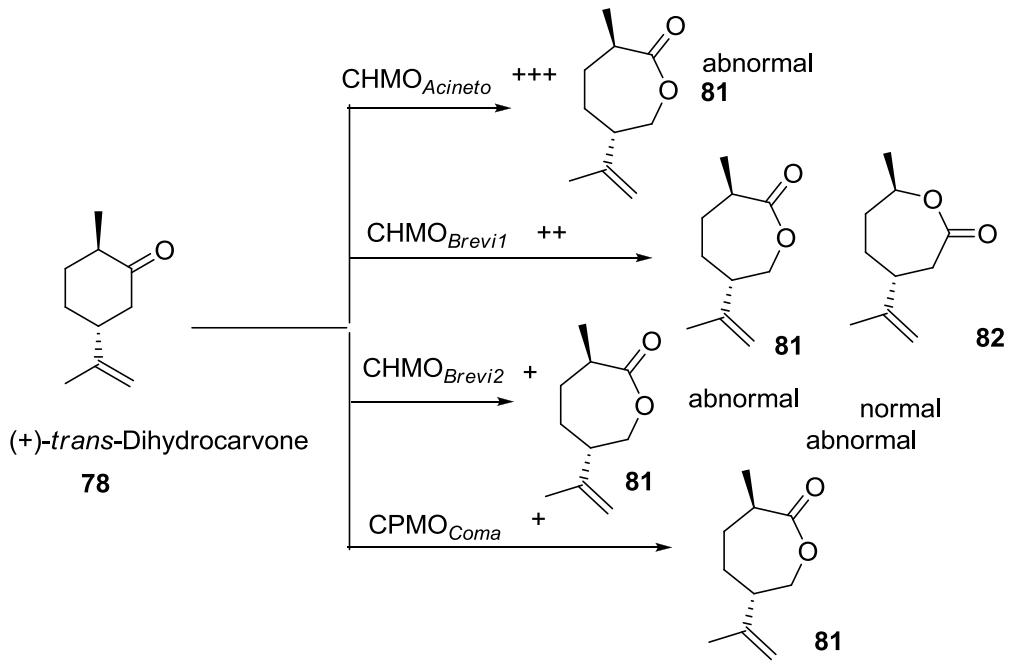


Figure 2.30 Baeyer Villiger monooxygenation of (+)-*trans*-dihydrocarvone (**78**)
(conversion according to GC: +++: >90%, ++: 50–90%, +: <50%)

---

[162] Mihovilovic, M. D.; Rudroff, F.; Grotzl, B.; Kapitan, P.; Snajdrova, R.; Rydz, J.; Mach, R. *Angew. Chem. Int. Ed.* **2004**, *44*, 3609–3613.

Biooxidation with "CPMO"-type enzymes ($CHMO_{Brevi2}$, $CPMO_{Coma}$) showed preferred formation of "abnormal" lactones, however, with low conversions.

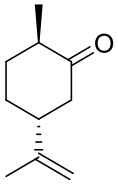
Enzyme	**Time**	 **(+)-*trans*-dihydrocarvone (78)**
$CHMO_{Acineto}$	1h	75%[a] (0:100)[b]
	3h	93% (0:100)
	6h	100% (0:100)
$CHMO_{Brevi\ 1}$	1h	15% (60:40)
	3h	51% (60:40)
	6h	55% (60:40)
$CHMO_{Brevi\ 2}$	1h	3% (0:100)
	3h	5% (0:100)
	6h	6% (0:100)
$CPMO_{Coma}$	1h	2% (0:100)
	3h	2.8% (0:100)
	6h	3% (0:100)

Table 2.27 Baeyer Villiger monooxygenation of (+)-*trans*-dihydrocarvone (**78**). a) conversion %, b) normal lactone: abnormal lactone (N:AB)

In contrast, the opposite (−)-*cis*-dihydrocarvone (**79**) and was transformed exclusively into "normal" lactones (−)-NL (**83**) with $CHMO_{Acineto}$ and $CHMO_{Brevi1}$ CCE. The (−)-*trans*-dihydrocarvone (**80**) was oxidized to normal lactones (**85**) with $CHMO_{Acineto}$ and $CHMO_{Brevi1}$ CCE. CPMO-type enzymes ($CHMO_{Brevi2}$, $CPMO_{Coma}$) gave poor conversion to abnormal lactones (**86**) from (−)-*trans*-dihydrocarvone (**80**). These results were obtained using CCE within this study and gave reproducible data compared to previous work on carvones in the group with different BVMOs using whole cell biotransformations.[141]

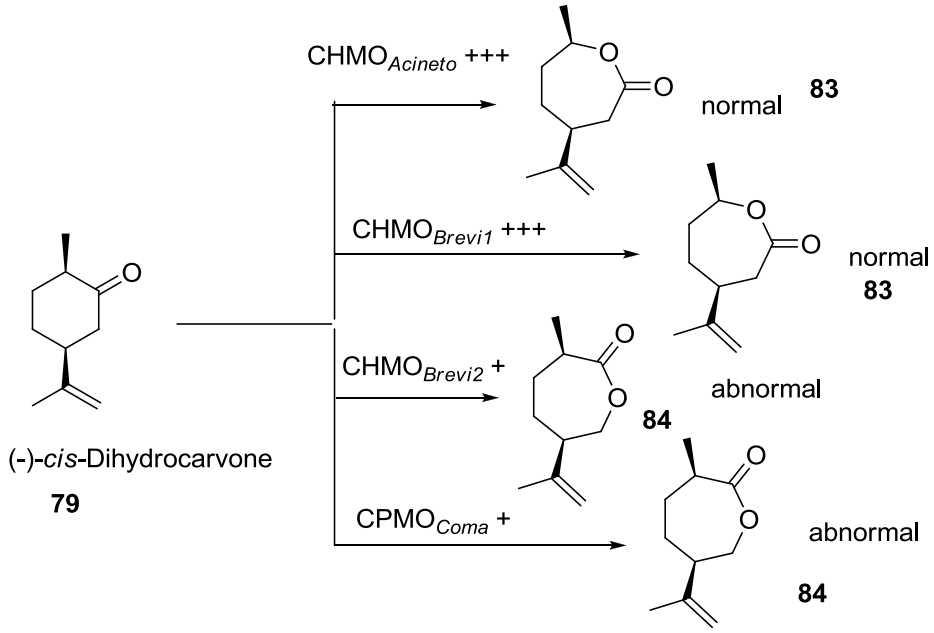


Figure 2.31 Baeyer Villiger monooxygenation of (−)-*cis*-dihydrocarvone (conversion according to GC: +++: >90%, ++: 50–90%, +: <50%)

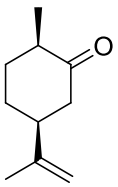
Enzyme	**Time**	 **(−)-*cis* dihydrocarvone (79)**
CHMO$_{Acineto}$	1h	99%[a] (98:2)[b]
	3h	100% (97:3)
	6h	100% (93:7)
CHMO$_{Brevi\ 1}$	1h	69% (99:1)
	3h	100% (99:1)
	6h	100% (99:1)
CHMO$_{Brevi\ 2}$	1h	14% (0:100)
	3h	25% (0:100)
	6h	28% (0:100)
CPMO$_{Coma}$	1h	1% (0:100)
	3h	2.7% (0:100)
	6h	4% (0:100)

Table 2.28 Baeyer Villiger monooxygenation of (−)-*cis*-dihydrocarvone (**79**). a) conversion %, b) normal lactone: abnormal lactone (N :AB)

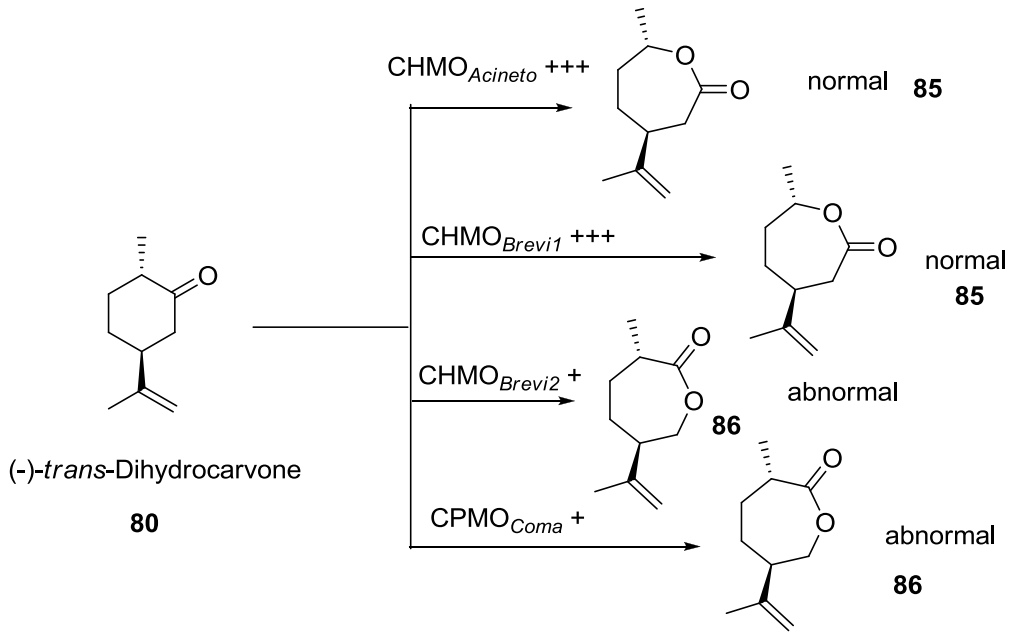


Figure 2.32 Baeyer Villiger monooxygenation of (−)-*trans*-dihydrocarvone (**80**) (conversion according to GC: +++: >90%, ++: 50–90%, +: <50%)

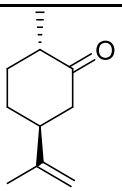
Enzyme	**Time**	 **(−)-*trans* dihydrocarvone (80)**
CHMO$_{Acineto}$	1h	78%[a] (2:98)[b]
	3h	100% (3:97)
	6h	100% (3:97)
CHMO$_{Brevi\ 1}$	1h	52% (4:96)
	3h	53% (4:96)
	6h	78% (4:96)
CHMO$_{Brevi\ 2}$	1h	16% (0:100)
	3h	17% (0:100)
	6h	18% (0:100)
CPMO$_{Coma}$	1h	traces (AB)
	3h	traces (AB)
	6h	traces (AB)

Table 2.29 Baeyer Villiger monooxygenation of (−)-*trans*-dihydrocarvone (**80**). a) conversion %, b) normal lactone: abnormal lactone (N :AB)

2.3.3 One-Pot Redox Reactions of Carvones

One pot redox biotransformations were performed in multi-well plates. Different combinations of EREDs and BVMOs were applied for the biotransformation. Redox screening experiments with ERED and BVMO CCE (5mg each) in sterile multi-well plates were performed in the presence of Tris HCl (pH 8, 50mM), $NADP^+$ (200µM), glucose-6-phosphate (4mM), glucose-6-phosphate dehydrogenase (1 unit) and substrate (2mM) at 28°C. Conversion of substrates was monitored by GC. The first combination investigated was SYE-4 and $CHMO_{Acineto}$ with two options, firstly adding both ERED and BVMO at the start of the experiment; secondly BVMO was added after 1h when major part of bioreduction with ERED was already over. At the end full conversion was observed after 6h in both cases so it makes no difference to add BVMO after certain time or both ERED and BVMO simultaneously (Figure 2.33). Consequently, all one-pot redox reactions were subsequently carried out by simultaneous addition of EREDs and BVMOs due to operational simplicity. The results for the screening experiments were summarized in Table 2.30.

Different combinations of redox cascade reactions was investigated for both enantiomerically pure carvones.

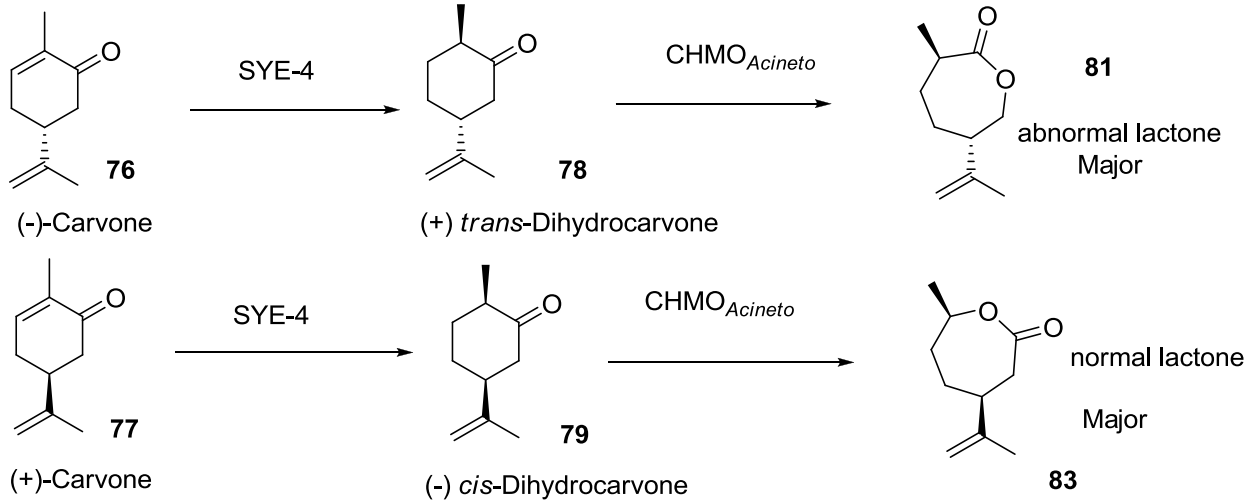


Figure 2.33 One-pot redox reactions of carvones with SYE-4 & $CHMO_{Acineto}$

Time	(+)-Carvone (77)		(−)-Carvone(76)	
	Both SYE-4 and $CHMO_{Acineto}$ add at start	$CHMO_{Acineto}$ added after 1h	Both SYE-4 and $CHMO_{Acineto}$ add at start	$CHMO_{Acineto}$ added after 1h
1h	10.5%[a] (99:1)[b]	100% (to (-)-dihydrocarvone) (91:9) (cis:trans)	12% (3:97)	59% (to (+)-dihydrocarvone) (3.5:96.5) (cis:trans)
6h	100% (97:3)	100% (95.5:4.5)	97% (3:97)	100% (1:99)

Table 2.30 One-pot redox reaction of carvones with SYE-4 & $CHMO_{Acineto}$.

a) conversion %, b) normal lactone: abnormal lactone (N :AB)

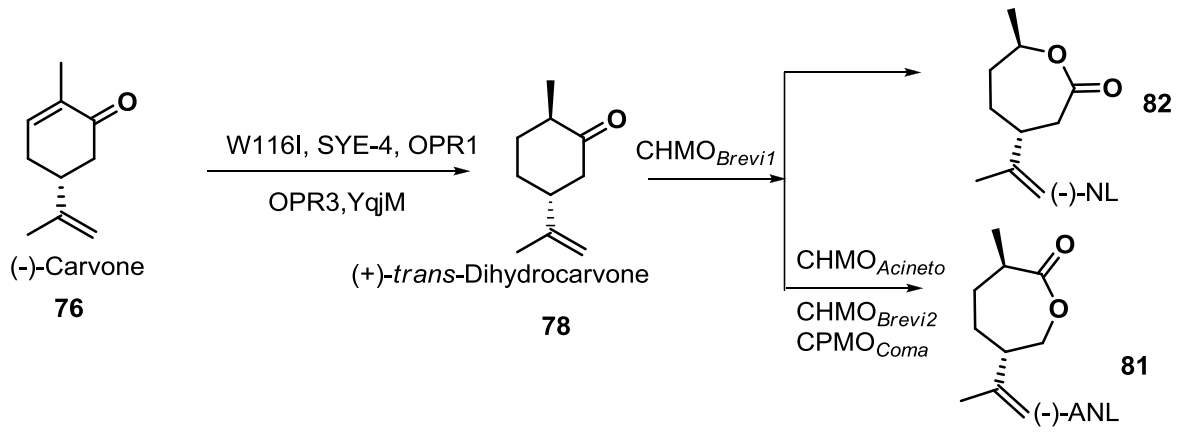


Figure 2.34 One-pot redox reactions of (−)-carvone (**42**)

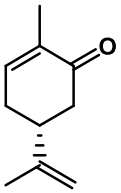
Enzymes	Time	 (−)-Carvone (76)			
		CHMO ***Acineto***	**CHMO** ***Brevi 1***	**CHMO** ***Brevi 2***	**CPMO** ***Coma***
SYE-4	3h	--	52%[a] (60:40)[b]	1% (0:100)	16% (0:100)
	6h	97% (3:97)	65% (62:38)	2.5% (0:100)	18% (0:100)
OPR1	3h	54% (0:100)	9% (65:35)	traces (0:100)	traces (0:100)
	6h	100% (0:100)	33% (67:33)	traces (0:100)	traces (0:100)
OPR3	3h	92% (0:100)	49% (59:41)	1% (0:100)	2.7% (0:100)
	6h	95% (1:99)	79% (61:39)	2% (0:100)	3.5% (0:100)
YqjM	3h	85% (0:100)	40% (68:32)	1% (0:100)	traces (0:100)
	6h	100% (0:100)	55% (70:30)	2% (0:100)	traces (0:100)
W116I	24h	82% (3:97)	35% (55:45)	traces (0:100)	traces (0:100)

Table 2.31 One-pot redox reactions of (−)-carvone (**76**) with EREDs & BVMOs.
a) conversion %, b) normal lactone: abnormal lactone (N :AB)

(−)-Carvone (**76**) was investigated for redox reactions with some interesting outcomes (Figure 2.34). Redox reaction of (−)-carvone (**76**) using $CHMO_{Acineto}$ for the second stage (almost 100% conversion with all EREDs during the first stage) gave selectively abnormal lactone (−)-ANL (**81**). In contrast, a mixture of normal (−)-NL (**82**) and abnormal lactones (−)-ANL (**81**) was observed when using $CHMO_{Brevi1}$ for the second stage with all redox combinations; with normal

lactone (−)-NL (**82**) as the major product in ~60:40 ratio. "CPMO"-type enzymes $CHMO_{Brevi2}$ and $CPMO_{Coma}$ showed similar behavior leading to abnormal lactone (−)-ANL (**81**), however, with low efficiency for the Baeyer-Villiger step. Screening experiment results of one-pot redox reaction of (−)-carvone were summarized in Table 2.31.

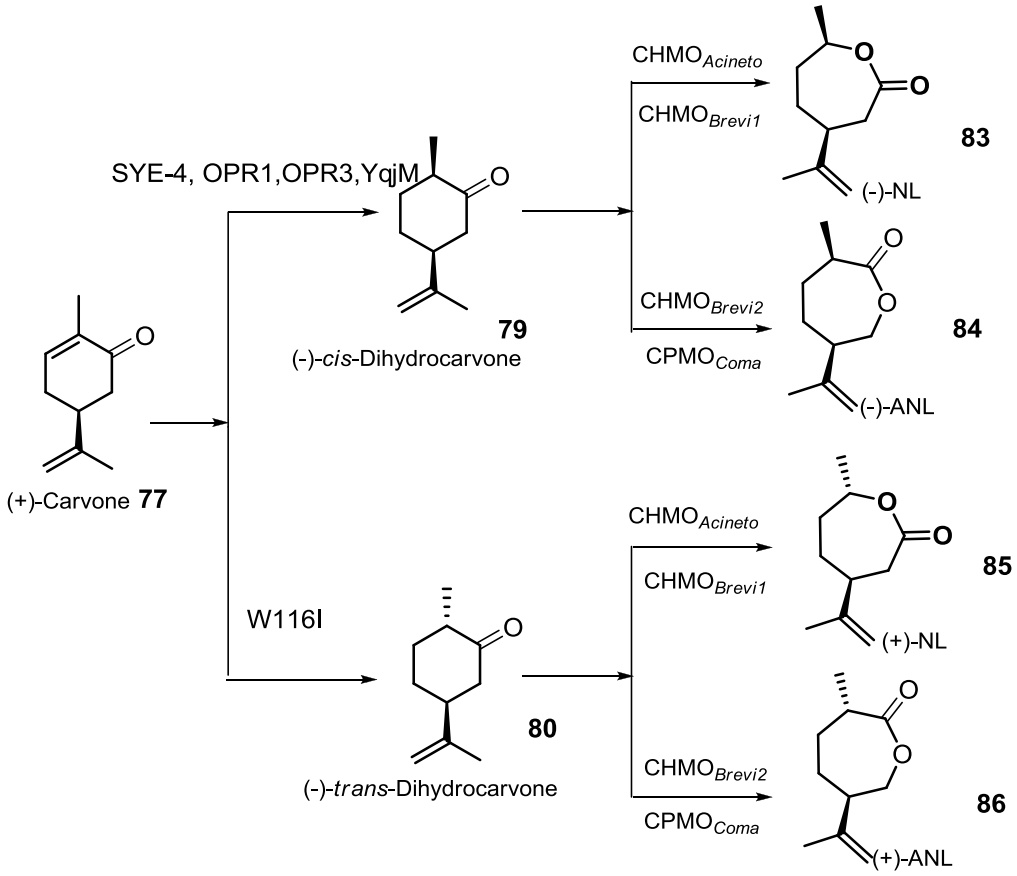


Figure 2.35 One-pot redox reactions of (+)-carvone (**77**)

(+)-Carvone (**77**) was converted fully into the normal lactone (−)-NL (**83**) in the presence of $CHMO_{Acineto}$ or $CHMO_{Brevi1}$ with EREDs (SYE-4, OPR1, OPR3, YqjM) after 6h. $CHMO_{Brevi2}$ and $CPMO_{Coma}$ ("CPMO"-type enzymes) in combination with EREDs (SYE-4, OPR1, OPR3, YqjM) gave (−)-ANL (**84**) with poor conversion. Mutant W116I requied 24h for completing this transformation towards (−)-*trans*-dihydrocarvone (**80**) and subsequently to (+)-NL (**85**) within presence of $CHMO_{Acineto}$ and $CHMO_{Brevi1}$. $CHMO_{Brevi2}$ and $CPMO_{Coma}$ ("CPMO"-type enzymes) in combination with mutant W116I gave abnormal lactones (+)-ANL (**86**) with low yields. The result for these bioscreening experiments were summarized in Table 2.32.

Using mutant W116I (in combination with BVMOs) on (−)-carvone (**76**) as substrate enabled access to both normal lactone (−)-NL (**82**) and abnormal lactones (−)-ANL (**81**) same like other ene-reductases. However, in redox reactions of (+)-carvone (**77**) mutant W116I (in combination with BVMOs) enabled formation of two new lactones (+)-NL (**85**) and (+)-ANL (**86**) which were not accessable with other ene-reductases.

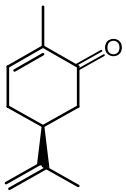
Enzyme	**Time**	 **(+)- Carvone (77)**			
		CHMO ***Acineto***	**CHMO** ***Brevi 1***	**CHMO** ***Brevi 2***	**CPMO** ***Coma***
SYE-4	3h	--	42%[a] (0:100)[b]	5.6% (0:100)	6% (0:100)
	6h	100% (99:1)	78% (0:100)	19% (0:100)	8% (0:100)
OPR1	3h	57% (98:2)	11% (100: 0)	1% (0:100)	2% (0:100)
	6h	100% (98:2)	25% (100: 0)	2% (0:100)	3% (0:100)
OPR3	3h	83% (98:2)	85% (100:0)	4.3% (0:100)	2% (0:100)
	6h	99% (75:25)	97% (79:21)	7.5% (0:100)	3% (0:100)
YqjM	3h	100% (91:9)	18% (100: 0)	2% (0:100)	traces (0:100)
	6h	100% (85:15)	34% (100: 0)	4% (0:100)	traces (0:100)
W116I[d]	24h	95% (99:1)	45% (99:1)	traces (0:100)	traces (0:100)

Table 2.32 One-pot redox reaction of (+)-carvone (**77**) with EREDs & BVMOs.

a) conversion %, b) normal lactone: abnormal lactone (N : AB), d) (+)-NL (**85**) & (+) ANL (**86**)

So by applying different combinations of redox enzymes both normal and abnormal lactones can be accessed from (+)-carvone (**77**) and (−)-carvone (**76**). The graphical representation of all the results was presented in Figures 2.36 & 2.37.

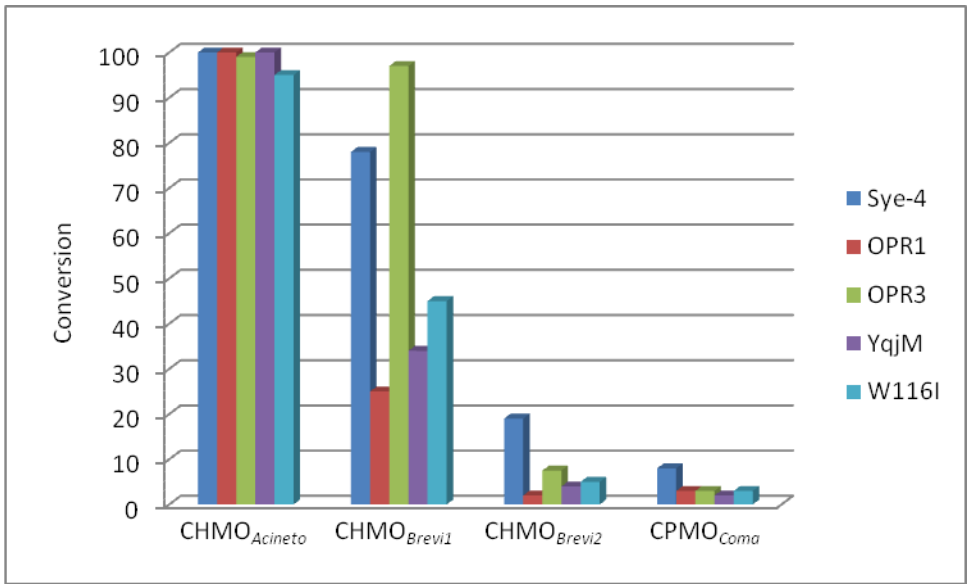


Figure 2.36 Graphical representations of redox reactions of (+)-Carvone (**77**)

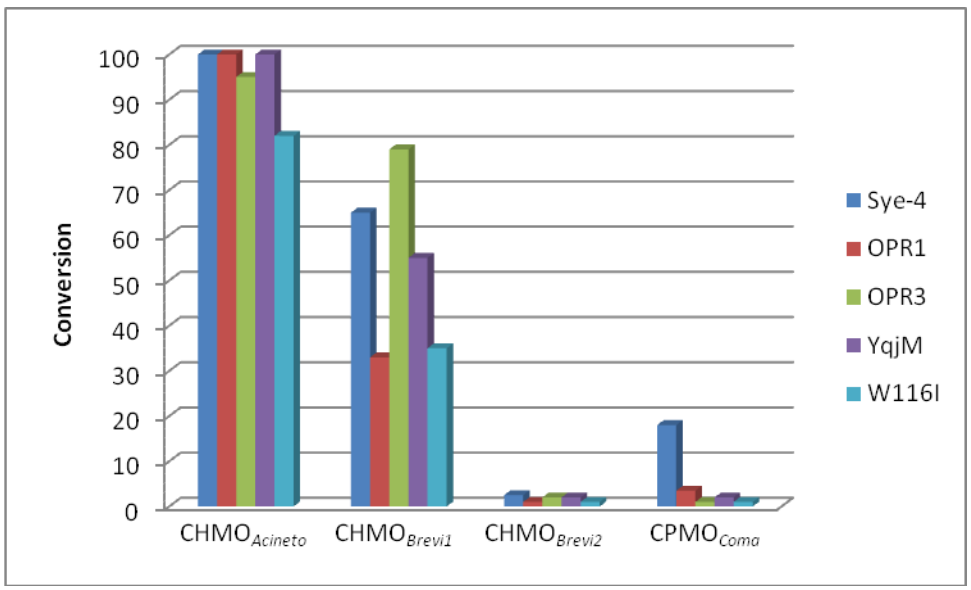


Figure 2.37 Graphical representations of redox reactions of (−)-carvone (**76**)

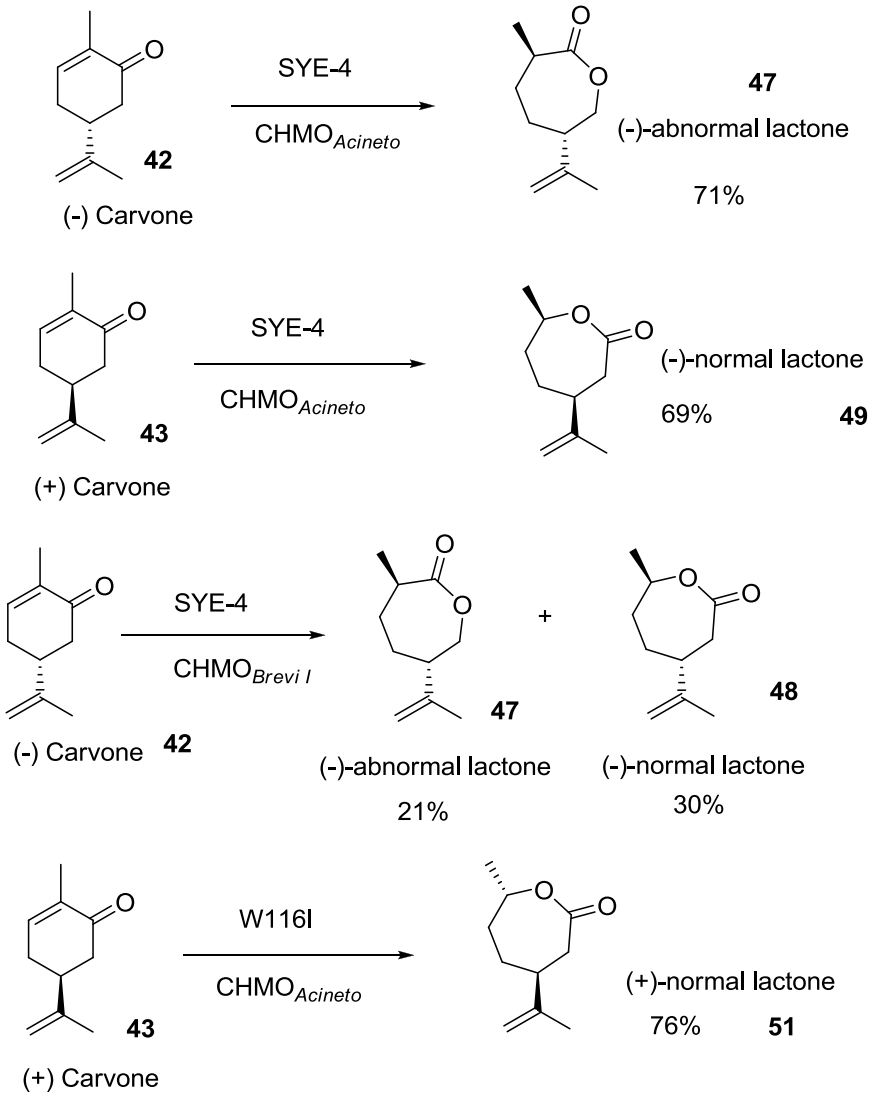


Figure 2.38 Scale up one-pot redox reactions of carvones

To confirm the results of the screening, representative biotransformations were also performed on preparative scale in order to isolate and characterize the redox products. Starting from (−)-carvone (**76**) abnormal lactone (−)-ANL (**81**) ($[\alpha]_D^{22}$ = −31.4 (c = 0.7, $CHCl_3$)) was obtained in 71% isolated yield by combination of SYE-4 and CHMO$_{Acineto}$; (+)-carvone gave normal lactones (−)-NL (**83**) ($[\alpha]_D^{22}$ = −5.7 (c = 0.2, $CHCl_3$)) in 69% isolated yield after 24h.

Preparative scale experiment of (−)-carvone with SYE-4 and CHMO$_{Brevi1}$ gave a mixture of normal lactone (−)-NL (**82**) ($[\alpha]_D^{22}$ = −39.6 (c = 0.96, $CHCl_3$); ref.[141] $[\alpha]_D^{20}$ = −42.1 (c = 1.12, $CHCl_3$)) and abnormal lactone (−)-ANL (**47**) ($[\alpha]_D^{22}$ = −35.2 (c = 1.13, $CHCl_3$); ref.[141] $[\alpha]_D^{20}$ = −35.8 (c = 1.6, $CHCl_3$)) in 30% and 21% isolated yields.

The new product (+)-NL (**51**) ($[\alpha]_D^{22}$ = +42.3 (c = 1.13, $CHCl_3$); ref.[163] $[\alpha]_D^{20}$ = +46.2 (c = 1.1, $CHCl_3$)) was obtained from the one pot redox combination of W116I and $CHMO_{Acineto}$ starting from (+)-carvone

[163] Alphand, V.; Furstoss, R. *Tetrahedron Asymmetry*. **1992**, *3*, 379–382.

2.4 Conclusions

In summary, *trans*-specific bioreductions with *Shewanella* Yellow Enzyme of different activated alkenes such as α,β-unsaturated ketones, α,β-unsaturated esters, and α-methylmaleimides were performed with high stereoselctivity generating new chiral centres. Both whole cell biotransformations and crude cell extract biotransformations were performed with SYE-3 and SYE-4 for these substrates.The α,β-unsaturated ketones (**1-5** & **8-9**) were the most difficult substrates with respect to enantioselectivity. Unsaturated diesters (**13** & **14**) having both the ester groups directly linked to the double bond were fully reduced to yield enantiomerically pure *R*-products. Whereas, diesters (**16** & **17**) resulted in little or almost no conversion with SYE-4, probably owing to the presence of a terminal methylene group; so *exo*-methylene analogs proved to be difficult substrates. The compounds with imide functionality (**22-29**) were investigated to extend the substrates tolerance of SYE CCE on more complex cyclic carboxylic acid derivatives. These α-methylmaleimides turned out to be excellent substrates for SYE-4, with almost 100% conversion and high enantioselectivity when using CCE. The tolerance for the functional groups proved to be remarkably broad. With compounds (**27** & **29**) bearing two C=C double bonds, only the alkene group of the α,β-unsaturated ketone function was reduced selctivity, where as non acivated double bond remained intact. As a whole, efficiency of the bioreduction in the cell lysate is better than the whole cells.

Baylis Hillman products were the new substrates for ERED mediated bioreduction studies. Applying general Baylis Hillman conditions it was possible to synthesize different adducts using aldehyde (aromatic and aliphatic), acrylate (methyl acrylate and acrylamide) and DABCO in good to moderate yields. These substrates were tested with *Shewanella* Yellow Enzyme (SYE-4), YqjM, OPR1 and OPR3 enzymes. Only YqjM was an efficient enzyme to convert this class of compounds with some interesting outcomes. In the bioreduction of Baylis Hillman products (**31-45**), the *syn*-diastereoisomer was obtained in excess as compare to *anti*-diastereoisomer with good conversions. Theoratically, a 50:50 *syn:anti* mixture was expected fron this transformation as we started with racemic starting material. At present there are two hypotheses for this *syn* selectivity:

1. Crude cell extracts from cells was used which may also contain some alcohol dehydrogeneases; such native enzymes of the expression host can facilitate the reversible alcohol-ketone interconversions (racemization process around the carbinol carbon).
2. According to the generally accepted mechanisms for EREDs, *syn* diastereoisomers are obtained from *R*-precursors and *anti* from *S* enantiomers, as the hydride attacks on the β-carbon followed by a shift of double bond in conjugation with carbonyl group. Then the proton capture from a tyrosine residue gives *anti* addition of hydrogen. There is a possibility of rearrangement after the attack of the hydride ion at the β-carbon leading to the loss of stereocentre in the *S* enantiomer. Reverse reaction of this rearrangement can give *R* configuration at the carbinol carbon which on accepting proton forms *syn* product.

Aza Baylis Hillman adducts with different substituents (**53, 56,64-67** and **69-70**) were also synthesized employing different strategies. Bioreduction of these substrates with Ene-reductases (*Shewanella* Yellow Enzyme SYE-4, YqjM, OPR1 and OPR3) gave different results with only *anti* diastereoisomer with high stereoselectivity. This biotransformation offers a biocatalytically novel entry towards the stereoselective synthesis of β-amino acids. There is not explanation yet for the *anti* selectivity in this transformation. The representative compounds from this series were scaled up for the characterization and absolute configuration assignments.

Regiodivergent biooxygenation by BVMOs ($CHMO_{Acineto}$, $CHMO_{Brevi1}$, $CHMO_{Brevi2}$ and $CPMO_{Coma}$) was coupled with ERED ((SYE-4, OPR1, OPR3, YqjM and mutant W116I) mediated bioreductions of enones with high stereoselectivity. Firstly, bioreduction of enantiomerically pure carvones was performed to get dihydrocarvones with high yields and selectivity. Secondly, biooxygenation of these dihydrocarvones with BVMOs was studied to get normal and abnormal lactones. At the end these two steps were combined in one pot cascade reactions to form different types of normal and abnormal lactones starting from simple carvones.

Experimental

3.1 General

Chemicals and microbial growth media were purchased from commercial suppliers and used without further purification. All solvents were distilled prior to use. Melting points were determined using a Kofler-type Leica Galen III micro hot stage microscope and are uncorrected. Screening experiments were performed in sterile multiwell plates. Flash column chromatography was performed on silica gel 60 from Merck (40–63 µm). NMR spectra were recorded on a Bruker AC 200 (200 MHz) spectrometer in deuterated solvents and chemical shifts are reported in ppm using TMS as internal standard. Peak assignment is based on correlation experiments. Ambiguous assignment is marked with an asterix. Harvesting of cells was performed by centrifugation using a Sigma 6K15 centrifuge (rotor 372/C). Centrifugations for removal of debris were done on Sigma 3K30 centrifuge (rotor 12155-H). Gerhard THO5 orbital thermoshakers were used for cultivating microbial cells. Ultrasonication was carried out by using a Bandeli. Enantiomeric purity was determined by chiral phase GC using a BGB 175 column (30 m × 0.25 mm ID, 0.25 µm film) on a Thermo Finnigan Trace or Focus chromatograph and compared to reference material obtained by chemical conversion on where applicable. GC-MS analyses were carried out on a GC-MS Thermo Scientific DSQ II with standard capillary column BGB5 (30 m × 0.25 mm ID, 0.25 µm film). Rotation values $[\alpha]_D^{20}$ were determined using a Anton Paar MCP 500 Polarimeter by the following equation: $[\alpha]_D^{20} = 100*\alpha/(c*l)$; c[g/100 mL], l[dm].

Dip reagent: I) 13.2 g conc. sulfuric acid

0.8 g cerium (IV)-ammonium nitrate

10.0 g phosphor molybdate

150 mL ethanol

II) 1.00g $KMnO_4$

20.0 g K_2CO_3

10.0 mL NaOH /5%

150 mL H_2O

Abbreviations:

AcOH	acetic acid
Amp	ampicilin
b.p.	boiling point
DABCO	1,4-diazabicyclo[2.2.2]octane
DCC	*N*,*N'*-dicyclohexylcarbodiimide
DMSO	dimethylsulfoxide
EtOAc	ethyl acetate
Et_2O	diethyl ether
EtOH	ethanol
IPTG	isopropyl-beta-thio-galactopyranoside
KRD	*Kugelrohr* distillation
LB	Luria-Bertani media
MeOH	methanol
MW	Microwave
m.p.	melting point
PBS	phosphate buffered saline
rt	room temperature
TB	terrific broth media
THF	tetrahydrofuran
TLC	thin layer chromatography

3.1.1 Biotransformations

Media for biotransformations

LB_{amp}		TB_{amp}	
10.0g	bacto-peptone	12g	bacto-tryptone
5.0g	yeast extract	24g	bacto-yeast
10.0	sodium chloride	4ml	glycerol
		16.4g	$K_2HPO_4*3H_2O$
		2.3g	KH_2PO_4
1000mL	deion. H_2O	1000mL	deion. H_2O
4mL	autoclaved at 121°C for 20 minutes, then add ampicilline stock solution	4mL	autoclaved at 121°C for 20 minutes, then add ampicilline stock solution

Phosphated-buffered Saline (10Mm PBS)

8.0g NaCl

0.2g KCL

1.44g Na_2HPO_4

0.24g KH_2PO_4

800mL deion. H_2O, adjust pH to 7.4 with HCl, add water to 1l, autoclave at 121^oC for 20minutes

Tris/HCl buffer (50mM)

3.0 g Tris(hydroxymethyl)-aminomethane (Tris)

400mL adjust pH to 7.4 with HCl, add water to 500mL, autoclave at 121^oC for 20minutes

Chloroamphenicol – stock solution

34mg/mL dissolved in ethanol, sterilization by filtration (0.2μm)

Ampicilline – stock solution

50mg/mL dissolved in deion. water, sterilization by filtration (0.2μm)

IPTG – solution

200mg/mLdissolved in deion. water, sterilization by filtration (0.2µm)

Preparation of frozen stocks

E.coli strain was incubated at 37°C on LB_{amp} plates for 12 hours. A single colony was selected and a 10 mL pre-culture was inoculated (10 mL LB_{amp}, shake flask, 120 rpm for 12hours at 37°C). After addition of 2 mL glycerol, the mixture was vortexed, transferred in 1 mL aliquots into Eppendorf vials and stored at -80°C.

3.2 Synthesis of Substrates and Reference Materials for Bioreductions with SYE CCE

3.2.1 Synthesis of α,β-Unsaturated Cyclic Ketones and Diesters

2-Methylcyclopent-2-enone (**1**), 3-methylcyclopent-2-enone (**2**), 3-methylcyclohex-2-enone (**3**), 1-cyclopentenylethanone (**4**) and 1-cyclohexenylethanone (**5**) were commercially available unsaturated ketones and used for the bioreduction. Compounds (**8** & **9)** were synthesized by using the reported protocol[142], spectral data and yields were comparable with reported values.

Similarly, different types of diesters were synthesized by esterification of 2-methylmaleic acid (**12**) and 2-methylenesuccinic acid (**15**)[117].

3.2.2 Synthesis of *N*-Substituted Imides

3.2.2.1 Condition Optimization for the Imide Synthesis

3.2.2.1.1 Procedure 1

Maleic anhydride (**21**) (5.10mmol) and benzyl amine (5.10mmol) were dissolved in glacial acetic acid (40mL) and the reaction mixture was refluxed under nitrogen along with stirring for one hour. Glacial acetic acid was removed by extraction of the reaction mixture with ethyl acetate or chloroform and water. Product was purified by column chromatography**.**

3.2.2.1.2 Procedure 2

A mixture of maleic anhydride (**21**) (5.10mmol) and benzyl amine (5.10mmol) was placed in a pressure-resistant test tube provided with a magnetic stirring bar and was sealed with a septum. The tube was subjected to MW irradiation with a power of 150 W for 5-30 min at 100°C. The reaction mixture turned red. After cooling, the reaction mixture was extracted with chloroform and washed with cold water, dried (Na_2SO_4), filtered and the solvent was removed, product was purified by column chromatography.

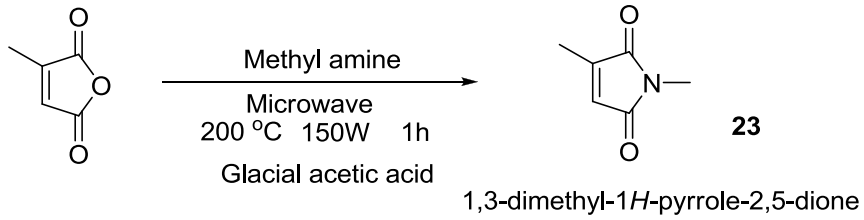
3.2.2.1.3 Procedure 3

A mixture of maleic anhydride derivative (**21**) (4.46 mmol) and amine (4.46 mmol) in glacial acetic acid was placed in a pressure-resistant test tube provided with a magnetic stirring bar and

sealed with a septum. The tube was subjected to MW irradiation with a power of 150 W for 1h at 200 ^{o}C. The reaction mixture turned red. After cooling, the reaction mixture was extracted with chloroform and washed with cold water, dried (Na_2SO_4), filtered and the solvent was removed; product was purified by column chromatography.

Similarly saturated imides for reference purpose were synthesized by the reaction of 2-methyl succinic anhydride with different amines under similar microwave conditions.

3.2.2.2 Synthesis of 1,3-Dimethyl-1H-pyrrole-2,5-dione (23)



The compound 1,3-dimethyl-1H-pyrrole-2,5-dione (**23**) was prepared according to the general procedure 3 using citraconic anhydride (500 mg, 4.46 mmol) and methyl amine (138 mg, 4.46 mmol) in 5mL of glacial acetic acid. The crude product was purified by column chromatography (silica gel) using ethyl acetate and petrol ether mixture (5-10%).

Product (**23**): 246 mg, 1.96 mmol

Yield: 45%

Chemical Formula: $C_6H_7NO_2$

Molecular Weight: 125.13

Appearance: Colorless oil

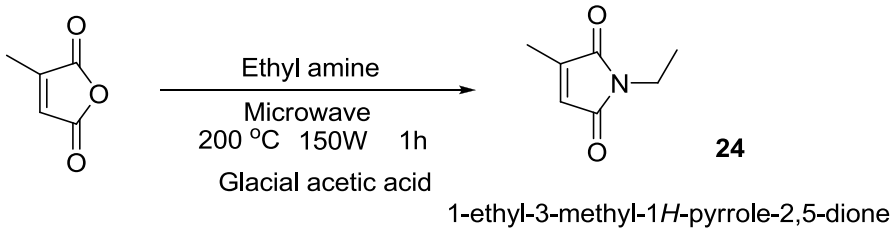
^{1}H-NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 2.08 (d, J = 1.8Hz, 3H), 2.99 (s, 3H), 6.34 (d, J = 1.8Hz, 1H).

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 10.8 (q), 23.6 (q), 127.2 (d), 145.7 (s), 170.8 (s), 171.8 (s).

3.2.2.3 Synthesis of 1-Ethyl-3-methyl-1H-pyrrole-2,5-dione (24)



The compound 1-ethyl-3-methyl-1H-pyrrole-2,5-dione (**24**) was prepared according to the general procedure 3 using citraconic anhydride (500 mg, 4.46 mmol) and ethyl amine (0.812 mL, 4.46 mmol) in 5mL of glacial acetic acid. The product was extracted with ethyl acetate, dried over Na_2SO_4 and evaporated under vacuum. The crude product was purified by column chromatography (silica gel) using ethyl acetate and petrol ether mixture (5-10%).

Product (**24**): 259 mg, 1.86 mmol

Yield: 43%

Chemical Formula: $C_7H_9NO_2$

Molecular Weight: 139.15

Appearance: Colorless oil

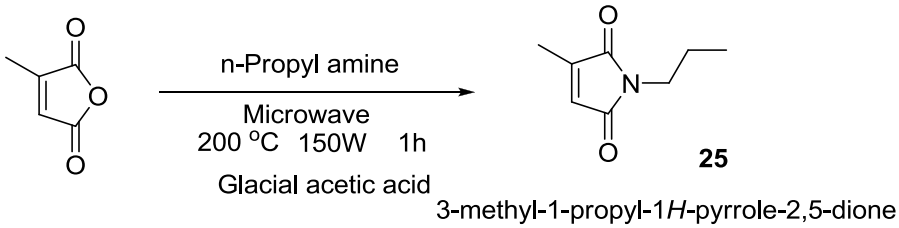
^{1}H-NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 1.17 (t, J = 7.2Hz, 3H), 2.08 (s, 3H), 3.54 (q, J = 7.2Hz, 2H), 6.32 (d, J = 1.6Hz, 1H).

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 10.7 (q), 13.8 (q), 32.6 (t), 127.1 (d), 145.4 (s), 170.6 (s), 171.6 (s).

3.2.2.4 Synthesis of 3-Methyl-1-propyl-1H-pyrrole-2,5-dione (25)



3-methyl-1-propyl-1*H*-pyrrole-2,5-dione

The compound 3-methyl-1-propyl-1H-pyrrole-2,5-dione (**25**) was prepared according to the general procedure 3 using citraconic anhydride (500 mg, 4.46 mmol) and n-propyl amine (255 mg, 4.46 mmol) in 5mL glacial acetic acid. The product was extracted with ethyl acetate, dried over Na_2SO_4 and evaporated under vacuum. The crude product was purified by column chromatography (silica gel) using ethyl acetate and petrol ether mixture (5-10%).

Product (**25**): 518 mg, 3.38 mmol

Yield: 75%

Chemical Formula: $C_8H_{11}NO_2$

Molecular Weight: 153.18

Appearance: Colorless oil

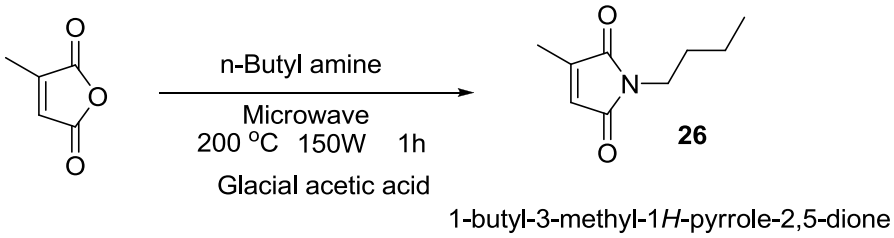
^{1}H-NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 0.89 (t, J = 7.4Hz, 3H), 1.55-1.65 (m, 2H), 2.09 (s, 3H), 3.45 (t, J = 6.8Hz, 2H), 6.33 (s, 1H).

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 10.7 (q), 11.0 (q), 21.7 (t), 39.3 (t), 127.0 (d), 145.3 (s), 170.8 (s), 171.8 (s).

3.2.2.5 Synthesis of 1-Butyl-3-methyl-1H-pyrrole-2,5-dione (26)



1-butyl-3-methyl-1*H*-pyrrole-2,5-dione

The compound 1-butyl-3-methyl-1H-pyrrole-2,5-dione (**26**) was prepared according to the general procedure 3 using citraconic anhydride (500 mg, 4.46 mmol) and n-butyl amine (319 mg, 4.46 mmol) in 5mL glacial acetic acid. The product was extracted with ethyl acetate, dried over Na_2SO_4 and evaporated under vacuum. The crude product was purified by column chromatography (silica gel) using ethyl acetate and petrol ether mixture (5-10%).

Product (**26**): 400 mg, 2.39 mmol

Yield: 54%

Chemical Formula: $C_9H_{13}NO_2$

Molecular Weight: 167.21

Appearance: Colorless oil

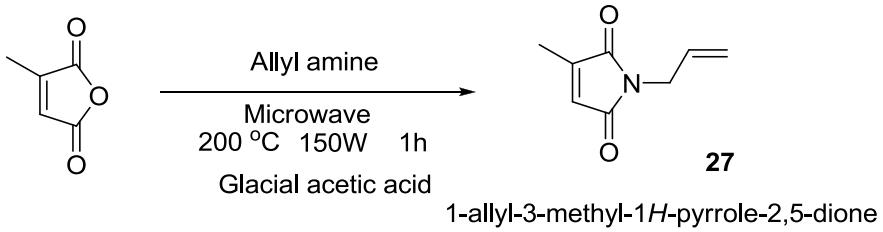
^{1}H-NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 0.91 (t, J = 7.2Hz, 3H), 1.21-1.38 (m, 2H), 1.48-1.63 (m, 2H), 2.08 (d, J = 1.8Hz, 3H), 3.48 (t, J = 7.2Hz, 2H), 6.42 (d, J = 1.8Hz, 1H).

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 10.8 (q), 13.4 (q), 19.8 (t), 30.5 (t), 37.5 (t), 127.1 (d), 145.3 (s), 170.8 (s), 171.8 (s).

3.2.2.6 Synthesis of 1-Allyl-3-methyl-1H-pyrrole-2,5-dione (27)



The compound 1-allyl-3-methyl-1H-pyrrole-2,5-dione (**27**) was prepared according to the general procedure 3 using citraconic anhydride (500 mg, 4.46 mmol) and allyl amine (253 mg, 4.46 mmol) in 5mL glacial acetic acid. The product was extracted with ethyl acetate, dried over Na_2SO_4 and evaporated under vacuum. The crude product was purified by column chromatography (silica gel) using ethyl acetate and petrol ether mixture (5-10%).

Product (**27**): 434 mg, 2.87 mmol

Yield: 64%

Chemical Formula: $C_8H_9NO_2$

Molecular Weight: 151.16

Appearance: Colorless liquid

^{1}H-NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 2.09 (d, J = 1.8Hz, 3H), 4.10 (dt, J = 5.6Hz, J = 1.4Hz, 2H), 5.11-5.21 (m, 4H), 5.70-5.89 (m, 1H), 6.36 (q, J = 1.6Hz, 1H).

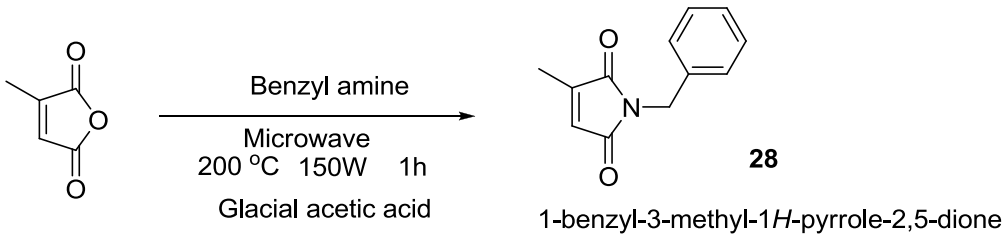
^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 10.8 (q), 39.8 (t), 117.1 (t), 127.2 (d), 131.7 (d), 145.6 (s), 170.2 (s), 171.2 (s).

GC-MS

m/z 151 (M^+, 100), 94 (53), 68 (71), 56 (53), 54 (73).

3.2.2.7 Synthesis of 1-Benzyl-3-methyl-1H-pyrrole-2,5-dione (28)



The compound 1-benzyl-3-methyl-1H-pyrrole-2,5-dione (**28**) was prepared according to the general procedure 3 using citraconic anhydride (500 mg, 4.46 mmol) and benzyl amine (476 mg, 4.46 mmol) in 5mL of glacial acetic acid. The product was extracted with ethyl acetate, dried over Na_2SO_4 and evaporated under vacuum. The crude product was purified by column chromatography (silica gel) using ethyl acetate and petrol ether mixture (5-10%).

Product (**28**): 640 mg, 3.18 mmol

Yield: 71%

Chemical Formula: $C_{12}H_{11}NO_2$

Molecular Weight: 201.22

Appearance: Colorless oil

^{1}H-NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 2.01 (d, J = 2.0Hz, 3H), 4.60 (s, 2H), 6.26 (d, J = 2.0Hz, 1H), 7.22-7.28 (m, 5H).

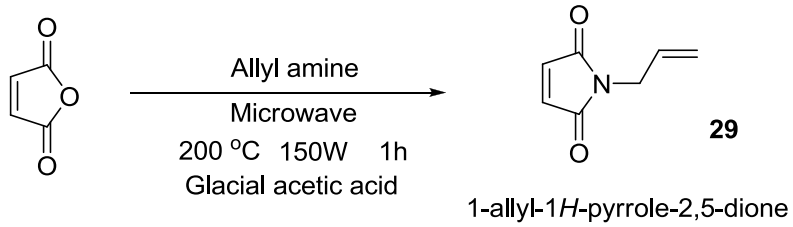
^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 10.9 (q), 41.4 (t), 127.3 (d), 127.7 (d), 128.3 (d), 128.6 (d), 136.5 (s), 145.6 (s), 170.4 (s), 171.4 (s).

GC-MS

m/z 201 (M^+, 100), 172 (31), 104 (70), 78 (50), 51 (29).

3.2.2.8 Synthesis of 1-Allyl-1H-pyrrole-2,5-dione (29)



The compound 1-allyl-1H-pyrrole-2,5-dione (**29**) was prepared according to the general procedure 3 maleic anhydride (500 mg, 5.1 mmol) and allyl amine (290 mg, 5.1 mmol) in 5mL of glacial acetic acid. The product was extracted with ethyl acetate, dried over Na_2SO_4 and evaporated under vacuum. The crude product was purified by column chromatography (silica gel) using ethyl acetate and petrol ether mixture (5-10%).

Product (**29**): 335 mg, 2.44 mmol

Yield: 48%

Chemical Formula: $C_7H_7NO_2$

Molecular Weight: 137.14

Appearance: Colorless oil

^{1}H-NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 4.06 (dt, J = 5.4Hz, J =1.4Hz, 2H), 5.06-5.15 (m, 2H), 5.63-5.80 (m, 1H), 6.65 (s, 2H).

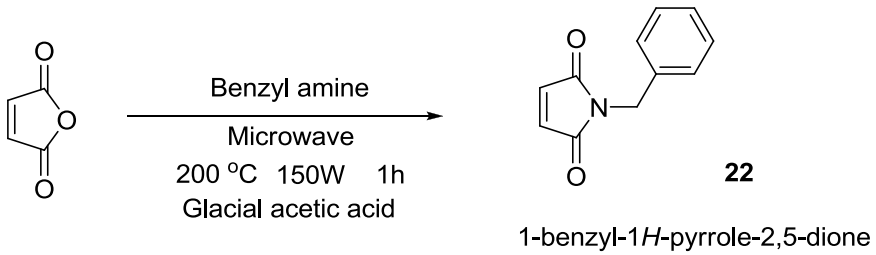
^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 39.8 (t), 117.6 (t), 131.4 (d), 134.1 (d), 170.3 (s).

GC-MS

m/z 137 (M^+, 100), 86 (11), 56 (12), 55(28).

3.2.2.9 Synthesis of 1-Benzyl-1H-pyrrole-2,5-dione (22)



The compound 1-benzyl-1H-pyrrole-2,5-dione (**22**) was prepared according to the general procedure 3 using maleic anhydride (500 mg, 5.1 mmol) and phenyl amine (545 mg, 5.1 mmol) in 5mL of glacial acetic acid. The product was extracted with ethyl acetate, dried over Na_2SO_4 and evaporated under vacuum. The crude product was purified by column chromatography (silica gel) using ethyl acetate and petrol ether mixture (5-10%).

Product (**22**): 496 mg, 2.65 mmol, Yield: 52%

Chemical Formula: $C_{11}H_9NO_2$

Molecular Weight: 187.19

Appearance: Colorless solid

m.p: 72-74^{o}C

^{1}H-NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 4.67 (s, 2H), 6.70 (s, 2H), 7.25-7.37 (m, 5H, Ar).

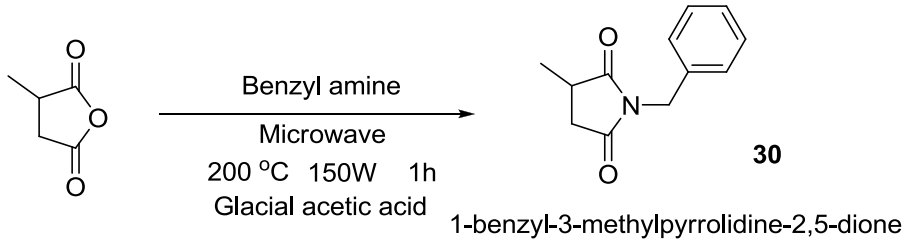
^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 41.4 (t), 127.3 (d), 128.3 (d), 128.7 (d), 134.1 (s), 136.2 (s), 170.4 (s).

GC-MS

m/z 187 (M^+, 100), 130 (44), 106 (91), 104(70), 77 (22), 65 (15).

3.2.2.10 Synthesis of 1-Benzyl-3-methylpyrrolidine-2,5-dione (30)



The compound 1-benzyl-3-methylpyrrolidine-2,5-dione (**30**) was prepared according to the general procedure 3 using 2-methyl succinic anhydride (500 mg, 4.38 mmol) and phenyl amine (465 mg, 4.38 mmol) in 5mL of glacial acetic acid. The crude product was purified by column chromatography (silica gel) using ethyl acetate and petrol ether mixture (5-10%).

Product (**30**): 345 mg, 1.69 mmol

Yield: 38%

Chemical Formula: $C_{12}H_{13}NO_2$

Molecular Weight: 203.24

Appearance: Colorless oil

^{1}H-NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 1.30 (d, J = 7.2Hz, 3H), 2.14-2.37 (m, 1H), 2.80-2.90 (m, 2H), 4.62 (s, 2H), 7.25-7.34 (m, 5H).

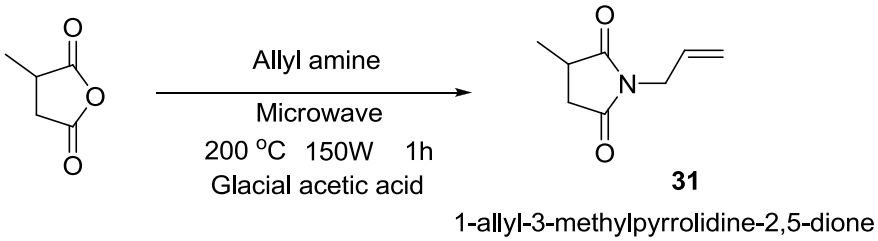
^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 16.7 (q), 34.7 (t), 36.4 (d), 42.3 (t), 127.9 (d), 128.6 (d), 128.7 (d), 135.8 (s), 176.1 (s), 180.2 (s).

GC-MS

m/z 203 (M^+, 100), 174 (20), 160 (72), 104(64), 77 (33), 65 (32).

3.2.2.11 Synthesis of 1-Allyl-3-methylpyrrolidine-2,5-dione (31)



The compound 1-allyl-3-methylpyrrolidine-2,5-dione (**31**) was prepared according to the general procedure 3 using 2-methyl succinic anhydride (500 mg, 4.38 mmol) and allyl amine (248 mg, 4.38 mmol) in 5mL of glacial acetic acid. The product was extracted with ethyl acetate, dried over Na_2SO_4 and evaporated under vacuum. The crude product was purified by column chromatography (silica gel) using ethyl acetate and petrol ether mixture (5-10%).

Product (**32**): 304 mg, 1.98 mmol

Yield: 42%

Chemical Formula: $C_8H_{11}NO_2$

Molecular Weight: 153.18

Appearance: Colorless oil

^{1}H-NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 1.35 (d, J = 8Hz, 3H), 2.30-2.37 (m, 1H), 2.87-3.02 (m, 2H), 4.10 (d, J = 6Hz, 2H), 5.15-5.30 (m, 2H), 5.69-5.86 (m, 1H).

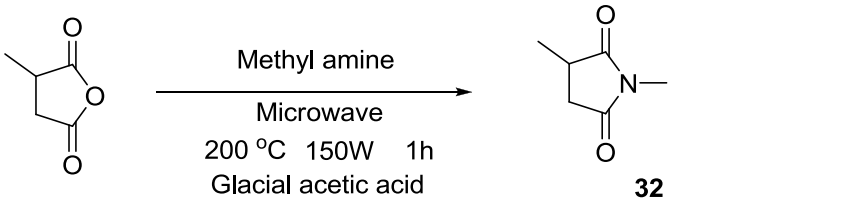
^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 16.5 (q), 34.5 (t), 36.2 (d), 40.6 (t), 117.8 (t), 130.7 (d), 176.2 (s), 180.2 (s).

GC-MS

m/z 153 (M^+, 1), 110 (12), 82(29), 70(39), 56 (100), 54 (98).

3.2.2.12 Synthesis of 1,3-Dimethylpyrrolidine-2,5-dione (32)



The compound 1,3-dimethylpyrrolidine-2,5-dione (**32**) was prepared according to the general procedure 3 using 2-methyl succinic anhydride (500 mg, 4.38 mmol) and methyl amine (0.49 mL, 4.38 mmol) in 5mL of glacial acetic acid. The product was extracted with ethyl acetate, dried over Na_2SO_4 and evaporated under vacuum. The crude product was purified by column chromatography (silica gel) using ethyl acetate and petrol ether mixture (5-10%).

Product (**32**): 463 mg, 3.46 mmol

Yield: 83%

Chemical Formula: $C_6H_9NO_2$

Molecular Weight: 127.14

Appearance: Colorless liquid

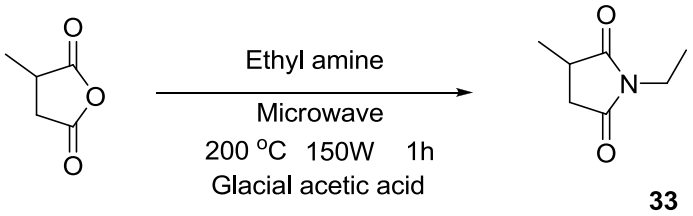
^{1}H-NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 1.34 (d, J = 7Hz, 3H), 2.27-2.37 (m, 1H), 2.85-2.89 (m, 2H), 2.98 (s, 3H).

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 16.7 (q), 24.8 (q), 34.7 (t), 36.4 (d), 176.6 (s), 180.6 (s).

3.2.2.13 Synthesis of 1-Ethyl-3-methylpyrrolidine-2,5-dione (33)



33

1-ethyl-3-methylpyrrolidine-2,5-dione

The compound 1-ethyl-3-methylpyrrolidine-2,5-dione (**33**) was prepared according to the general procedure 3 using 2-methyl succinic anhydride (500 mg, 4.38 mmol) and ethyl amine (0.72 mL, 4.38 mmol) in 5mL of glacial acetic acid. The product was extracted with ethyl acetate, dried over Na_2SO_4 and evaporated under vacuum. The crude product was purified by column chromatography (silica gel) using ethyl acetate and petrol ether mixture (5-10%).

Product (**33**): 478 mg, 3.39 mmol

Yield: 77%

Chemical Formula: $C_7H_{11}NO_2$

Molecular Weight: 141.17

Appearance: Colorless oil

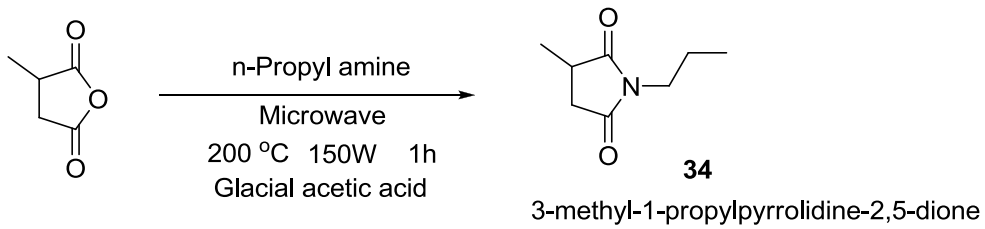
^{1}H-NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 1.16 (t, J = 7.2Hz, 3H), 1.34 (d, J = 7.2Hz, 3H), 2.26-2.35 (m, 1H), 2.79-2.89 (m, 2H), 3.54 (q, J = 7.2Hz, 2H).

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 12.9 (q), 16.6 (q), 33.5 (t), 34.5 (t), 36.3 (d), 176.2 (s), 180.3 (s).

3.2.2.14 Synthesis of 3-Methyl-1-propylpyrrolidine-2,5-dione (34)



The compound 3-methyl-1-propylpyrrolidine-2,5-dione (**34**) was prepared according to the general procedure 3 using 2-methyl succinic anhydride (500 mg, 4.38 mmol) and n-propyl amine (255 mg, 4.38 mmol) in 5mL of glacial acetic acid. The product was extracted with ethyl acetate, dried over Na_2SO_4 and evaporated under vacuum. The crude product was purified by column chromatography (silica gel) using ethyl acetate and petrol ether mixture (5-10%).

Product (**34**): 518 mg, 3.34 mmol

Yield: 75%

Chemical Formula: $C_8H_{13}NO_2$

Molecular Weight: 155.19

Appearance: Colorless oil

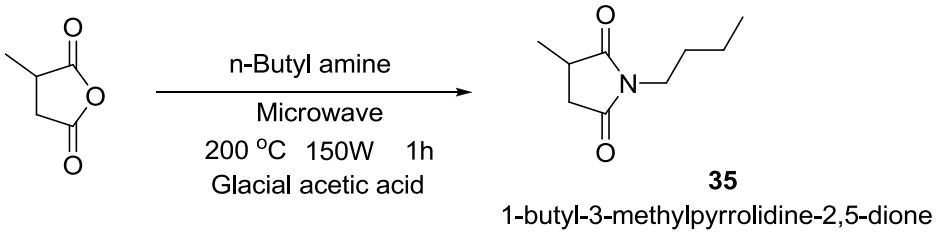
^{1}H-NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 0.78 (t, J = 7.4Hz, 3H), 1.22 (d, J = 3.2Hz, 3H), 1.41-1.54 (m, 2H), 2.16-2.24 (m, 1H), 2.72-2.84 (m, 2H), 3.35 (t, J = 7Hz, 2H).

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 11.1 (q), 16.7 (q), 20.9 (t), 34.5 (t), 36.2 (t), 40.2 (d), 176.5 (s), 180.6 (s).

3.2.2.15 Synthesis of 1-Butyl-3-methylpyrrolidine-2,5-dione (35)



The compound 1-butyl-3-methylpyrrolidine-2,5-dione (**35**) was prepared according to the general procedure 3 using 2-methyl succinic anhydride (500 mg, 4.38 mmol) and n-butyl amine (319 mg, 4.38 mmol) in 5mL of glacial acetic acid. The product was extracted with ethyl acetate, dried over Na_2SO_4 and evaporated under vacuum. The crude product was purified by column chromatography (silica gel) using ethyl acetate and petrol ether mixture (5-10%).

Product (**35**): 600 mg, 3.55 mmol

Yield: 80%

Chemical Formula: $C_9H_{15}NO_2$

Molecular Weight: 169.22

Appearance: Colorless oil

^{1}H-NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 0.80 (t, J = 7Hz, 3H), 1.22 (d, J = 6Hz, 3H), 1.13-1.46(m, 4H), 2.15-2.42 (m, 1H), 2.72-2.87 (m, 2H), 3.36 (t, J = 7.2Hz, 2H).

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 13.4 (q), 16.6 (q), 19.9 (t), 29.6 (t), 34.4 (t), 36.2 (t), 38.3 (d), 176.3 (s), 180.5 (s).

3.3 Bioreductions with SYE-3 and SYE-4 Ene-Reductases

3.3.1 Bioreductions with SYE-3 and SYE-4 BL21 (DE3) Expressing Cells

An Erlenmeyer flask containing sterile TB medium supplemented with chloroamphenicol, (34µg/mL) was incubated with 2% vol of overnight culture SYE *E. coli* BL21(DE3) grown on $LB_{chloamph}$ media up to O.D. of 1 within 3h at 28°C. Production of enzyme was induced by IPTG (0.5mM), substrates (Fig 2.9) were added after 1h. Samples were analyzed with chiral GC and GC-MS.

3.3.2 Typical Procedure for Crude Cell Extract Preparation

Cell lysate was isolated from SYE-3 and SYE-4 *E.coli* BL21 (DE3) cells. An Erlenmeyer flask containing sterile TB medium (200mL) supplemented with chloroamphenicol (34µg/ml) (200µL) was incubated with 2% vol of overnight culture grown on LB medium (chloroamphenicol) up to O.D. of 1 within 3h at 28°C. Production of enzyme was induced by IPTG (0.5mM) (160µL) and the flask was shaken (120 rpm) at 28°C for 24h. The cells pellet was collected by centrifugation (6000 rpm for 15min) and resuspended in PBS (30mL), again centrifuged (6000 rpm for 4 min) and pellet was resuspended in PBS (5mL). Protease inhibitor (phenyl methane sulfonyl fluoride) was added (1µL/mL). Cells were ruptured by sonication at 0^{o}C (pulse for 10 seconds after every 1 min about six times). After sonication, suspension was centrifuged (10,000 rpm for 15 min) to obtain cell lysate, which was stored at -20°C. Bradford assay was conducted to calculate the concentration of protein in cell lysate.

3.3.3 Typical Procedure Bradford Assay for Protein Concentration

For estimation of protein concentration Bio-Rad Protein Assay was conducted. Bovine serum albumin (BSA) was used as standard. Standard curve was prepared each time the assay is performed in the range between 0-1000 µg/mL.

1. Place 0.8mL of standards or samples in clean, dry test tubes. Place 0.8Ml sample buffer in blank test tube.
2. Add 0.2M Dye Reagent Concentrate.
3. Vortex (avoid excess foaming); or mix several times by gentle inversion of the test tube.
4. After a period of 5 minutes to one hour, measure OD_{590} versus reagent blank.

5. Plot OD_{590} versus concentration of standards. Read unknowns from the standard curve.

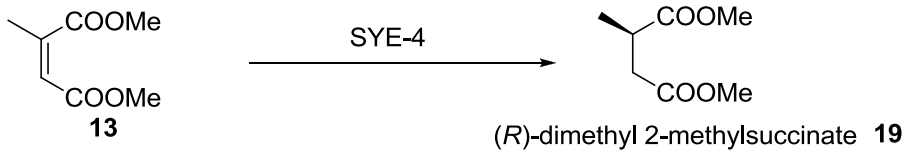
3.3.4 Typical Procedure for Bioreduction Screening Experiments

Biotransformation (200µL) with SYE protein (5mg, 100µL of CCE) in sterile multiwell plates (500µL each well) was performed in the presence of Tris HCl (pH 8, 50mM, 90.5 µL), $NADP^+$ (200µM, 4µL), glucose-6-phosphate (4mM, 80µL), glucose-6-phosphate dehydrogenease (1 unit, 0.24µL) and substrate (2mM, 0.8µL (stock solution (0.5M) in EtOH:H_2O=2:1) at 28°C for 6h. Samples were collected after 1h, 3h and 6h. Product was extracted with ethyl acetate containing internal standard (methyl benzoate). Samples were analyzed by chiral GC and GC-MS.

3.3.5 Typical Procedure for Bioreduction on Preparative Scale with SYE-4 Protein

Biotransformation with SYE-4 protein in sterile baffled Erlenmeyer flask was performed in the presence of Tris HCl (pH 8, 18mL, 50mM), $NADP^+$ (80 µL, 100mM stock solution), glucose-6-phosphate (1.6 mL, 100 mM stock solution), glucose-6-phosphate dehydrogenease (68 µL), SYE-4 CCE (5.6mL CCE, 35.5mg/mL) and substrate (0.5M solution (10-30mg) in EtOH:H_2O=2:1) at 28°C for 24h in orbital thermoshakers (750 rpm). Product was extracted with ethyl acetate and purified by column chromatography. Samples were analyzed by chiral GC and GC-MS.

3.3.6 Synthesis of (*2R*)-Dimethyl-2-methylsuccinate (19)



Dimethyl 2-methylmaleate (**13**) (20mg in ethanol: water (2:1)) was reduced by using Tris HCl (pH 8, 18mL, 50mM), $NADP^+$ (80 µL, 100mM stock solution), glucose-6-phosphate (1.6 mL, 100 mM stock solution), glucose-6-phosphate dehydrogenease (68 µL) and SYE-4 CCE (5.6mL, 35.5mg/mL) at 28°C to (*2R*)-Dimethyl 2-methylsuccinate (**19**) in 24h. The product was extracted with ethyl acetate, dried over Na_2SO_4 and evaporated under vacuum. The crude product was purified with column chromatography using ethyl acetate and petrol ether mixture (5-10%).

Product (**19**): 18 mg, 0.11 mmol, Yield: 85%

Chemical Formula: $C_7H_{12}O_4$, Molecular Weight: 160.17

Appearance: Colorless oil

$[\alpha]_D^{22}$ =+ 5.3 (c = 2.9, $CHCl_3$) (lit[164] $[\alpha]_D^{20}$ =+ 4.5 (c = 2.9, $CHCl_3$)

^{1}HNMR (200 MHz; $CDCl_3$; Me_4Si)

δ 1.23 (d, J = 7.2Hz, 3H, $CHCH_3$), 2.35-2.47 (m, 1H,CHCO), 2.68-2.94 (m, 2H, CH_2CO), 3.68 (s, 3H, $COOCH_3$), 3.70 (s, 3H, $COOCH_3$).

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 16.8 (q,), 35.6 (t), 37.3 (d), 51.5 (q), 51.7 (q), 172.0 (s), 175.5 (s).

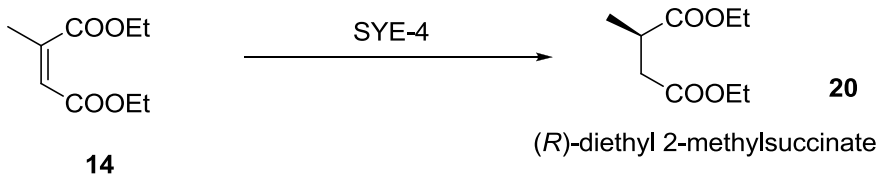
GC-MS

m/z 160 (M^+, 1), 127 (100), 99 (30),

---

[164] Salaun, J.; Karkour, B.; Ollivier, J. *Tetrahedron*, **1989,** 45, 10, 3151-3162.

3.3.7 Synthesis of (*2R*)-Diethyl 2-methylsuccinate (20)



Diethyl 2-methylmaleate (**14**) (20mg in ethanol: water (2:1)) was reduced by using Tris HCl (pH 8, 18mL, 50mM), $NADP^+$ (80 µL, 100mM stock solution), glucose-6-phosphate (1.6 mL, 100 mM stock solution), glucose-6-phosphate dehydrogenease (68 µL) and SYE-4 CCE (5.6mL, 35.5mg/mL) at 28°C to (*2R*)-diethyl 2-methylsuccinate (**20**) in 24h. The product was extracted with ethyl acetate, dried over Na_2SO_4 and evaporated under vacuum. The crude product was purified with column chromatography using ethyl acetate and petrol ether mixture (5-10%).

Product (**20**): 16 mg, 0.085 mmol**,** Yield: 83%

Chemical Formula: $C_9H_{16}O_4$

Molecular Weight: 188.22

Appearance: Colorless oil

$([\alpha]_D^{22}$ = + 3.1 (c = 0.9, $CHCl_3$).

^{1}HNMR (200 MHz; $CDCl_3$; Me_4Si)

δ 1.22 (d, J= 7Hz, 3H, CH$\underline{CH_3}$), 1.26 (dt, J= 7.2Hz J= 1.2Hz, 6H, $CH_2\underline{CH_3}$), 2.33-2.44 (m, 1H, CH), 2.66-2.92 (m, 2H, CH_2), 4.14 (dq, J=7.2Hz, J=2.4Hz, 4H, COO$\underline{CH_2}CH_3$),

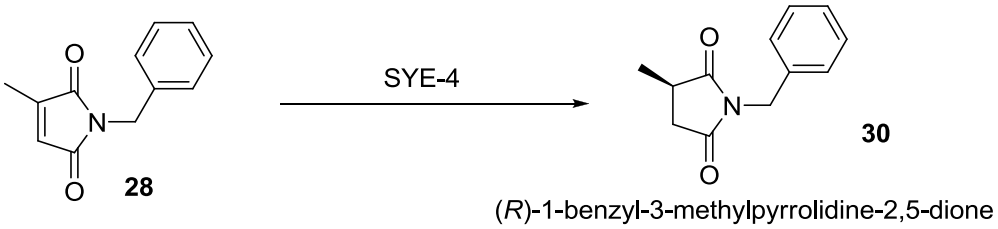
^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 16.0(q , O$CH_2\underline{CH_3}$), 16.8(q, CH$\underline{CH_3}$), 35.7(d, CH), 37.6(t, CH$\underline{CH_2}$), 60.3(t, O$\underline{CH_2}CH_3$), 60.4(t, O$\underline{CH_2}CH_3$), 171.6(s, $\underline{C}$OOEt), 175.1(s, $\underline{C}$OOEt).

GC-MS

m/z 188 (M^+, 1), 143 (100), 115 (78), 87 (23), 73 (25).

3.3.8 Synthesis of (3*R*)-1-Benzyl-3-methylpyrrolidine-2,5-dione (30)



1-Benzyl-3-methyl-1H-pyrrole-2,5-dione **(28)** (30mg in ethanol: water (2:1)) was reduced by using Tris HCl (pH 8, 18mL, 50mM), $NADP^+$ (80 µL, 100mM stock solution), glucose-6-phosphate (1.6 mL, 100 mM stock solution), glucose-6-phosphate dehydrogenease (68 µL) and SYE-4 CCE (5.6mL, 35.5mg/mL) at 28°C to (3*R*)-1-Benzyl-3-methylpyrrolidine-2,5-dione (**30**) in 24h. The product was extracted with ethyl acetate, dried over Na_2SO_4 and evaporated under vacuum. The crude product was purified with column chromatography using ethyl acetate and petrol ether mixture (5-10%).

Product (**30**): 24mg, 0.11mmol, Yield: 76%

Chemical Formula: $C_{12}H_{13}NO_2$ Molecular Weight: 203.24

$[\alpha]_D^{22}$ = +17.7 (c = 2.4, $CHCl_3$) (lit[165] $[\alpha]_D^{27}$ = +19.7 (c = 0.73, $CHCl_3$))

Appearance: Colorless oil

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 1.30 (d, J = 7.2Hz, 3H, CH_3), 2.14-2.37 (m, 1H, CHCO), 2.80-2.90 (m, 2H, CH_2CO), 4.62 (s, 2H, CH_2N), 7.25-7.34 (m, 5H, ArH).

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 16.7(q, CH_3), 34.7(t, $\underline{CH_2}CO$), 36.4(d, $\underline{CH}CO$), 42.3(t, CH_2N), 127.9(d, C2'/ C2), 128.6(d, C3'/ C3), 128.7(d, C4'/ C4), 135.8(s, C1), 176.1(s, COO), 180.2(s, COO).

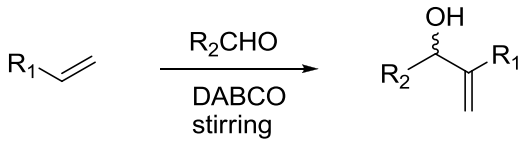
GC-MS

m/z 203 (M^+, 100), 174 (23), 160 (70), 104 (61), 91 (58).

---

[165] Maximiliano, S.; Susana, A. Z. *Tetrahedron: Asymmetry*, **2010,** *21*, 5, 535-539.

3.4 Synthesis of Baylis Hillman Adducts



3.4.1 General Procedures for the Synthesis of Baylis Hillman Adducts

3.4.1.1 Procedure 1

Methyl acrylate (2.18 g, 30 mmol), the corresponding aldehyde (30 mmol) and DABCO (312 mg, 2.55 mmol) were stirred without any solvent for seven days at room temperature. After dilution with diethyl ether the mixture was washed with 2M HCl, saturated $NaHCO_3$ and water. The organic phase was dried (Na_2SO_4) and concentrated to remove the solvent and the excess of methyl acrylate. The obtained crude products were purified by chromatography to yield the desired racemic mixtures.

3.4.1.2 Procedure 2

Following the procedure of Hu et al.[148] methyl acrylate (2.18 g, 30 mmol), the corresponding aldehyde (10 mmol), and DABCO (1.38 g, 10 mmol) were dissolved in 10 mL of dioxane/water (1:1 v/v) and the mixture was stirred at ambient temperature. After completion of the reaction methyl tert.-butyl ether (100 mL) and water (50 mL) were added to the mixture. After phase separation the organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The crude product was purified by column chromatography to yield the desired products.

3.4.1.3 Procedure 3

Acrylamide (1.80 g, 25.4 mmol), the corresponding aldehyde (25.4 mmol), and DABCO (2.84 g, 25.4 mmol) were dissolved in 10 mL of dioxane/water (1:1 v/v) and the mixture was stirred at ambient temperature. After completion of the reaction ethyl acetate was added to the mixture. After phase separation the organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The crude product was purified by column chromatography to yield the desired products.

3.4.1.4 Procedure 4[150]

Quinuclidine (8.1 mmol) and methanol (1.25 mL) were added to a stirred mixture of the substrate aldehyde (16.5 mmol) and Michael acceptor (16.5 mmol). The homogeneous reaction mixture was stirred at ambient temperature and reaction progress was monitored by GC-MS. Upon completion or as indicated, the reaction mixture was purified by flash column chromatography on silica gel, eluting with ethyl acetate and petroleum ether or by recrystallization to give the desired product.

3.4.1.5 Procedure 5[166]

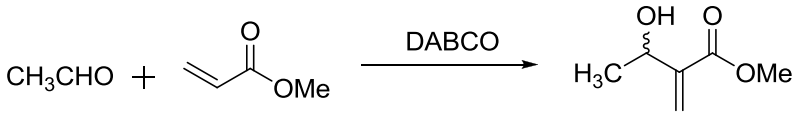
A 1 mL reaction vessel equipped with a stirring bar was charged with acrylamide (568 mg, 8.0 mmol), DABCO (896 mg, 8.0 mmol), and phenol (188 mg, 2.0 mmol). tBuOH/H_2O (3:7) solvent mixture (500 μL) and the aldehyde (8.0 mmol) were then added *via* syringe. The resulting homogeneous mixture was stirred at 55°C for 24h and allowed to cool. Column chromatography of the reaction mixture yielded the required Baylis-Hillman adducts.

---

[166] Cornelia, F.; Eimear, M. F.; Stephen, J. C. *J. Org. Chem.*, **2004,** *69,* 19, 6496-6499.

3.4.2 Synthesis of Substrates for Bioreduction of Baylis Hillman Adducts

3.4.2.1 Synthesis of Methyl 3-hydroxy-2-methylenebutanoate (33)



33

methyl 3-hydroxy-2-methylenebutanoate

Methyl 3-hydroxy-2-methylenebutanoate (**33**) was prepared by using general procedure of Baylis Hillman reaction (procedure 1) with methyl acrylate (2.18 g, 30 mmol), acetaldehyde (1.68 mL 30 mmol) and DABCO (312 mg, 2.55 mmol). The crude product was purified with column chromatography using 5% ethyl acetate and petrol ether mixture.

Product (**33**): 1.73 g, 13.3 mmol

Yield: 53%

Chemical Formula: $C_6H_{10}O_3$

Molecular Weight: 130.14

Appearance: Colorless oil

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 1.24 (dd, J = 6.6Hz, J = 1.6Hz, 3H), 3.29 (bs, 1H), 3.66 (s, 1H), 4.51 (q, J = 6Hz, 1H), 5.75 (d, J = 2Hz, 1H), 6.09 (s, 1H).

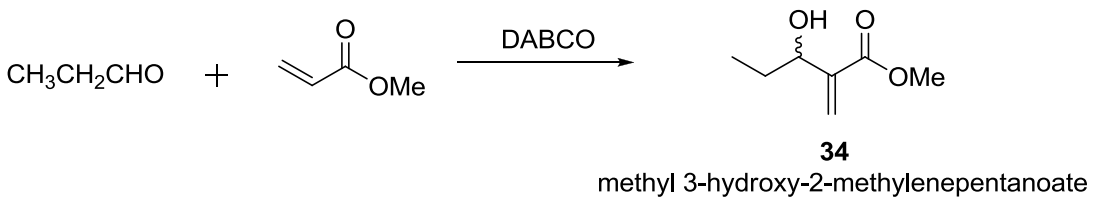
^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 22.2 (q), 51.7 (q), 66.4 (d), 123.8 (t), 143.8 (s), 166.9 (s).

GC-MS

m/z 130 (M^+, 1), 115 (80), 98 (32), 83 (100), 55 (40).

3.4.2.2 Synthesis of Methyl 3-hydroxy-2-methylenepentanoate (34)



34

methyl 3-hydroxy-2-methylenepentanoate

Methyl 3-hydroxy-2-methylenepentanoate (**34**) was prepared by using general procedure of Baylis Hillman reaction (procedure 1) with methyl acrylate (2.18 g, 30 mmol), propanaldehyde (2.1 mL 30 mmol) and DABCO (312 mg, 2.55 mmol). The crude product was purified with column chromatography using 5% ethyl acetate and petrol ether mixture.

Product (**34**): 2.12 g, 14.7 mmol

Yield: 58%

Chemical Formula: $C_7H_{12}O_3$

Molecular Weight: 144.17

Appearance: Colorless oil

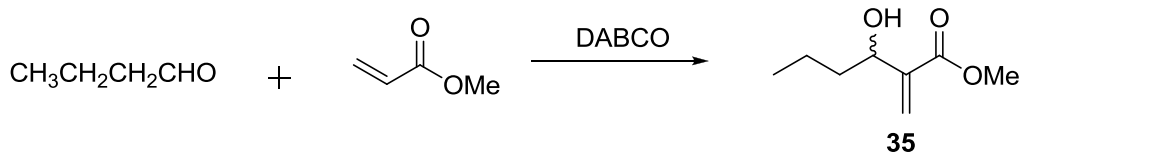
^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 0.77 (dt , J = 2.8Hz, J = 7.4Hz, 3H), 1.37-1.60 (m, 2H), 3.31 (bs, 1H), 3.61 (s, 3H), 4.21 (t, J = 6.2Hz, 1H), 5.69 (s, 1H), 6.08 (s, 1H)

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 9.7 (q), 29.0 (t), 51.6 (q), 71.8 (d), 124.6 (t), 142.5 (s), 166.8 (s).

3.4.2.3 Synthesis of Methyl 3-hydroxy-2-methylenehexanoate (35)



35

methyl 3-hydroxy-2-methylenehexanoate

Methyl 3-hydroxy-2-methylenehexanoate (**35**) was prepared by using general procedure of Baylis Hillman reaction (procedure 1) with methyl acrylate (2.18 g, 30 mmol), butyraldehyde (2.71 mL 30 mmol) and DABCO (312 mg, 2.55 mmol). The crude product was purified with column chromatography using 10% ethyl acetate and petrol ether mixture.

Product (**35**): 1.91 g, 12.0 mmol

Yield: 49%

Chemical Formula: $C_8H_{14}O_3$

Molecular Weight: 158.19

Appearance: Colorless oil

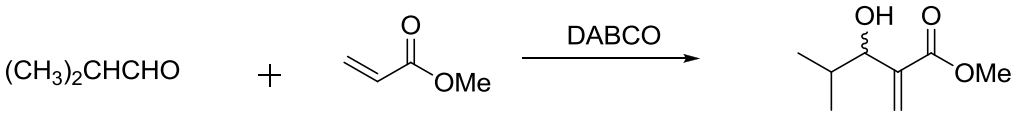
^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 0.86 (t, J = 7.2Hz, 3H), 1.23-1.159 (m, 4H), 2.91 (bs, 1H), 3.71 (s, 3H), 4.35 (t, J = 6Hz, 1H), 5.75 (d, J = 1.2Hz, 1H), 6.15 (s, 1H)

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 13.7 (q), 18.9 (t), 38.0 (t), 51.7 (q), 71.0 (d), 124.7 (t), 142.7 (s), 167.0 (s).

3.4.2.4 Synthesis of Methyl 3-hydroxy-4-methyl-2-methylenepentanoate (36)



36

methyl 3-hydroxy-4-methyl-2-methylenepentanoate

Methyl 3-hydroxy-4-methyl-2-methylenepentanoate (**36**) was prepared by using general procedure of Baylis Hillman reaction (procedure 1) with methyl acrylate (2.18 g, 30 mmol), isobutyraldehyde (2.71 mL 30 mmol) and DABCO (312 mg, 2.55 mmol). The crude product was purified with column chromatography using 10% ethyl acetate and petrol ether mixture.

Product (**36**): 2.70 g, 17.0 mmol

Yield: 61%

Chemical Formula: $C_8H_{14}O_3$

Molecular Weight: 158.19

Appearance: Colorless oil

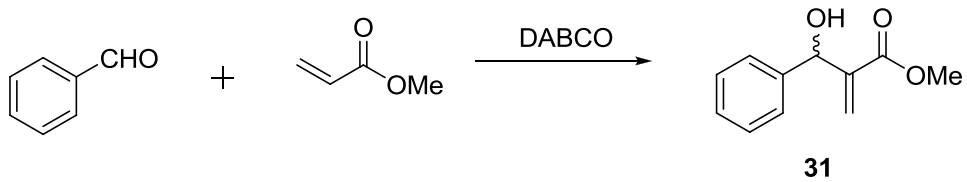
^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 0.84 (d, J = 6.8Hz, 3H), 0.90 (d, J = 6.6Hz, 3H), 1.79-2.00 (m, 1H), 3.73 (s, 3H), 4.05 (d, J = 6.8Hz, 1H), 5.74 (s, 1H), 6.22 (d, J = 1.2Hz, 1H).

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 17.4 (q), 19.5 (q), 32.6 (d), 51.8 (q), 76.4 (d), 126.0 (t), 141.4 (s), 167.1 (s).

3.4.2.5 Synthesis of Methyl 2-(hydroxy(phenyl)methyl)acrylate (31)



31

methyl 2-(hydroxy(phenyl)methyl)acrylate

Methyl 2-(hydroxy(phenyl)methyl)acrylate (**31**) was prepared by using general procedure of Baylis Hillman reaction (procedure 1) with methyl acrylate (2.18 g, 30 mmol), benzaldehyde (3.05 mL 30 mmol) and DABCO (312 mg, 2.55 mmol). The crude product was purified with column chromatography using 15% ethyl acetate and petrol ether mixture.

Product (**31**): 3.10 g, 16.1 mmol

Yield: 64%

Chemical Formula: $C_{11}H_{12}O_3$

Molecular Weight: 192.21

Appearance: Colorless viscous oil

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 3.43 (bs, 1H), 3.63 (s, 3H), 5.49 (s, 1H), 5.83 (t, J = 1.2Hz, 1H), 6.28 (s, 1H), 7.25-7.32 (m, 5H).

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 51.8 (q), 72.7 (d), 125.7 (t), 126.7 (d), 127.7 (d), 128.3 (d), 141.4 (s), 142.2 (s), 166.6 (s).

3.4.2.6 Synthesis of Methyl 2-(hydroxy(4-methoxyphenyl)methyl)acrylate (37)

37

methyl 2-(hydroxy(4-methoxyphenyl)methyl)acrylate

Methyl 2-(hydroxy(4-methoxyphenyl)methyl)acrylate (**37**) was prepared by using general procedure of Baylis Hillman reaction (procedure 1) with methyl acrylate (2.18 g, 30 mmol), *p*-anisaldehyde (4.08 g 30 mmol) and DABCO (312 mg, 2.55 mmol). The crude product was purified with column chromatography using 15% ethyl acetate and petrol ether mixture.

Product (**37**): 2.69 g, 12.1 mmol

Yield: 47%

Chemical Formula: $C_{12}H_{14}O_4$

Molecular Weight: 222.24

Appearance: Colorless solid

mp: 61-65^{o}C (lit[147] 62–63^{o}C)

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 3.00 (bs, 1H), 3.69 (s, 3H), 3.77 (s, 3H), 5.05 (s, 1H), 5.85 (t, J = 1Hz, 1H), 6.30 (s, 1H), 6.85 (td, J = 8.6Hz, J = 2.8Hz, 2H), 7.26 (td, J = 8.8Hz, J = 2.8 Hz, 2H).

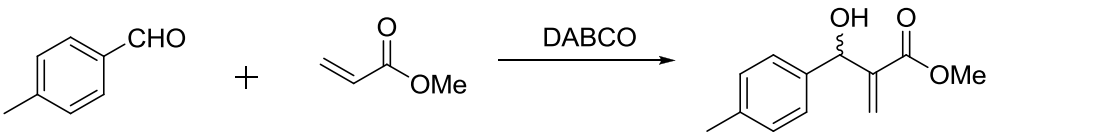
^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 51.9 (q), 55.2 (q),72.6 (d), 113.8 (d), 125.4 (t) 127.9 (d), 133.5 (d), 142.2 (d), 159.1 (s), 166.7 (s).

GC-MS

m/z 222 (M^+, 30), 134 (100), 108 (41), 77 (22).

3.4.2.7 Synthesis of Methyl 2-(hydroxy(p-tolyl)methyl)acrylate (38)



38

methyl 2-(hydroxy(*p*-tolyl)methyl)acrylate

Methyl 2-(hydroxy(p-tolyl)methyl)acrylate (**38**) was prepared by using general procedure of Baylis Hillman reaction (procedure 1) with methyl acrylate (2.18 g, 30 mmol), *p*-tolylaldehyde (3.6 g 30 mmol) and DABCO (312 mg, 2.55 mmol). The crude product was purified with column chromatography using 15% ethyl acetate and petrol ether mixture.

Product (**38**): 3.91 g, 18.9 mmol

Yield: 76%

Chemical Formula: $C_{12}H_{14}O_3$

Molecular Weight: 206.24

Appearance: Colorless viscous oil

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 2.32 (s, 3H), 2.85 (bs, 1H), 3.69 (s, 3H), 5.51 (s, 1H), 5.87 (t, J = 1.2Hz, 1H), 6.31 (s, 1H), 7.11-7.26 (m, 4H).

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 21.1 (q), 51.9 (q), 72.9 (d), 125.7 (t), 126.5 (d), 129.1 (d), 137.5 (s), 138.4 (s), 142.1 (s), 166.7 (s).

GC-MS

m/z 206 (M^+, 30), 174 (29), 146 (37), 118 (100), 90 (57).

3.4.2.8 Synthesis of Methyl 2-((4-chlorophenyl)(hydroxy)methyl)acrylate (39)

39

methyl 2-((4-chlorophenyl)(hydroxy)methyl)acrylate

Methyl 2-((4-chlorophenyl)(hydroxy)methyl)acrylate (**39**) was prepared by using general procedure of Baylis Hillman reaction (procedure 1) with methyl acrylate (2.18 g, 30 mmol), *p*-cholobenzaldehyde (4.2 g 30 mmol) and DABCO (312 mg, 2.55 mmol).The crude product was purified with column chromatography using 15% ethyl acetate and petrol ether mixture.

Product (**39**): 3.95 g, 17.4 mmol, Yield: 69%

Chemical Formula: $C_{11}H_{11}ClO_3$, Molecular Weight: 226.66

Appearance: Colorless solid

m.p: 40-42°C (lit[167] 40.7°C)

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 3.16 (bs, 1H), 3.70 (s, 3H), 5.50 (s, 1H), 5.83 (t, J = 1Hz, 1H), 6.32 (d, J = 0.6Hz, 1H), 7.27-7.29 (m, 4H).

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 52.0 (q), 72.5 (d), 126.2 (t), 128.0 (d), 128.5 (d), 133.5 (s), 139.0 (s), 141.7 (s), 166.5 (s).

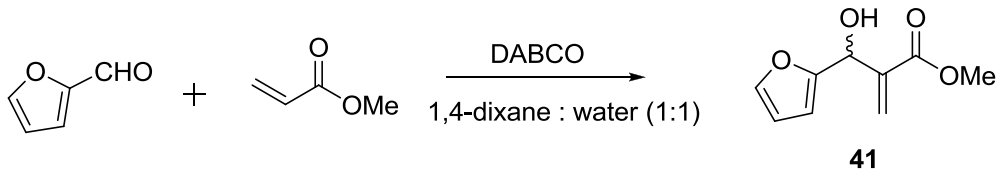
GC-MS

m/z 228 (M^++2, 12), 226 (M^+, 29), 165 (64), 140 (46), 138 (100), 77 (48).

---

[167] Jeong, Y.; Ryu, J. *J. Org. Chem.*, **2010**, *75*, 4183–4191.

3.4.2.9 Synthesis of Methyl 2-(furan-2-yl(hydroxy)methyl)acrylate (41)



41

methyl 2-(furan-2-yl(hydroxy)methyl)acrylate

Methyl 2-(furan-2-yl(hydroxy)methyl)acrylate (**41**) was prepared by using general procedure of Baylis Hillman reaction (procedure 2) with methyl acrylate (2.18 g, 30 mmol), furfural (960 mg 10 mmol), DABCO (1.38 g, 10 mmol) (1,4-dixane : water 1:1, 50mL). The crude product was purified with column chromatography using 20% ethyl acetate and petrol ether mixture.

Product (**41**): 2.83 g, 15.5 mmol

Yield: 61%

Chemical Formula: $C_9H_{10}O_4$

Molecular Weight: 182.17

Appearance: Yellow oil

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 2.99 (bs, 1H), 3.75 (s, 3H), 5.59 (s, 1H), 5.95 (s, 1H), 6.24 (d, J = 3.2Hz, 1H), 6.31-6.34 (m, 1H), 6.38 (s, 1H), 7.36 (t, J = 1.0Hz, 1H).

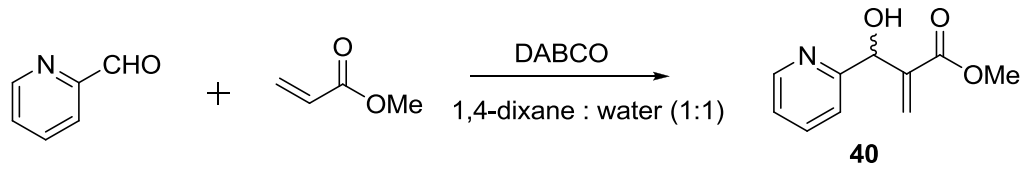
^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 51.9 (q), 66.6 (d), 107.1 (d), 110.3 (d), 126.5 (t), 139.6 (d), 142.2 (s), 154.2 (s), 166.4 (s).

GC-MS

m/z 182 (M^+, 25), 122 (81), 95 (100), 55 (23).

3.4.2.10 Synthesis of Methyl 2-(hydroxy(pyridin-2-yl)methyl)acrylate (40)



40

methyl 2-(hydroxy(pyridin-2-yl)methyl)acrylate

Methyl 2-(hydroxy(pyridin-2-yl)methyl)acrylate (**40**) was prepared by using general procedure of Baylis Hillman reaction (procedure 2) with methyl acrylate (2.18 g, 30 mmol), 2-pyridylaldehyde (1.07 g 10 mmol), DABCO (1.38 g, 10 mmol) (1,4-dixane : water 1:1, 50mL). The crude product was purified with column chromatography using 30% ethyl acetate and petrol ether mixture.

Product (**40**): 2.59 g, 13.4 mmol, Yield: 53%

Chemical Formula: $C_{10}H_{11}NO_3$, Molecular Weight: 193.20

Appearance: Yellow solid

mp: 54 ^oC (lit[147] 50–51 ^oC)

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 3.69 (s, 3H), 4.25 (bs, 1H), 5.60 (s, 1H), 5.94 (d, J = 1.0Hz, 1H), 6.33 (s, 1H), 7.14-7.27 (m, 1H), 7.39 (d, J = 7.8Hz, 1H), 7.64 (dt, J = 1.8Hz, J = 7.8Hz, 1H), 8.49 (d, J = 4.4Hz, 1H).

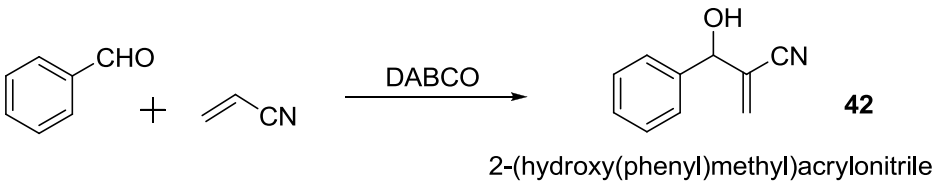
^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 51.8 (q), 72.0 (d), 121.3 (d), 122.6 (d), 126.7 (t), 136.8 (d), 141.6 (s), 148.2 (d),159.6 (s), 166.4 (s).

GC-MS

m/z 193 (M^+, 2), 175 (100), 117 (31), 140 (46), 78 (34).

3.4.2.11 Synthesis of 2-(Hydroxy(phenyl)methyl)acrylonitrile (42)



2-(hydroxy(phenyl)methyl)acrylonitrile

2-(Hydroxy(phenyl)methyl)acrylonitrile (**42**) was prepared by using general procedure of Baylis Hillman reaction (procedure 1) with acrylonitrile (1.35 g, 25.4 mmol), benzaldehyde (2.58 mL 25.4 mmol) and DABCO (312 mg, 2.55 mmol) . The crude product was purified with column chromatography using 20% ethyl acetate and petrol ether mixture.

Product (**42**): 3.18 g, 20 mmol

Yield: 79%

Chemical Formula: $C_{10}H_9NO$

Molecular Weight: 159.18

Appearance: Viscous liquid

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 3.27 (bs, 1H), 5.23 (d, J = 1Hz, 1H), 5.98 (s, 1H), 6.07 (d, J = 1Hz, 1H), 7.33-7.42 (m, 5H).

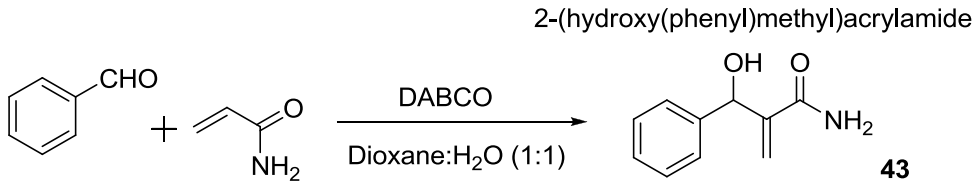
^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 73.9 (d), 117.0 (s), 126.4 (s), 126.5 (d), 128.7 (d), 128.8 (d), 129.8 (t), 139.4 (s).

GC-MS

m/z 159 (M^+, 23), 106 (100), 79 (72), 76 (60), 51 (22).

3.4.2.12 Synthesis of 2-(Hydroxy(phenyl)methyl)acrylamide (43)



2-(Hydroxy(phenyl)methyl)acrylamide (**43**) was prepared by using general procedure of Baylis Hillman reaction (procedure 3) with acrylamide (1.80 g, 25.4 mmol), benzaldehyde (2.58 mL 25.4 mmol), DABCO (2.84 g, 25.4 mmol) (1,4-dixane : water 1:1, 10mL). The crude product was purified with column chromatography using 50% ethyl acetate and petrol ether mixture.

Product (**43**):1.93 g, 10.9 mmol

Yield: 43%

Chemical Formula: $C_{10}H_{11}NO_2$

Molecular Weight: 177.20

Appearance: Colorless solid

m.p: 97-100°C (lit[149] 97-99 °C)

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 3.31 (bs, 1H), 4.74 (s, 2H), 5.60 (d, J = 6.8Hz, 1H), 5.96 (s, 1H), 7.24-7.40 (m, 5H).

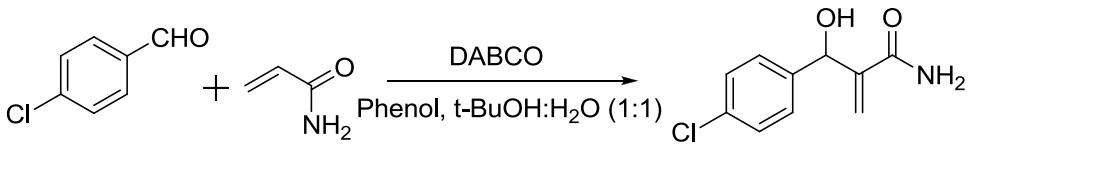
^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 72.7 (d), 120.7 (t), 125.9 (d), 126.7 (d), 127.6 (s), 141.6 (s), 145.0 (s), 168.6 (s).

GC-MS

m/z 177 (M^+, 76), 132 (44), 105 (100), 76 (62), 55 (16).

3.4.2.13 Synthesis of 2-((4-Chlorophenyl)(hydroxy)methyl)acrylamide (44)



44

2-((4-chlorophenyl)(hydroxy)methyl)acrylamide

2-((4-Chlorophenyl)(hydroxy)methyl)acrylamide (**44**) was prepared by using general procedure of Baylis Hillman reaction (procedure 5) with acrylamide (568 mg, 8.0 mmol), DABCO (896 mg, 8.0 mmol) and phenol (188 mg, 2.0 mmol) tBuOH/H_2O solvent mixture (500 µL) and the *p*-cholorobenzaldehyde (1.65g 8.0 mmol). The crude product was purified with column chromatography using 50% ethyl acetate and petrol ether mixture.

Product (**44**): 1.27 g, 6.01 mmol, Yield: 51%

Chemical Formula: $C_{10}H_{10}ClNO_2$

Molecular Weight: 211.64

Appearance: Colorless solid

m.p: 131-133°C (lit[149]132-134 °C)

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 3.37 (bs, 1H), 5.47 (s, 1H), 5.61 (s, 1H), 5.80 (s, 1H), 6.99 (bs, 2H, NH_2), 7.27-7.45 (m, 4H).

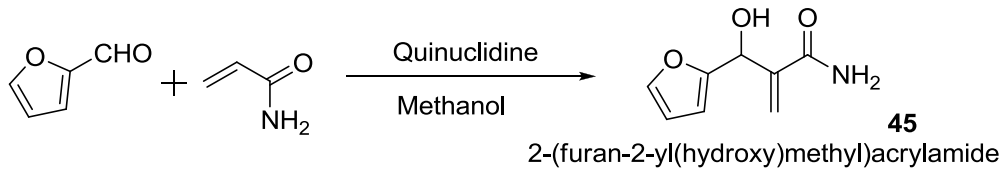
^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 73.0 (d), 120.3 (t), 130.5 (d), 131.2 (d), 134.1 (s), 145.0 (s), 149.7 (s), 171.2 (s).

GC-MS

m/z 212 (M^++2, 17), 210 (M^+, 50), 138 (100), 77 (42), 51 (11).

3.4.2.14 Synthesis of 2-(Furan-2-yl(hydroxy)methyl)acrylamide (45)



2-(Furan-2-yl(hydroxy)methyl)acrylamide (**45**) was prepared by using general procedure of Baylis Hillman reaction (procedure 4) using furfurladehyde (1.55 g, 16.5 mmol) acrylamide (1.15 g 16.5 mmol) quinuclidine (750 mg, 8.1 mmol) and methanol (1.25 mL). The crude product was purified with column chromatography using 30% ethyl acetate and petrol ether mixture.

Product (**45**): 1.63 g, 9.7 mmol

Yield: 61%

Chemical Formula: $C_8H_9NO_3$

Molecular Weight: 167.16

Appearance: Colorless solid

m.p: 81-82^{o}C (lit[150] 83-84^{o}C)

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 3.32 (bs, 1H), 5.47 (d, J = 5.4Hz, 1H), 5.63 (s, 1H), 5.72 (d, J = 5.8Hz, 1H), 5.85 (s, 1H), 6.11 (d, J = 2.8Hz, 1H), 6.33 (d, J = 1.6Hz, 1H), 7.02 (s, 1H), 7.54 (s, 2H).

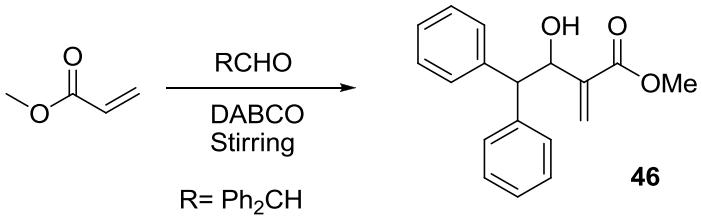
^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 62.8 (d), 104.4 (d), 108.2 (d), 116.3 (t), 140.1 (d), 143.0 (s), 153.8 (s), 166.5 (s).

GC-MS

m/z 167 (M^+, 27), 122 (100), 95 (68), 51 (11).

3.4.2.15 Synthesis of Methyl 3-hydroxy-2-methylene-4,4-diphenylbutanoate (46)



methyl 3-hydroxy-2-methylene-4,4-diphenylbutanoate

Methyl 3-hydroxy-2-methylene-4,4-diphenylbutanoate (**46**) was prepared by using general procedure of Baylis Hillman reaction (procedure 1) with methyl acrylate (2.18 g, 30 mmol), diphenyl acetaldehyde (5 g 30 mmol) and DABCO (312 mg, 2.55 mmol). The crude product was purified with column chromatography using 10% ethyl acetate and petrol ether mixture.

Product (**46**): 3.30 g, 11.7 mmol, Yield: 46%

Chemical Formula: $C_{18}H_{18}O_3$

Molecular Weight: 282.33

Appearance: Colorless solid

m.p: 95-99 ^oC (lit[168] 99-100^oC)

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 2.73 (bs, 1H), 3.61 (s, 3H), 4.31 (d, J = 8Hz, 1H), 5.16 (d, J = 8.2Hz, 1H), 5.58 (s, 1H), 6.08 (s, 1H), 7.11-7.36 (m, 10H).

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

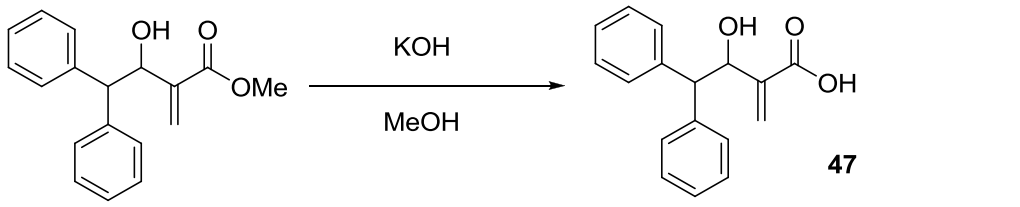
δ 51.9 (d), 57.1 (q), 74.1 (d), 126.6 (t), 126.8 (d), 127.3 (d), 128.5 (d), 128.6 (s), 129.2 (s), 140.7 (s), 141.8 (s), 166.9 (s).

---

[168] Martnez, I.; Andrews, A. E.; Emch, J. D.; Ndakala, A. J.; Wang, J.; Howell, A. R. *Org. Lett.*, **2003**, *5*, 4, 399-402.

3.4.3 Synthesis of Baylis Hillman Adduct Derivatives

3.4.3.1 Synthesis of 3-Hydroxy-2-methylene-4,4-diphenylbutanoic acid (47)



46 **47**

3-hydroxy-2-methylene-4,4-diphenylbutanoic acid

3-Hydroxy-2-methylene-4,4-diphenylbutanoic acid (**47**) was prepared by stirring the solution of Baylis Hillman ester (**46**) (3 g, 10.63 mmol) in methanol (20mL) with KOH (2M, 10mL) for 24h at room temperature. Methanol was removed by evaporation and the residue was acidified with 2N HCl to pH 2. The mixture was diluted with diethyl ether and the aqueous layer was extracted three times with diethyl ether. Combined organic layers were dried (Na_2SO_4) and evaporated. The product was almost pure and used in next step without purification.

Product (**47**): 2.1 g, 7.8 mmol, Yield: 74%

Chemical Formula: $C_{17}H_{16}O_3$

Molecular Weight: 268.31

Appearance: Colorless solid

m.p: 102-103^oC

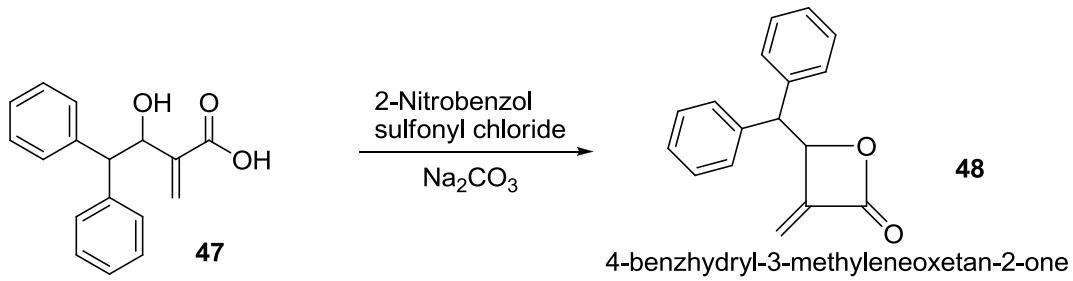
^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 3.3 (bs, 1H), 4.38 (d, J = 8Hz, 1H), 5.22 (d, J = 8Hz, 1H), 5.71 (s, 1H), 6.30 (d, 1H), 7.20-7.37(m, 10H).

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 53.2 (d), 76.1 (d), 125.3 (t) 126.2 (d), 127.4 (d), 129.5 (d), 129.9 (s), 130.2 (s), 142.7 (s), 143.6 (s), 167.9 (s).

3.4.3.2 Synthesis of 4-Benzhydryl-3-methyleneoxetan-2-one (48)



Anhydrous Na_2CO_3 (5g, 37.3mmol) was added to the stirred solution of acid (**47**) (1g, 3.73mmol) in dichloromethane. After 15 minutes 2-nitrobenzol sulfonyl chloride (1.61g, 5.59 mmol) was added and stirring was continued at room temperature for 2 days. The mixture was diluted with dichloromethane and water and stirred for 15 min. Organic layer was separated and the aques layer was extracted with dichloromethane. Organic layer was dried with Na_2SO_4 and concentrated and purified by chromatography using 5-10% ethyl acetate:petrol ether mixture to obtain the product 4-benzhydryl-3-methyleneoxetan-2-one (**48**).

Product (**48**): 425mg, 1.7 mmol, Yield: 45%

Chemical Formula: $C_{17}H_{14}O_2$

Molecular Weight: 250.29

Appearance: Colorless solid

m.p: 83-90^{o}C (lit[168]82-83^{o}C)

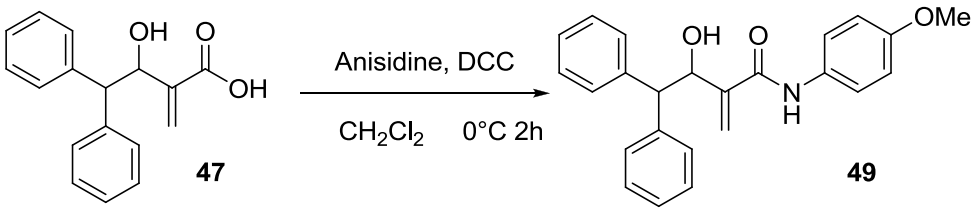
^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 4.22 (d, J = 9.6 Hz, 1H), 4.73 (t, J = 3 Hz, 1H,), 5.55 (td, J = 1.6 Hz, J = 9.4 Hz, 1 H), 5.74 (t, J = 1.8 Hz, 1H), 7.22-7.29 (m, 10H).

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 54.5 (d), 80.0 (d), 117.2 (t) 127.2 (d), 127.7 (t), 128.3 (d), 128.8 (d), 129.0 (d), 139.1 (s), 139.5 (s), 144.8 (s), 163.4 (s).

3.4.3.3 Synthesis of 3-Hydroxy-N-(4-methoxyphenyl)-2-methylene-4,4-diphenylbutanamide (49)



3-hydroxy-*N*-(4-methoxyphenyl)-2-methylene-4, 4-diphenylbutanamide

A solution of *N*,*N'*-dicyclohexylcarbodiimide **(**DCC) (338 mg, 1.86 mmol) in dichloromethane was added slowly at 0 ^{o}C to a solution of acid (**47**) (490 mg, 1.86 mmol) and *p*-anisidine (228 mg, 1.86 mmol) in dichloromethane (20 mL). The mixture was stirred for 30 min at 0^{o}C and warmed to 20^{o}C and stirred for another 3h. The colorless precipitate was filtered and washed with dichloromethane. The solvent was evaporated (20°C, 10 torr) and the crude product was purified with MPLC using dichloromethane and diethyl ether (3:7).

Product (**49**): 205 mg, 0.54 mmol, Yield: 30%

Chemical Formula: $C_{24}H_{23}NO_3$

Molecular Weight: 373.44

Appearnace: Colorless solid

m.p: 134-137^{o}C

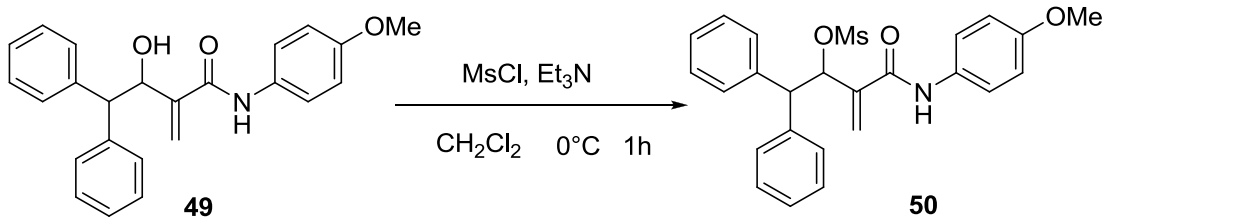
^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 3.29 (bs, 1H), 3.73 (s, 3H), 4.07 (q, J = 5.4Hz, 1H), 4.30 (d, J = 9.6Hz, 1H), 5.25 (s, 1H), 5.66 (s, 1H), 7.17-7.39 (m, 14H).

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 55.5 (d), 57.1 (q), 76.3 (d), 114.1 (d), 122.2 (d), 122.9 (t), 126.7 (d), 128.5 (d), 128.7 (d), 130.6 (s), 141.7 (s), 144.0 (s), 156.6 (s), 166.0 (s).

3.4.3.4 Synthesis of 3-(4-Methoxyphenylcarbamoyl)-1,1-diphenylbut-3-en-2-yl methane sulfonate (50)



49 **50**

3-(4-methoxyphenylcarbamoyl)-1,1-diphenylbut-3-en-2-yl methanesulfonate

Mesetyl chloride (100 mg, 0.8 mmol) and triethylamine (80 mg, 0.8 mmol) were added to a solution of amide (**50**) (150 mg, 0.40 mmol) in dichloromethane (10 mL) at 0^{o}C. After stirring for 2h, the reaction mixture was warmed to 20^{o}C. The reaction mixture was washed with water, the organic layer was separated and dried with Na_2SO_4. The crude product was obtained by evaporation and purified by recrystallization using cold dichloromethane.

Product (**50**): 203 mg, 0.44 mmol

Yield: 82%

Chemical Formula: $C_{25}H_{25}NO_5S$

Molecular Weight: 451.53

Appearance: Colorless solid

m.p: 188-190^{o}C

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

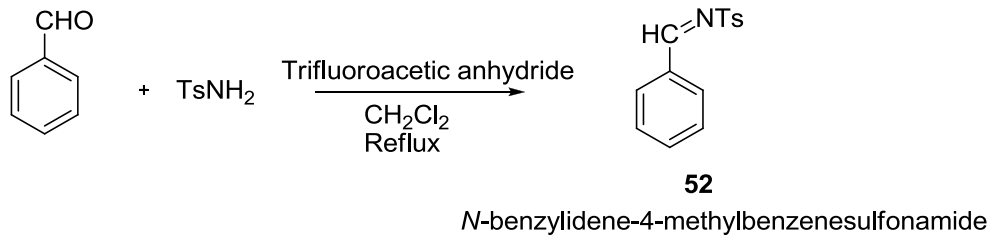
δ 2.45 (s, 3H), 3.79 (s, 3H), 4.73 (d, J = 8.8Hz, 1H), 5.29 (s, 1H), 5.69 (d, J = 7.4Hz, 1H), 6.05 (d, J = 8.8Hz, 1H), 6.85 (d, J = 7Hz, 2H), 7.21-7.46 (m, 12H).

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 37.9 (q), 55.1 (d), 55.5 (q), 83.7 (d), 114.1 (d), 121.1 (d), 121.7 (t), 127.2 (d), 128.7 (d), 129.0(d), 130.2 (s), 139.4 (s), 143.3 (s), 156.7 (s), 164.9 (s).

3.4.4 Synthesis of Aza-Baylis Hillman Products

3.4.4.1 Synthesis of *N*-Benzylidene-4-methylbenzenesulfonamide (52)



52

N-benzylidene-4-methylbenzenesulfonamide

Trifluoroacetic anhydride (1.54m, 11mmol) was added to the stirred solution of benzaldehyde (1.06g, 10mmol) and *p*-tosylamid (1.88g 11mmol) in 50mL of dichloromethane and heated to reflux for 12h. The reaction mixture was poured into cold water and extracted with CH_2Cl_2, dried with Na_2SO_4. The crude product (**52**) was purified with flash chromatography using 10% ethyl acetate and petrolether mixture.

Product(**52**): 1.29 g, 4.9 mmol, Yield: 59%

Chemical Formula: $C_{14}H_{13}NO_2S$

Molecular Weight: 259.32

Appearance: Colorless solid

m.p: 110-114 ^oC (lit[169] 112–113^oC)

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 2.35 (s, 3H), 7.19-7.87 (m, 9H), 8.96 (s, 1H)

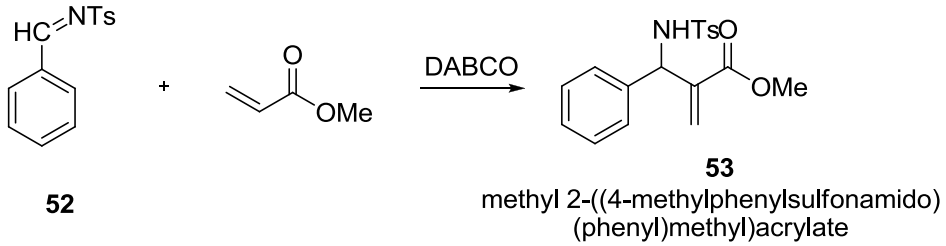
^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 21.5 (q), 126.4 (t) 128.5 (d), 129.7 (d), 130.1 (d), 133.7 (s), 139.0 (s), 143.6 (s), 187.2 (d).

---

[169]Ka, Y. L.; Chang, G. L.; Jae, N. K. *Tetrahedron Letters.* **2003**, *44*, 1231–1234.

3.4.4.2 Synthesis of Methyl 2-((4-methylphenylsulfonamido)(phenyl)methyl)acrylate (53)



52

53
methyl 2-((4-methylphenylsulfonamido)(phenyl)methyl)acrylate

Methyl 2-((4-methylphenylsulfonamido)(phenyl)methyl)acrylate (**53**) was prepared according to the general procedure of Baylis Hillman reactions (procedure 1) using methyl acrylate (150mg, 1.9 mmol), *N*-benzylidene-4-methylbenzenesulfonamide (**52**) (500 mg, 1.9 mmol) and DABCO (20 mg, 0.15 mmol) and stirring for 24h .The product was purified using 10-20% ethyl acetate and petrol ether mixture.

Product (**53**): 210 mg, 0.6 mmol, Yield: 35%

Chemical Formula: $C_{18}H_{19}NO_4S$, Molecular Weight: 345.41

Appearance: Colorless solid

m.p: 72-76 ^oC (lit[170] 76–78^oC)

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 2.32 (s, 3H), 3.51 (s, 3H), 3.70 (s, 1H), 5.23 (d, J = 8.8 Hz, 1H), 5.75 (s, 1H), 6.14 (s, 1H), 7.06-7.18 (m, 7H), 7.59 (d, J = 8.2Hz, 2H).

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 21.5 (q), 52.0 (q), 58.9 (d), 124.2 (t) 126.4 (d), 127.2 (d), 127.7 (d), 128.5 (d), 129.4 (d), 137.6 (s), 138.6 (s),143.3 (s), 165.7 (s).

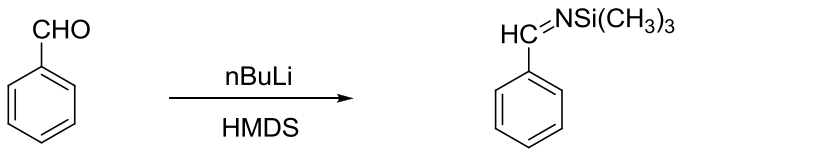
GC-MS

m/z 345 (M^+, 1), 260 (5), 190 (100), 91 (33).

---

[170] Shi, M.; Xu, Y.; Shi, Y. *Chem. Eur. J.* **2005,** *11*, 1794–1802.

3.4.4.3 Synthesis of *N*-Benzylidene-1,1,1-trimethylsilanamine (54)



54

N-benzylidene-1,1,1-trimethylsilanamine

n-BuLi (8mL, 24mmol, 2.94M solution in hexane) was added slowly to a dry flask containing 1,1,1,3,3,3-hexamethyldisilazane (5.33mL, 25.2mmol) under argon cooled to 0°C. The reaction was warmed to room temperature for 15min, then again cooled to 0°C. Benzaldehyde (2.43g 23mmol) was slowly added and stirred for 30 min at room temperature. Hexane was evaporated and the resulting slurry was distilled at vacuum (1.4mm, b.p 100°C) to get pale yellow liquid *N*-benzylidene-1,1,1-trimethylsilanamine (**54**).

Product (**54**): 3 g, 16.9 mmol

Yield: 76%

Chemical Formula: $C_{10}H_{15}NSi$

Molecular Weight: 177.32

Appearance: Pale yellow liquid

b.p: 100°C at 1.4mm Hg

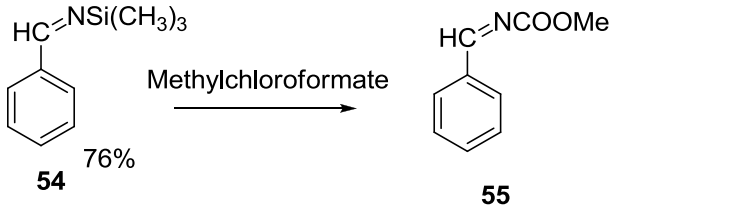
^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 0.14 (s, 9H), 7.28-7.32 (m, 3H), 7.65-7.70 (m, 2H), 8.85 (s, 1H).

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 6.7 (q), 128.2 (d), 129.3 (d), 130.1 (s), 134.3 (s), 142.5 (d).

3.4.4.4 Synthesis of Methyl benzylidenecarbamate (55)



76%

54 **55**

methyl benzylidenecarbamate

A solution of methyl chloroformate (1.6g, 16.9mmol) in dichloromethane (15mL) was added dropwise to a solution of silylimine (3g, 16.9mmol) (**54**) in dry dichloromethane (15mL) at 0^{o}C. Then the reaction mixture was refluxed for 1h (or until yellow color disppeared). The solvent was removed and the crude product was purified with distillation (0.06mm Hg, b.p 75-80^{o}C)

Product (**55**): 1.96 g, 12.0 mmol

Yield: 71%

Chemical Formula: $C_9H_9NO_2$

Molecular Weight: 163.17

Appearance: Colorless liquid

b.p: 75-80^{o}C at 0.06mm Hg

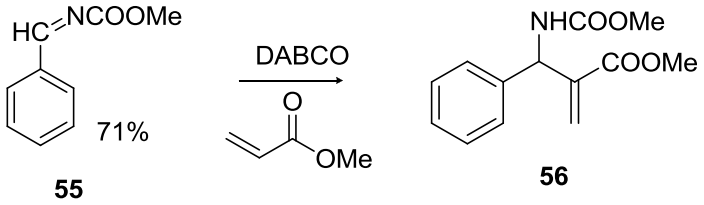
^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 3.69 (s, 3H), 7.22-7.36 (m, 3H), 7.69-7.72 (m, 2H), 8.73 (s, 1H).

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 52.4 (q), 126.2 (d) 128.6 (d), 129.0 (d), 130.4 (s), 133.9 (s), 138.5 (d).

3.4.4.5 Synthesis of Methyl 2-((methoxycarbonylamino)(phenyl)methyl)acrylate (56)



methyl 2-((methoxycarbonylamino)(phenyl)methyl)acrylate

The methyl benzylidenecarbamate (**55**) (700mg, 4.4mmol) and DABCO (100 mg, 0.9 mmol) were dissolved in methyl acrylate (2mL) and stirred overnight. Excess methyl acrylate was removed and the residue was dissolved in $CHCl_3$, washed with 2N HCl and $NaHCO_3$. The organic layer was dried and evaporated, the product was purified by columm chromatography using 6:1 ethyl acetate petrol ether mixture to afford methyl 2-((methoxycarbonylamino)(phenyl)methyl)acrylate (**56**).

Product (**56**): 413 mg, 1.65 mmol, Yield: 59%

Chemical Formula: $C_{13}H_{15}NO_4$, Molecular Weight: 249.26

Appearance: Colorless solid

m.p: 118-122 ^oC (lit[157] 120–121^oC)

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 3.60 (s, 3H), 3.63 (s, 3H), 5.55 (s, 1H), 5.85 (s, 1H), 6.30 (s, 1H), 7.17-7.30 (m, 5H).

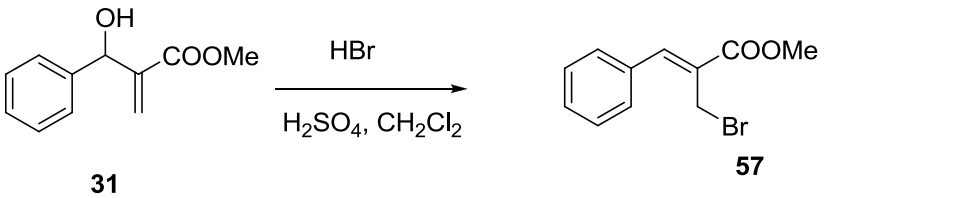
^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 52.0 (q), 52.3 (q), 56.7 (d), 126.0 (t), 126.4 (d), 127.6 (d), 128.5 (d), 139.5 (s), 141.1 (s), 156.2 (s), 166.0 (s).

GC-MS

m/z 249 (M^+, 7), 217 (54), 173 (100), 115 (72), 77 (40).

3.4.4.6 Synthesis of Methyl 2-(bromomethyl)-3-phenylacrylate (57)



31 **57**

Methyl 2-(bromomethyl)-3-phenylacrylate

conc. HBr solution (48%, 2.7 ml) was added dropwise to a the stirred solution of Baylis Hillman ester (**31**) (1.58 g, 8.22 mmol) in 20mL of CH_2Cl_2 followed by conc. H_2SO_4 solution (2.38 ml) at 0^oC. After stirring overnight at room temperature, the mixture was carefully diluted with CH_2CI_2 and H_2O. The aqeous phase was extracted twice with CH_2CI_2, the combined organic phase washed twice with H_2O, dried (Na_2SO_4), and evaporated, and the residual oil was purified by flash chromatography using 5-10% ethylacetate and petrol ether mixture.

Product (**57**): 1.64g, 6.45 mmol

Yield: 79%

Chemical Formula: $C_{11}H_{11}BrO_2$

Molecular Weight: 255.11

Appearance: Colorless oil

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 3.88 (s, 3 H), 4.39 (s, 2H), 7.40-7.49 (m, 3H), 7.55-7.59 (m, 2H), 7.83 (s, 1H).

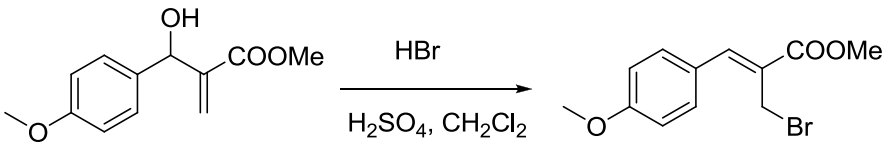
^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 26.8 (t), 52.5 (q), 128.6 (d), 128.9 (d), 129.6 (s), 134.2 (s), 142.9 (d), 166.6 (s).

GC-MS

m/z 255 (M^+, 5), 253 (M^+, 5), 174 (69), 114 (100).

3.4.4.7 Synthesis of Methyl 2-(bromomethyl)-3-(4-methoxyphenyl)acrylate (58)



37 **58**

Methyl 2-(bromomethyl)-3-(4-methoxyphenyl)acrylate

The compound (**58**) was prepared by using the same protocol as described in the synthesis of compound (**57**), using Baylis Hillman ester (**37**) (0.5 g, 2.25 mmol) in 10mL of CH_2Cl_2, HBr solution (48%, 0.7 ml) and conc. H_2SO_4 solution (0.6 ml). The crude product was purified by flash chromatography using 5-10% ethylacetate and petrol ether mixture.

Product (**58**): 468mg, 1.64 mmol

Yield: 73%

Chemical Formula: $C_{12}H_{13}BrO_3$

Molecular Weight: 285

Appearance: Colorless solid

m.p: 57-59 ºC (lit[171] 59.6-60.1ºC)

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 3.85 (s, 3H), 3.87 (s, 3H), 4.44 (s, 2H), 6.98 (td, J = 2 Hz, J = 8.8 Hz,2H), 7.57 (td, J = 2.8 Hz, J = 8.8 Hz, 2H), 7.78 (s, 1H).

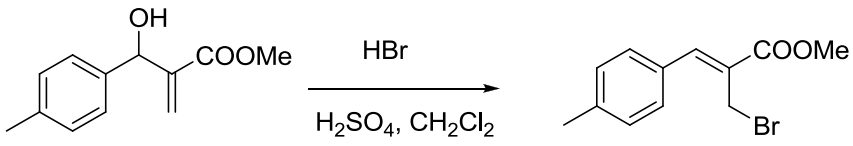
^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 27.5 (t), 52.4 (q), 55.4 (q), 114.4 (d), 126.1 (d), 126.7 (d), 132.0 (s), 142.9 (d),160.8 (s), 166.9 (s).

---

[171] Misael, F.; Luciano, F.; Marcus, M. S. *J. Braz. Chem. Soc.* **2009**, *20*, 3, 564-568.

3.4.4.8 Synthesis of Methyl 2-(bromomethyl)-3-p-tolylacrylate (59)



38 **59**

Methyl 2-(bromomethyl)-3-*p*-tolylacrylate

The compound (**59**) was prepared by using the same protocol as described in the synthesis of compound (**57**), using Baylis Hillman ester (**38**) (1.2 g, 5.82 mmol) in 20mL of CH_2Cl_2, HBr solution (48%, 1.9 ml) and conc. H_2SO_4 solution (1.7 ml). The crude product was purified by flash chromatography using 5-15% ethylacetate and petrol ether mixture.

Product (**59**): 1.08g, 4.01 mmol, Yield: 69%

Chemical Formula: $C_{12}H_{13}BrO_2$

Molecular Weight: 269.13

Appearance: Colorless oil

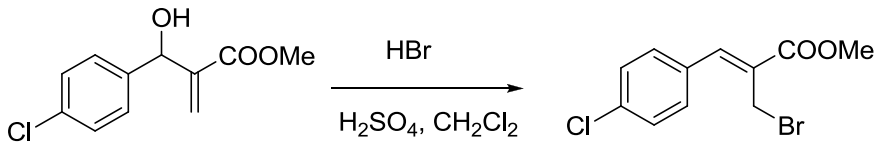
^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 2.36 (s, 3H), 3.85 (s, 3H), 4.39 (s, 2H), 7.24 (d, J = 7.8 Hz, 2H), 7.46 (d, J = 8.0 Hz, 2H), 7.78 (s, 1H).

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 21.4 (q), 27.1 (t), 52.3 (q), 127.6 (s), 129.6 (d), 129.9 (d), 131.3 (s), 140.0 (s),143.0 (d), 166.6 (s).

3.4.4.9 Synthesis of Methyl 2-(bromomethyl)-3-(4-chlorophenyl)acrylate (60)



39 **60**

Methyl 2-(bromomethyl)-3-(4-chlorophenyl)acrylate

The compound (**60**) was prepared by using the same protocol as described in the synthesis of compound (**57**), using Baylis Hillman ester (**39**) (1.85 g, 8.1 mmol) in 20mL of CH_2Cl_2, HBr solution (48%, 2.7 ml) and conc. H_2SO_4 solution (2.38 ml). The crude product was purified by flash chromatography using 5-15% ethylacetate and petrol ether mixture.

Product (**60**): 1.91g, 6.6 mmol, Yield: 81%

Chemical Formula: $C_{11}H_{10}BrClO_2$

Molecular Weight: 289.55

Appearance: Colorless oil

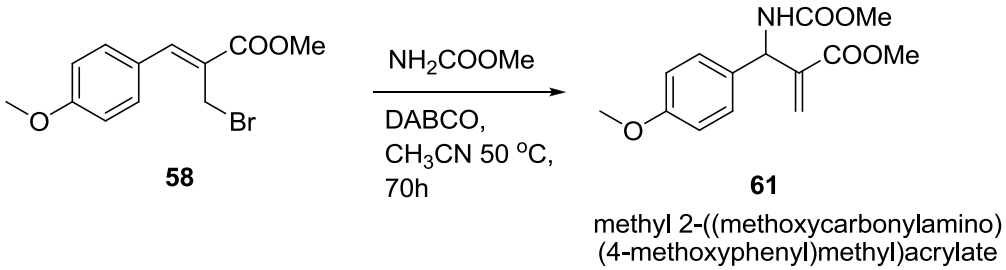
^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 3.87(s, 3H), 4.34(s, 2H), 7.38-7.52 (m, 4H), 7.74 (s, 1H).

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 26.3 (t), 52.5 (q), 129.1 (d), 130.9 (d), 132.6 (s), 135.6 (s), 141.3 (d), 166.2 (s).

3.4.4.10 Synthesis of Methyl 2-((methoxycarbonylamino) (4-methoxyphenyl) methyl) acrylate (61)



DABCO (67.5 mg, 0.6 mmol) was added to a stirred solution of Baylis-Hillman bromide (**58)** (142 mg, 0.5 mmol) in CH_3CN (3 mL) and stirring was continued at room temperature for 30 min. NaOH (24 mg, 0.6 mmol) and methyl carbamate (45 mg, 0.6 mmol) were added and the reaction mixture was heated to 50^{o}C for 70 h. After the dilution with diethyl ether, the organic layer was washed with water and dried. Crude product was purified by column chromatography (ethyl acetate/petrol ether, 20%) to obtain (**61**) as clear oil.

Product (**61**): 59 mg, 0.2 mmol, Yield: 43%

Chemical Formula: $C_{14}H_{17}NO_5$

Molecular Weight: 279.29

Appearance: Colorless oil

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 3.67 (s, 3H), 3.69 (s, 3H), 3.78 (s, 3H), 5.66 (bs, 1H), 5.90 (s, 1H), 6.35 (s, 1H), 6.84 (td, J = 2.8 Hz, J = 8.8 Hz, 2H), 7.20 (td, J = 2.6 Hz, J = 8.6Hz, 2H), 7.26 (bs, 1H, NH).

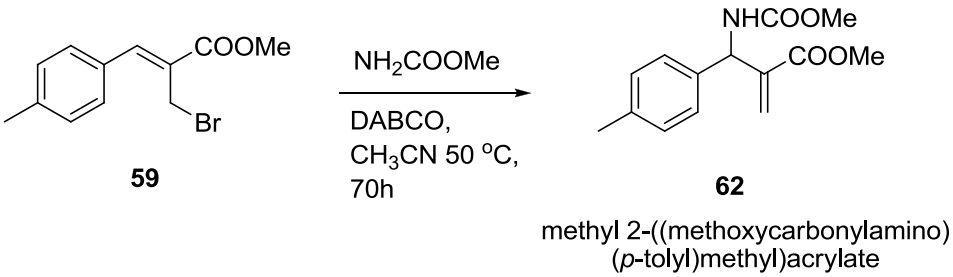
^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 51.9 (q), 52.3 (q), 55.2 (d), 56.1 (q), 114.0 (d), 126.4 (t) 127.6 (d), 131.6 (s), 139.9 (s), 156.1 (s), 159.0 (s), 166.1 (s).

GC-MS

m/z 279 (M^+, 62), 220 (80), 204 (98), 71(70), 70 (100).

3.4.4.11 Synthesis of Methyl 2-((methoxycarbonylamino)(p-tolyl)methyl)acrylate (62)



The compound (**62**) was synthesized by using the same procedure for the synthesis of (**61**). Baylis-Hillman bromide (**59)** (450 mg, 1.6 mmol) in CH_3CN (4 mL) was added to DABCO (215 mg, 1.92 mmol) and stirred at room temperature for 30 min. NaOH (76.8 mg, 1.92 mmol) and methyl carbamate (144 mg, 1.92 mmol) were added to the reaction mixture and heated to 50^{o}C for 70 h. After the usual aqueous workup and column chromatographic purification process (ethyacetate/petrolether, 20%) obtained (**83**) as colorless oil.

Product (**62**): 206 mg, 0.73 mmol, Yield: 47%

Chemical Formula: $C_{14}H_{17}NO_4$

Molecular Weight: 263.29

Appearance: Colorless oil

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 2.31 (s, 3H), 3.67 (s, 3H), 3.69 (s, 3H), 5.69 (bs, 1 H), 5.90 (s, 1H), 6.35 (s, 1H), 7.09-7.19 (m, 4H).

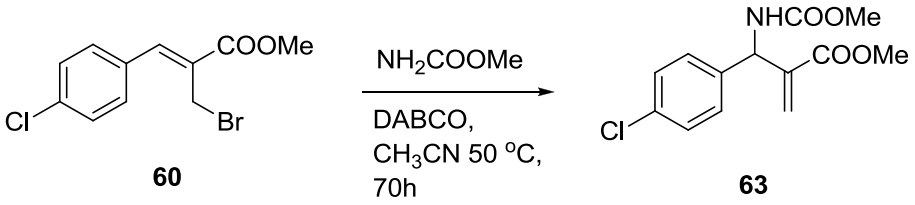
^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 21.0 (q), 51.9 (q), 56.4 (q), 126.3 (d), 126.7 (t), 129.3 (d), 136.6 (s), 137.2 (s), 139.9 (s), 156.2 (s), 166.1 (s).

GC-MS

m/z 263 (M^+, 18), 188 (100), 118 (35), 91(33).

3.4.4.12 Synthesis of Methyl 2-((4-chlorophenyl)(methoxycarbonylamino)methyl)acrylate (63)



methyl 2-((4-chlorophenyl)(methoxycarbonylamino) methyl)acrylate

The compound (**63**) was synthesized by using same procedure used for the synthesis of (**61**). Baylis-Hillman bromide (**60)** (510 mg, 1.77 mmol) in CH_3CN (5 mL) was added to DABCO (238 mg, 2.12 mmol) and stirred at room temperature for 30 min. NaOH (84.8 mg, 2.12 mmol) and methyl carbamate (159 mg, 2.12 mmol) were added to the reaction mixture and heated to 50^oC for 70 h. After the usual aqueous workup and column chromatographic purification process (ethyacetate/petrolether, 20%) obtained (**63**) as colorless oil.

Product (**63**): 259 mg, 0.91 mmol,Yield: 52%

Chemical Formula: $C_{13}H_{14}ClNO_4$

Molecular Weight: 283.71

Appearance: Colorless oil

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 3.68 (s, 3H), 3.70 (s, 3H), 5.70 (bs, 1H), 5.93 (s, 1H), 6.38(s 1H), 7.19-7.31 (m, 4H).

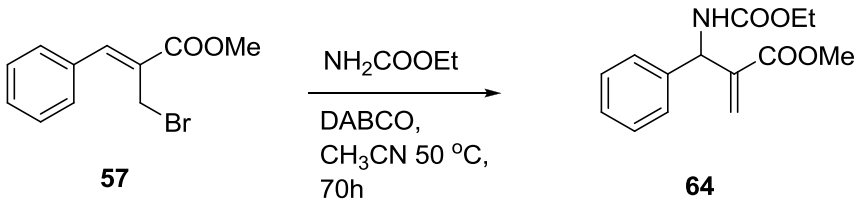
^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 52.0 (q), 52.4 (q), 56.3 (d), 126.3 (t), 127.7 (d), 128.3 (s), 128.7 (d), 133.4 (s), 138.2 (s), 156.8 (s), 166.2 (s).

GC-MS

m/z 283 (M^+, 16), 222 (78), 207 (100), 138 (35).

3.4.4.13 Synthesis of Methyl 2-((ethoxycarbonylamino)(phenyl)methyl)acrylate (64)



57 **64**

methyl 2-((ethoxycarbonylamino)(phenyl)methyl)acrylate

The compound (**64**) was synthesized by using same procedure used for the synthesis of (**61**). Baylis-Hillman bromide (**57)** (100 mg, 0.4 mmol) in CH_3CN (2 mL) was added to DABCO (54 mg, 0.48 mmol) and stirred at room temperature for 30 min. NaOH (19.2 mg, 0.48 mmol) and ethyl carbamate (42.8 mg, 0.48 mmol) were added to the reaction mixture and heated to 50^oC for 70 h. After the usual aqueous workup and column chromatographic purification process (ethyacetate/petrolether, 15%) obtained (**64**) as colorless oil.

Product (**64**): 40 mg, 0.15 mmol, Yield: 39%

Chemical Formula: $C_{14}H_{17}NO_4$

Molecular Weight: 263.29

Appearance: Colorless oil

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 1.24(t, J = 7.2 Hz, 3H), 3.67 (s, 3H), 4.15 (q, J =7.2 Hz, 2H), 5.71 (s, 1H), 5.92 (s, 1H), 6.37 (bd, J = 0.6 Hz, 1H), 7.26-7.31 (m, 5H).

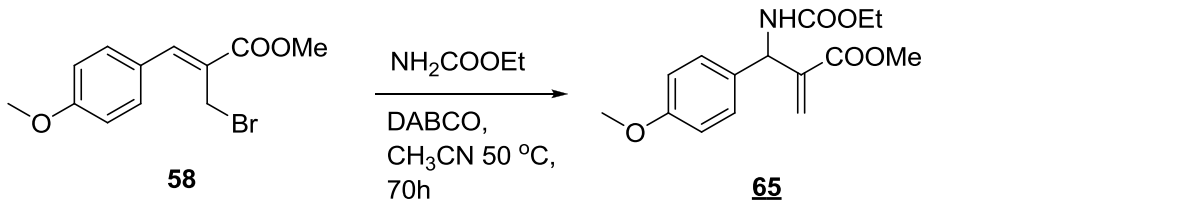
^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 14.6 (q), 51.9 (q), 56.5 (d), 61.9 t), 126.4 (t) 126.9 (d), 127.5 (d), 128.6 (d), 139.6 (s), 139.7 (s), 155.8 (s), 166.0 (s).

GC-MS

m/z 263 (M^+, 16), 204 (76), 188 (100), 118 (21).

3.4.4.14 Synthesis of Methyl 2-((ethoxycarbonylamino)(4-methoxyphenyl)methyl)acrylate (65)



58 65

methyl 2-((ethoxycarbonylamino)(4-methoxyphenyl)methyl)acrylate

The compound (**65**) was synthesized by using same procedure used for the synthesis of (**61**). Baylis-Hillman bromide (**58)** (142 mg, 0.5 mmol) in CH_3CN (3 mL) was added to DABCO (67.5 mg, 0.6 mmol) and stirred at room temperature for 30 min. NaOH (24 mg, 0.6 mmol) and ethyl carbamate (53.4 mg, 0.6 mmol) were added to the reaction mixture and heated to 50^{o}C for 70 h. After the usual aqueous workup and column chromatographic purification process (ethyacetate/petrolether, 15-20%) obtained (**65**) as colorless oil.

Product (**65**): 71 mg, 0.24 mmol, Yield: 49%

Chemical Formula: $C_{15}H_{19}NO_5$, Molecular Weight: 293.32

Appearance: Colorless oil

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 1.24 (t, J = 7.2 Hz, 3H, $CH_2\underline{CH_3}$), 3.67 (s, 3H, $COO\underline{CH_3}$), 3.78 (s, 3H, OCH_3), 4.15 (q, J =7.2 Hz, 2H, OCH_2), 5.65 (bs, 1H, $\underline{CH}NH$), 5.90 (s, 1H, $=CH_2$), 6.35 (s, 1H, $=CH_2$), 6.84 (d, J =8.6 Hz, 2H, H2/H2' ArH), 7.16-7.26 (m, 2H, H3/H3' ArH).

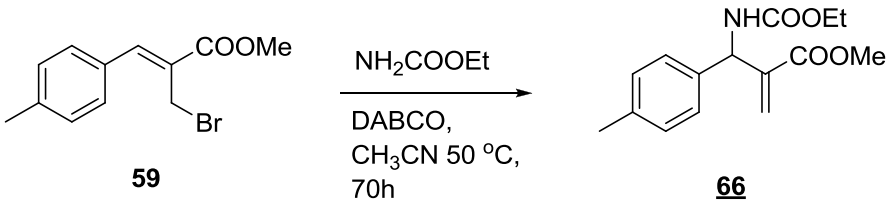
^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 14.6 (q, $OCH_2\underline{CH_3}$), 51.9 (q, $COO\underline{CH_3}$), 55.2 (q, $O\underline{CH_3}$), 56.0 (d, $\underline{CH}NH$), 61.1 (t, $O\underline{CH_2}$), 113.9 (d, C2/C2'), 126.3 (t, $=\underline{CH_2}$) 127.7 (d, C3/C3'), 131.7 (s, C4), 140.0 (s, $CH_2=\underline{C}$), 155.7 (s, C1), 159.0 (s, $\underline{C}OOEt$), 166.1 (s, $\underline{C}OOMe$).

GC-MS

m/z 293 (M^+, 10), 204 (100), 160 (42), 134 (85), 77(56).

3.4.4.15 Synthesis of Methyl 2-((ethoxycarbonylamino)(p-tolyl)methyl)acrylate (66)



59 66

methyl 2-((ethoxycarbonylamino)(*p*-tolyl)methyl)acrylate

The compound (**66**) was synthesized by using same procedure used for the synthesis of (**61**). Baylis-Hillman bromide (**59)** (450 mg, 1.6 mmol) in CH_3CN (4 mL) was added to DABCO (215 mg, 1.92 mmol) and stirred at room temperature for 30 min. NaOH (76.8 mg, 1.92 mmol) and ethyl carbamate (170.8 mg, 1.92 mmol) were added to the reaction mixture and heated to 50^oC for 70 h. After the usual aqueous workup and column chromatographic purification process (ethyacetate/petrolether, 15-20%) obtained (**66**) as colorless oil.

Product (**66**): 213 mg, 0.76 mmol, Yield: 46%

Chemical Formula: $C_{15}H_{19}NO_4$, Molecular Weight: 277.32

Appearance: Colorless oil

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 1.24 (t, J = 7.2 Hz, 3H, $CH_2\underline{CH_3}$), 2.31 (s, 3H, $Ar\underline{CH_3}$), 3.67 (s, 3H, $COO\underline{CH_3}$), 4.13 (q, J =7.2 Hz, 2H, OCH_2), 5.67 (bs, 1H, $\underline{CH}NH$), 5.91 (s, 1H, $=CH_2$), 6.35 (s, 1H, $=CH_2$), 7.09-7.19 (m, 4H, ArH).

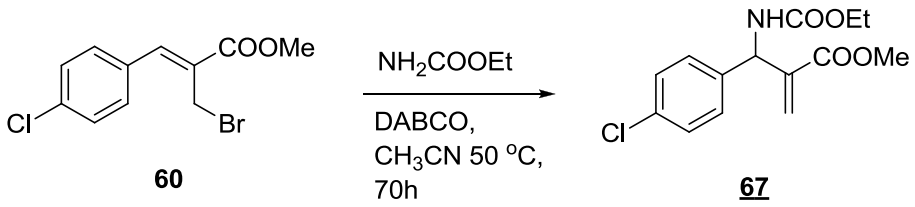
^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 14.6 (q, $OCH_2\underline{CH_3}$), 21.0 (q, $Ar\underline{CH_3}$), 51.9 (q, $COO\underline{CH_3}$), 56.3 (d, $\underline{CH}NH$), 61.1 (t, OCH_2), 126.3 (d, C2/C2'), 126.5 (t, $=CH_2$), 129.3 (d, C3/C3'), 136.3 (s, C1), 137.2 (s, C4), 138.2 (s, $\underline{C}=CH_2$),139.9 (s, $\underline{C}OOEt$), 166.1 (s, $\underline{C}OOMe$).

GC-MS

m/z 277 (M^+, 20), 204 (100), 188 (82), 118 (30).

3.4.4.16 Synthesis of Methyl 2-((4-chlorophenyl)(ethoxycarbonylamino)methyl)acrylate (67)



60 67

methyl 2-((4-chlorophenyl)(ethoxycarbonylamino)methyl)acrylate

The compound (**67**) was synthesized by using same procedure used for the synthesis of (**61**). Baylis-Hillman bromide (**60)** (510 mg, 1.77 mmol) in CH_3CN (5 mL) was added to DABCO (238 mg, 2.12 mmol) and stirred at room temperature for 30 min. NaOH (84.8 mg, 2.12 mmol) and ethyl carbamate (188.6 mg, 2.12 mmol) were added to the reaction mixture and heated to 50^oC for 70 h. After the usual aqueous workup and column chromatographic purification process (ethyacetate/petrolether, 15-20%) obtained (**67**) as colorless oil.

Product (**67**): 235 mg, 0.79 mmol, Yield: 45%

Chemical Formula: $C_{15}H_{19}NO_4$, Molecular Weight: 297.73

Appearance: Colorless oil

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 1.24 (t, J = 7.0 Hz, 3H, $CH_2\underline{CH_3}$), 3.68 (s, 3H, $COO\underline{CH_3}$), 4.15 (q, J =7.2 Hz, 2H, OCH_2), 5.70 (bs, 1H, <u>CH</u>NH), 5.93 (s, 1H, $=CH_2$), 6.38 (s, 1H, $=CH_2$), 7.24-7.31 (m, 4H, ArH).

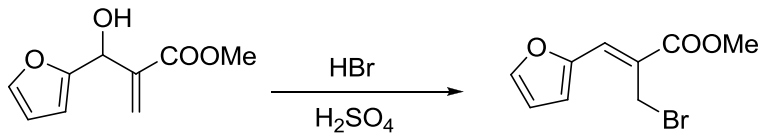
^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 14.5 (q, $OCH_2\underline{CH_3}$), 52.0 (q, $COO\underline{CH_3}$), 56.1 (d, <u>CH</u>NH), 61.2 (t, OCH_2), 127.4 (t, $=CH_2$), 127.7 (d, C2/C2'), 127.9 (d, C3/C3'), 128.5 (s, C1), 133.3 (s, C4), 138.3 (s, <u>C</u>$=CH_2$), 155.7 (s, <u>C</u>OOEt), 165.9 (s, <u>C</u>OOMe).

GC-MS

m/z 297 (M^++2, 5), 297 (M^+, 7), 224 (100), 207 (81), 192 (34).

3.4.4.17 Synthesis of Methyl 2-(bromomethyl)-3-(furan-2-yl)acrylate (68)



41 **68**

methyl 2-(bromomethyl)-3-(furan-2-yl)acrylate

To a the stirred solution of Baylis Hillman ester (**41**) (1.49 g, 8.18 mmol) in 20mL of CH_2Cl_2 was added dropwise conc. HBr solution (48%, 2.7 ml) and then conc. H_2SO_4 solution (2.38 ml) at -20^oC. After stirring for 30 minutes at -20 ^oC, then the mixture was carefully diluted with CH_2CI_2 and H_2O. The aqeous phase was extracted twice with CH_2CI_2, the combined organic phase washed twice with H_2O dried (Na_2SO_4), and evaporated, and the residual oil purified by flash chromatography using 5-10% ethylacetate and petrol ether mixture.

Product (**68**): 1.58 mg, 6.46 mmol, Yield: 79%

Chemical Formula: $C_9H_9BrO_3$

Molecular Weight: 245.07

Appearance: Brown oil

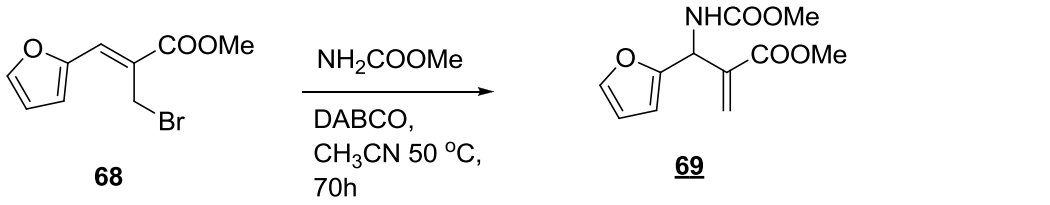
^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 3.84 (s, 3H), 4.71 (s, 2H), 6.55 (q, J =1.8 Hz, 1H), 6.84 (d , J =3.6 Hz, 1H), 7.49 (s, 1H), 7.65 (d, J =1.6 Hz, 1H).

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 26.6 (t), 52.5 (q), 114.7 (d), 120.3 (d), 124.7 (d), 128.3 (s), 146.1 (d), 152.1 (s), 166.7 (s).

3.4.4.18 Synthesis of Methyl 2-(furan-2-yl(methoxycarbonylamino)methyl)acrylate (69)



68 69

methyl 2-(furan-2-yl(methoxycarbonylamino)methyl)acrylate

The compound (**69**) was synthesized by using same procedure used for the synthesis of (**61**). Baylis-Hillman bromide (**68)** (120 mg, 0.48 mmol) in CH_3CN (3 mL) was added to DABCO (65.7 mg, 0.58 mmol) and stirred at room temperature for 30 min. NaOH (23.5 mg,0.58 mmol) and methyl carbamate (44.1 mg, 0.58 mmol) were added to the reaction mixture and heated to 50^oC for 70 h. After the usual aqueous workup and column chromatographic purification process (ethyacetate/petrolether, 15-20%) obtained (**69**) as colorless oil.

Product (**69**): 69 mg, 0.28 mmol, Yield: 59%

Chemical Formula: $C_{11}H_{13}NO_5$, Molecular Weight: 239.22

Appearance: Brown oil

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 3.70 (s, 3H, $COOCH_3$), 3.74 (s, 3H, $COOCH_3$), 5.76(bs, 1H, CHNH), 5.92 (s, 1H, $=CH_2$), 6.18 (d, J = 3.2 Hz, 1H, ArH/$=CH_2$), 6.29-7.39 (m, 3H, ArH/ $=CH_2$), 7.26 (s, 1H, NH), 7.32 (d, J = 1.6 Hz, 1H, CHO, ArH).

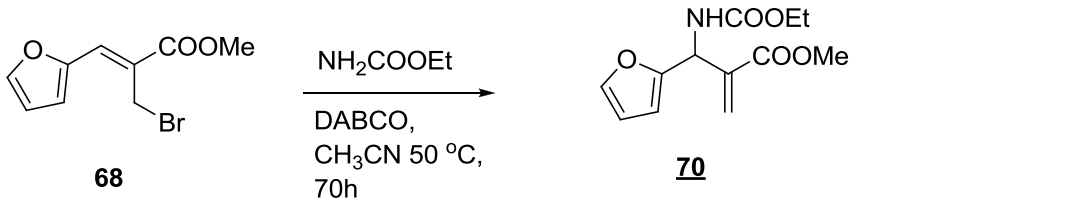
^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 52.3 (q, $COOCH_3$), 56.6 (q, $COOCH_3$), 59.3 (d, CHNH) 106.5 (d, C2), 110.4 (d, C3), 120.7 (t, $=CH_2$), 141.2 (s, $C=CH_2$), 142.2 (d, C3), 152.7 (s, C1), 156.6 (s, $NHCOOCH_3$), 165.7 (s, $COOCH_3$).

GC-MS

m/z 239 (M^+, 14), 180 (56), 163 (100), 94 (19).

3.4.4.19 Synthesis of Methyl 2-((ethoxycarbonylamino)(furan-2-yl)methyl)acrylate (70)



68 **70**

methyl 2-((ethoxycarbonylamino)(furan-2-yl)methyl)acrylate

The compound (**70**) was synthesiszed by using same procedure used in the synthesis of (**61**). Baylis-Hillman bromide (**68)** (120 mg, 0.48 mmol) in CH_3CN (3 mL) was added DABCO (65.7 mg, 0.58 mmol) and stirred at room temperature for 30 min. To the reaction mixture NaOH (23.5 mg,0.58 mmol) and ethyl carbamate (52.4 mg, 0.58 mmol) was added and heated to 50^oC for 70 h. After the usual aqueous workup and column chromatographic purification process (ethyacetate/petrolether, 15-20%) obtained (**70**) as colorless oil.

Product (**70**): 69 mg, 0.27 mmol, Yield: 56%

Chemical Formula: $C_{12}H_{15}NO_5$, Molecular Weight: 253.25

Appearance: Brown oil

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 1.25 (t, J = 7.0 Hz, 3H, O$\underline{CH_2}CH_3$), 3.74 (s, 3H, COOCH_3), 4.13 (q, J =5.2 Hz, 2 H, $\underline{CH_2}CH_3$), 5.76 (bs, 1H, $\underline{CH}$NH), 5.92 (s, 1H, =CH_2), 6.18 (d, J = 3.2 Hz, 1H, ArH/=CH_2), 6.27-7.36 (m, 3H, ArH/ =CH_2), 7.26 (s, 1H, NH), 7.32 (d, J = 1.6 Hz, 1H, ArH).

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 14.5 (q, O$CH_2\underline{CH_3}$), δ 52.3 (q, COO$\underline{CH_3}$), 56.6 (t, O$\underline{CH_2}CH_3$), 58.3 (d, $\underline{C}$HNH) 104.5 (d, C2), 112.3 (d, C3), 120.3 (t, =$\underline{CH_2}$), 142.2 (s, $\underline{C}$=CH_2), 142.8 (d, C3), 150.7 (s, C1), 156.7 (s, NH$\underline{C}$OOCH_3),165.6 (s, $\underline{C}$OOCH_3).

GC-MS

m/z 253 (M^+, 17), 163 (100), 148 (64), 94 (26).

3.4.5 Bioreduction of Baylis Hillman Products

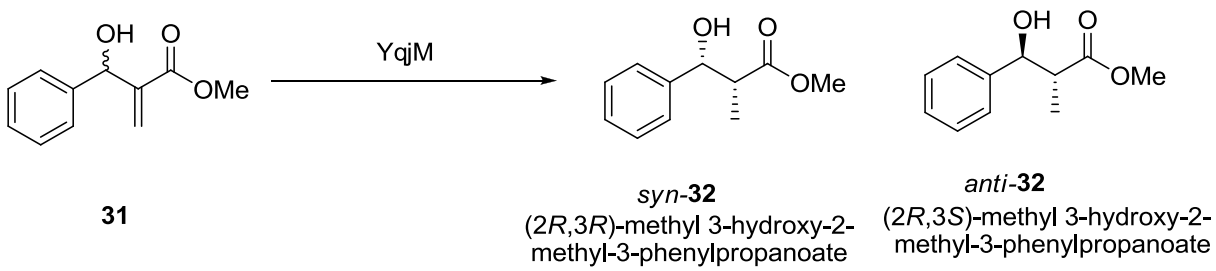
3.4.5.1 Typical Procedure for Bioreduction Screening Experiments

Cell lysate was isolated from ERED producing cells (SYE-4, OPR1, OPR3, YqjM) BL21 (DE3). An Erlenmeyer flask containing sterile TB medium (200mL) supplemented with antibiotic was incubated with 2% vol of overnight culture grown on LB medium up to O.D. of 1 within 3h at 30°C. Production of enzyme was induced by IPTG (0.5mM) (160µL) and the flask was shaken (120 rpm) at 30°C for 24h. The cells pellet collected by centrifugation (6000 rpm for 15min) was suspended in PBS (30mL), centrifuged (6000 rpm for 4 min) and pellet was resuspended in PBS (5mL). Protease inhibitor (phenyl methane sulfonyl fluoride) was added to 1µL/mL. Cells were ruptured by sonication (pulse for 10 seconds after every 1 min about six times). After sonication, suspension was centrifuged (10,000 rpm for 15 min) to obtain cell lysate, which was stored at -20°C. Bradford assay (procedure described above) was conducted to calculate the concentration of protein in cell lysate. Biotransformation (200µL) with EREDs protein (5mg, 100µL) in sterile multiwell plates (500µL each well) was performed in the presence of Tris HCl (pH 8, 50mM, 90.5 µL), $NADP^+$ (200µM, 4µL), glucose-6-phosphate (4mM, 80µL), glucose-6-phosphate dehydrogenease (1 unit, 0.24µL) and substrate (2mM, 0.8µL (stock solution in EtOH:H_2O(2:1)) at 30°C for 48h in orbital thermoshakers (750 rpm). Samples were collected after 12h, 24h and 48h. Product was extracted with ethyl acetate containing internal standard (methyl benzoate). Samples were analyzed by chiral GC and GC-MS.

3.4.5.2 Typical Procedure for Bioreduction of Baylis Hillman Products on Preparative Scale

Bioreduction of Baylis Hillman products with YqjM CCE in sterile baffled Erlenmeyer flask was performed in the presence of Tris HCl (pH 8, 50mM), $NADP^+$ (100 mM stock solution), glucose-6-phosphate (100 mM stock solution), glucose-6-phosphate dehydrogenease, YqjM CCE and substrate (0.5M solution (100 mg) in EtOH:H_2O (2:1)) at 30°C. Product was extracted with diethyl ether and purified by column chromatography. Samples were analyzed by chiral GC and GC-MS.

3.4.5.3 Synthesis of (*2R,3R*)-Methyl 3-hydroxy-2-methyl-3-phenylpropanoate (*syn*-32)



31

syn-**32**
(2*R*,3*R*)-methyl 3-hydroxy-2-methyl-3-phenylpropanoate

anti-**32**
(2*R*,3*S*)-methyl 3-hydroxy-2-methyl-3-phenylpropanoate

(*2R,3R*)-Methyl 3-hydroxy-2-methyl-3-phenylpropanoate (*syn*-**32**) and (*2R,3S*)-methyl 3-hydroxy-2-methyl-3-phenylpropanoate (*anti*-**32**) were prepared by using Tris HCl (pH 8, 30mL, 50mM), $NADP^+$ (200 µL, 100 mM stock solution), glucose-6-phosphate (4mL, 100 mM stock solution), glucose-6-phosphate dehydrogenease (48 µL), YqjM CCE (10mL, 15mg/mL) and methyl 2-(hydroxy(phenyl)methyl)acrylate (**31**) (100 mg, 0.52 mmol) (0.5M solution (100 mg) in EtOH:H_2O (2:1)) at 30°C. Mixture of *syn* and *anti* products was separated by column chromatography using 20% ethyl acetate petrol ether mixture

Product (*syn*-**32**): 50 mg, 0.25 mmol

Yield: 50%

Chemical Formula: $C_{11}H_{14}O_3$

Molecular Weight: 194.23

Appearance: Colorless oil

Enantiomeric excess (ee): 99% (Chiral GC)

$[\alpha]_D^{22}$ = +21.8 (c = 1, $CHCl_3$) (lit[172] $[\alpha]_D^{23}$ = +23.1 (c = 1.5, $CHCl_3$)

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 1.12 (d, J = 7.2Hz, 3H, $\underline{CH_3}CH$), 2.72-2.85 (m, 1H, $CH_3\underline{CH}$), 2.97 (bs, 1H, OH), 3.67 (s, 3H, $COO\underline{CH_3}$) 5.09 (d, J = 3.4Hz, 1H, $\underline{CH}OH$), 7.25-7.35 (m, 5H, ArH).

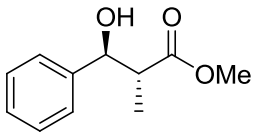
^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

---

[172] Gennari, C.; Colombo, L.; Bertolini, G.; Schimperna, G. *J. Org. Chem.* **1987**, 52, 2754-2760.

δ 14.5 (q, $\underline{CH_3}CH$), 47.1 (d, $CH_3\underline{CH}$), 51.9 (q, $COO\underline{CH_3}$), 76.4 (d, $\underline{CH}OH$), 126.6 (d, C4), 128.1 (d, C2), 128.5 (d, C3), 141.5 (s, C1), 176.3 (s, $\underline{C}OOCH_3$).

3.4.5.4 Synthesis of (*2R,3S*)-Methyl 3-hydroxy-2-methyl-3-phenylpropanoate (*anti*-32)



anti-**32**

(2*R*,3*S*)-methyl 3-hydroxy-2-methyl-3-phenylpropanoate

Product (*anti*-**32)**: 28 mg, 0.25 mmol

Yield: 28%

Chemical Formula: $C_{11}H_{14}O_3$

Molecular Weight: 194.23

Appearance: Colorless oil

Enantiomeric excess (ee): 98% (Chiral GC)

$[\alpha]_D^{22}$ = -62.5 (c = 0.24, $CHCl_3$) (lit[172] $[\alpha]_D^{22}$ = -57.1 (c = 0.12, $CHCl_3$)

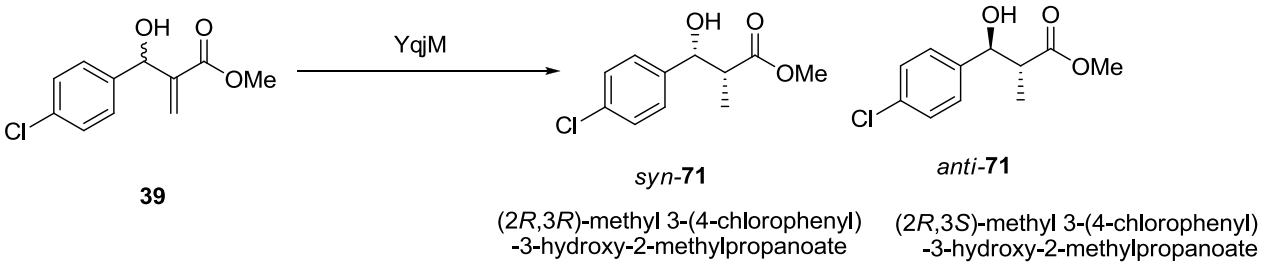
^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 1.00 (d, J = 7.2Hz, 3H, $\underline{CH_3}CH$), 2.74-2.89 (m, 1H, $CH_3\underline{CH}$), 2.98 (bs, 1H, OH), 3.72 (s, 3H, $COO\underline{CH_3}$), 4.74 (d, J = 8.6Hz, 1H, $\underline{CH}OH$), 7.26-7.35 (m, 5H, ArH).

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 10.7 (q, $\underline{CH_3}CH$), 46.3 (d, $CH_3\underline{CH}$), 51.9 (q, $COO\underline{CH_3}$), 73.6 (d, $\underline{CH}OH$), 125.9 (d, C4), 127.5 (d, C2), 128.2 (d, C3), 141.3 (s, C1), 176.2 (s, $\underline{C}OOCH_3$).

3.4.5.5 Synthesis of (*2R,3R*)-Methyl 3-(4-chlorophenyl)-3-hydroxy-2-methylpropanoate (*syn*-71)



39

syn-**71**

(2*R*,3*R*)-methyl 3-(4-chlorophenyl) -3-hydroxy-2-methylpropanoate

anti-**71**

(2*R*,3*S*)-methyl 3-(4-chlorophenyl) -3-hydroxy-2-methylpropanoate

(*2R,3R*)-methyl 3-(4-chlorophenyl)-3-hydroxy-2-methylpropanoate (*syn*-**71**) and (*2R,3S*)-methyl 3-(4-chlorophenyl)-3-hydroxy-2-methylpropanoate (*anti*-**71**) were prepared by using Tris HCl (pH 8, 30mL, 50mM), $NADP^+$ (200 µL, 100 mM stock solution), glucose-6-phosphate (4mL, 100 mM stock solution), glucose-6-phosphate dehydrogenease (48 µL), YqjM CCE (10mL, 15mg/mL) and Methyl 2-((4-chlorophenyl)(hydroxy)methyl)acrylate (**39**) (100 mg, 0.44 mmol) (0.5M solution (100 mg) in EtOH:H_2O (2:1)) at 30°C. Mixture of *syn* and *anti* products was separated by column chromatography using 20% ethyl acetate petrol ether mixture

Product (*syn*-**71**): 43 mg, 0.18 mmol, Yield: 43%

Chemical Formula: $C_{11}H_{13}ClO_3$, Molecular Weight: 228.67

Appearance: Yellow oil

Enantiomeric excess (ee): 97% (Chiral GC)

$[\alpha]_D^{22}$ = +12.4 (c = 1.1, $CHCl_3$) (lit[173] $[\alpha]_D^{22}$ = +17.2 (c = 1.8, $CHCl_3$)

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 1.11 (d, J = 7.2Hz, 3H, $\underline{CH_3}CH$), 2.68-2.81 (m, 1H, $CH_3\underline{CH}$), 3.08 (bs, 1H, OH), 3.68 (s, 3H, $COO\underline{CH_3}$) 5.07 (t, J = 3.4Hz, 1H, $\underline{CH}OH$), 7.24-7.34 (m, 4H, ArH).

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 10.6 (q, $\underline{CH_3}CH$), 46.1 (d, $CH_3\underline{CH}$), 52.0 (q, $COO\underline{CH_3}$), 72.9 (d, $\underline{CH}OH$), 127.3 (d, C2/C2'), 128.4 (d, C3/C3'), 133.2 (s, C1), 139.8 (s, C4), 176.1 (s, $\underline{C}OOCH_3$).

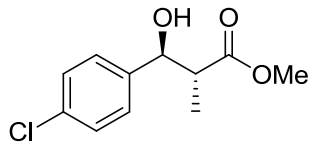
---

[173]Miguel, C.; Juan.; Eva, F.; Florenci, G.; Alberto, J. M. *Tetrahedron: Asymmetry*. **2000**, *11*, 3211-3220.

GC-MS

m/z 228 (M^+, 2), 140 (39), 87 (100), 76 (28).

3.4.5.6 Synthesis of (2R,3S)-Methyl 3-(4-chlorophenyl)-3-hydroxy-2-methylpropanoate (*anti*-71)



anti-**71**

(2*R*,3*S*)-methyl 3-(4-chlorophenyl)
-3-hydroxy-2-methylpropanoate

Product (*anti*-**71**): 37 mg, 0.16 mmol, Yield: 37%

Chemical Formula: $C_{11}H_{13}ClO_3$, Molecular Weight: 228.67

Appearance: Yellow oil

Enantiomeric excess (ee): 96% (Chiral GC)

$[\alpha]_D^{22}$ = -5.4 (c = 0.8, $CHCl_3$)

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 1.02 (d, J = 7.2Hz, 3H, $\underline{CH_3}CH$), 2.70-2.84 (m, 1H, $CH_3\underline{CH}$), 3.12 (s, 1H, OH), 3.72 (s, 3H, $COO\underline{CH_3}$) 4.73 (d, J = 8.4Hz, 1H, $\underline{CH}OH$), 7.24-7.34 (m, 4H, ArH).

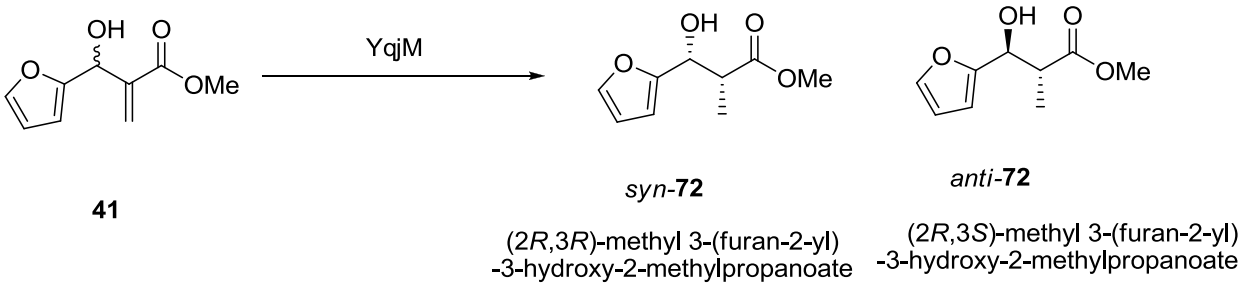
^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 16.0 (q, $\underline{CH_3}CH$), 46.6 (d, $CH_3\underline{CH}$), 53.6 (q, $COO\underline{CH_3}$), 77.2 (d, $\underline{CH}OH$), 129.6 (d, C2/C2'), 130.2 (d, C3/C3'), 135.4 (s, C1), 141.6 (s, C4), 177.7 (s, $\underline{C}OOCH_3$).

GC-MS

m/z 228 (M^+, 2), 140 (39), 87 (100), 76 (28).

3.4.5.7 Synthesis of (2R,3R)-Methyl 3-(furan-2-yl)-3-hydroxy-2-methylpropanoate (*syn*-72)



41

syn-**72**

(2*R*,3*R*)-methyl 3-(furan-2-yl) -3-hydroxy-2-methylpropanoate

anti-**72**

(2*R*,3*S*)-methyl 3-(furan-2-yl) -3-hydroxy-2-methylpropanoate

(*2R,3R*)-methyl 3-(furan-2-yl)-3-hydroxy-2-methylpropanoate (*syn*-**72**) and (*2R,3S*)-methyl 3-(furan-2-yl)-3-hydroxy-2-methylpropanoate (*anti*-**72**) was prepared by using Tris HCl (pH 8, 30mL, 50mM), $NADP^+$ (200 µL, 100 mM stock solution), glucose-6-phosphate (4mL, 100 mM stock solution), glucose-6-phosphate dehydrogenease (48 µL), YqjM CCE (10mL, 15mg/mL) and methyl 2-(furan-2-yl(hydroxy)methyl)acrylate (**41**) (100 mg, 0.54 mmol) (0.5M solution (100 mg) in EtOH:H_2O (2:1)) at 30°C. Mixture of *syn* and *anti* products was separated by column chromatography using 20% ethyl acetate petrol ether mixture

Product (*syn*-**72**): 79 mg, 0.43 mmol, Yield: 79 %

Chemical Formula: $C_9H_{12}O_4$

Molecular Weight: 184.19

Appearance: Yellow oil

Enantiomeric excess (ee): 98% (Chiral GC)

$[\alpha]_D^{20}$ = +14.76 (c = 0.55 $CHCl_3$) (lit[174] $[\alpha]_D^{20}$ = +14.7 (c = 1.64 $CHCl_3$) .

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 1.15 (d, J = 7.2Hz, 3H, $\underline{CH_3}CH$), 2.87-2.93 (m, 1H, $CH_3\underline{CH}$), 3.63 (s, 3H, $COO\underline{CH_3}$), 3.66 (bs, 1H, OH) 4.94 (t, J = 5.2Hz, 1H, $\underline{CH}OH$), 6.20-6.27 (m, 2H, ArH). 7.28-7.29 (m, 1H, ArH).

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

---

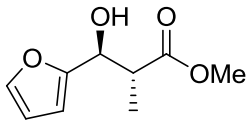
[174] Kusakabe, M.; Sato, F. *J. Org.* Chem. **1989,** *54*, 3486-3487.

δ 11.7 (q, $\underline{CH_3}CH$), 44.0 (d, $CH_3\underline{CH}$), 52.0 (q, $COO\underline{CH_3}$), 68.0 (d, $\underline{CH}OH$), 106.7(d, C2), 110.2(d, C3), 141.9 (d, C4), 154.1 (s, C5), 175.3 (s, $\underline{C}OOCH_3$).

GC-MS

m/z 184 (M^+, 25), 124 (81), 97 (100), 57 (23).

3.4.5.8 Synthesis of (2R,3S)-methyl 3-(furan-2-yl)-3-hydroxy-2-methylpropanoate (a*nti*-72)



anti-**72**

(2*R*,3*S*)-methyl 3-(furan-2-yl)
-3-hydroxy-2-methylpropanoate

Product (*anti*-**72**): 9 mg, 0.04 mmol, Yield: 9 %

Chemical Formula: $C_9H_{12}O_4$, Molecular Weight: 184.19

Appearance: Yellow oil

Enantiomeric excess (ee): 97% (Chiral GC)

$[\alpha]_D^{20}$ = -1.2 (c = 0.41, $CHCl_3$)

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 1.09 (d, J = 7.2Hz, 3H, $\underline{CH_3}CH$), 2.95-3.07 (m, 1H, $CH_3\underline{CH}$), 3.10 (bs,1H, OH), 3.73 (s, 3H; $COO\underline{CH_3}$), 4.78 (d, J = 8.0Hz, 1H, $\underline{CH}OH$), 6.28-6.35 (m, 2H, ArH). 7.27-7.39 (m, 1H, ArH).

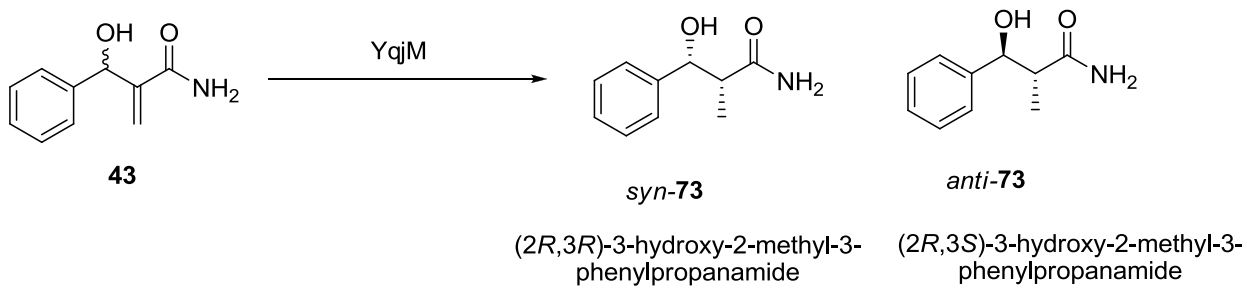
^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 14.2 (q, $\underline{CH_3}CH$), 44.5 (d, $CH_3\underline{CH}$), 52.0 (q, $COO\underline{CH_3}$), 69.7 (d, $\underline{CH}OH$), 107.5(d, C2), 110.2(d, C3), 142.3 (d, C4), 154.0 (s, C5), 175.9 (s, $\underline{C}OOCH_3$).

GC-MS

m/z 184 (M^+, 25), 124 (81), 97 (100), 57 (23).

3.4.5.9 Synthesis of (2R,3R)-3-Hydroxy-2-methyl-3-phenylpropanamide (*syn*-73)



43

syn-**73**

(2*R*,3*R*)-3-hydroxy-2-methyl-3-phenylpropanamide

anti-**73**

(2*R*,3*S*)-3-hydroxy-2-methyl-3-phenylpropanamide

(*2R,3R*)-3-hydroxy-2-methyl-3-phenylpropanamide (*syn*-**73**) and (*2R,3S*)-3-hydroxy-2-methyl-3-phenylpropanamide (*anti*-**73**) were prepared by using Tris HCl (pH 8, 30mL, 50mM), $NADP^+$ (200 µL, 100 mM stock solution), glucose-6-phosphate (4mL, 100 mM stock solution), glucose-6-phosphate dehydrogenease (48 µL), YqjM CCE (10mL, 15mg/mL) and 2-(hydroxy(phenyl)methyl)acrylamide (**43**) (100 mg, 0.56 mmol) (100 mg, 0.54 mmol) (0.5M solution (100 mg) in EtOH:H_2O (2:1)) at 30°C. Mixture of *syn* and *anti* products was separated by column chromatography using 20-40% ethyl acetate petrol ether mixture.

Product (*syn*-**73**): 23 mg, 0.12 mmol, Yield: 23 %

Chemical Formula: $C_{10}H_{13}NO_2$

Molecular Weight: 179.22

Appearance: Colorless solid

m.p: 130-135 °C, (lit[175] 133.5-135°C)

Enantiomeric excess (ee): 98% (Chiral GC)

$[\alpha]_D^{20}$ = +13.33 (c = 1.21, $CHCl_3$)

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 1.03 (d, J = 7.2Hz, 3H, $\underline{CH_3}CH$), 2.46-2.59 (m, 1H, $CH_3\underline{CH}$), 3.64 (bs, 1H, OH), 5.02 (d, J = 3.2 Hz, 1H; $\underline{CH}OH$), 7.19 (s, 2H, NH_2), 7.25-7.31 (m, 5H, ArH).

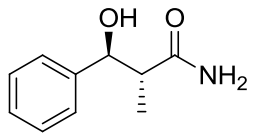
---

[175] Koga, K.; Yamada, S. *Chemical & Pharmaceutical Bulletin*, **1972**, *20*, 3, 526-388.

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 11.1 (q, $\underline{CH_3}CH$), 46.5 (d, $CH_3\underline{CH}$), 73.6 (d, $\underline{CH}OH$), 122.4 (d, C4), 125.9 (d, C3/C3'), 126.2 (d, C2/C2'), 141.4 (s, C1), 178.5 (s, $\underline{C}OO\ NH_2$).

3.4.5.10 Synthesis of (2R,3S)-3-hydroxy-2-methyl-3-phenylpropanamide (a*nti*-73)



anti-**73**

(2*R*,3*S*)-3-hydroxy-2-methyl-3-phenylpropanamide

Product (*anti*-**73**): 25 mg, 0.13 mmol

Yield: 25 %

Chemical Formula: $C_{10}H_{13}NO_2$

Molecular Weight: 179.22

Appearance: Colorless solid

m.p: 132-137 ^{o}C, (lit[175] 133.5-135^{o}C)

$[\alpha]_D^{20}$ = -30.26 (c = 1.24, $CHCl_3$)

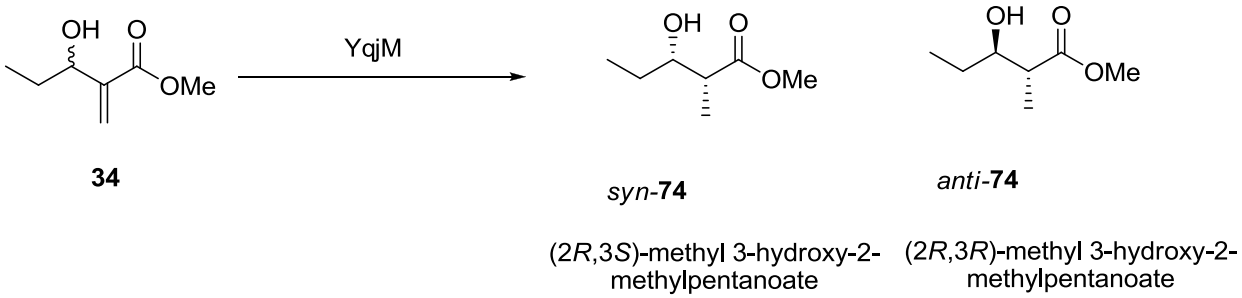
^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 1.03 (d, J = 7.2Hz, 3H, $\underline{CH_3}CH$), 2.47-2.61 (m, 1H, $CH_3\underline{CH}$), 3.46 (bs, 1H, OH), 4.67 (d, J = 7.4 Hz, 1H; $\underline{CH}OH$), 7.19 (s, 2H, NH_2), 7.25-7.29 (m, 5H, ArH).

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 14.2 (q, $\underline{CH_3}CH$), 47.3 (d, $CH_3\underline{CH}$), 60.4 (d, $\underline{CH}OH$), 126.4 (d, C4), 127.9 (d, C3/C3'), 128.5 (d, C2/C2'), 142.2 (s, C1), 171.1 (s, $\underline{C}OONH_2$).

3.4.5.11 Synthesis of (*2R,3S*)-Methyl 3-hydroxy-2-methylpentanoate (s*yn*-74)



34

syn-**74**

(2*R*,3*S*)-methyl 3-hydroxy-2-methylpentanoate

anti-**74**

(2*R*,3*R*)-methyl 3-hydroxy-2-methylpentanoate

(*2R,3S*)-methyl 3-hydroxy-2-methylpentanoate (*syn*-**74**) and (*2R,3R*)-methyl 3-hydroxy-2-methylpentanoate (*anti*-**74**) were prepared by using Tris HCl (pH 8, 30mL, 50mM), $NADP^+$ (200 µL, 100 mM stock solution), glucose-6-phosphate (4mL, 100 mM stock solution), glucose-6-phosphate dehydrogenease (48 µL), YqjM CCE (10mL, 15mg/mL) and methyl 3-hydroxy-2-methylenepentanoate (**34**) (100 mg, 0.69 mmol) (0.5M solution (100 mg) in EtOH:H_2O (2:1)) at 30°C. Mixture of *syn* and *anti* products was separated by column chromatography using 20-40% ethyl acetate petrol ether mixture.

Product (*syn*-**74**): 74 mg, 0.5 mmol, Yield: 73 %

Chemical Formula: $C_7H_{14}O_3$

Molecular Weight: 146.18

Appearance: colourless liquid

$[\alpha]_D^{20}$ = -2.92 (c = 0.21, $CHCl_3$) (lit[176] $[\alpha]_D^{20}$ = -3.5 (c = 0.2, CH_2Cl_2)

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 0.87 (t, J = 7.2Hz, 3H, $\underline{CH_3}CH_2$), 1.18 (d, J = 7.4Hz, 3H, $\underline{CH_3}CH$), 1.28-1.56 (m, 2H, $CH_3\underline{CH_2}$), 2.38-2.56 (m, 1H, $CH_3\underline{CH}$), 3.34 (bs, 1H, OH), 3.71 (s, 3H, $COO\underline{CH_3}$), 3.85-3.99 (m, 1H, $\underline{CH}OH$).

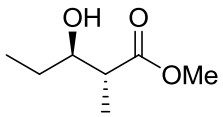
^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

---

[176]Jose, L. V.; Dolores, B.; Esther, D.; Monica, R.; Luisa, C. *J. Org. Chem.* **2000,** *65*, 3754-3760.

δ 9.9 (q, $\underline{CH_3}CH_2$), 15.7 (q, $\underline{CH_3}CH$), 29.2 (t, $CH_3\underline{CH_2}$), 44.8 (d, $CH_3\underline{CH}$), 52.2 (q, $COO\underline{CH_3}$), 76.3 (d, $\underline{CH}OH$), 168.2 (s, $\underline{C}OOCH_3$).

3.4.5.12 Synthesis of (*2R,3R*)-Methyl 3-hydroxy-2-methylpentanoate (a*nti*-74)



anti-**74**

(2*R*,3*R*)-methyl 3-hydroxy-2-methylpentanoate

Product (anti-**74**): 12 mg, 0.07 mmol

Yield: 11 %

Chemical Formula: $C_7H_{14}O_3$

Molecular Weight: 146.18

Appearance: colourless liquid

$[\alpha]_D^{20}$= -10.9(c = 0.92, $CHCl_3$)

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

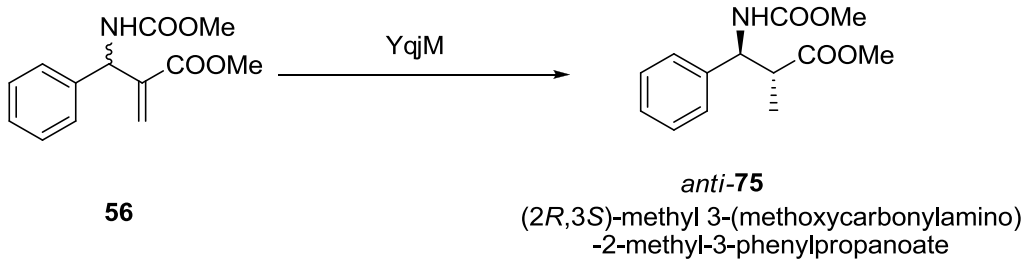
δ 0.89 (dt , J = 2.8Hz, J = 7.4Hz, 3H, $\underline{CH_3}CH_2$), 1.23 (t, J = 7.4Hz, 3H, $\underline{CH_3}CH$), 1.32-1.59 (m, 2H, $CH_3\underline{CH_2}$), 2.36-2.46 (m, 1H, $CH_3\underline{CH}$), 3.33 (bs, 1H, OH), 3.73 (s, 3H; $COO\underline{CH_3}$), 3.75-3.79 (m, 1H, $\underline{CH}OH$).

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 9.8 (q, $\underline{CH_3}CH_2$), 15.3 (q, $\underline{CH_3}CH$), 29.0 (t, $CH_3\underline{CH_2}$), 43.8 (d, $CH_3\underline{CH}$), 54.1 (q, $COO\underline{CH_3}$), 74.2 (d, $\underline{CH}OH$), 169.1 (s, $\underline{C}OOCH_3$).

3.4.6 Scale up Bioreduction of Aza Baylis Hillman Products

3.4.6.1 Synthesis of (*2R,3S*)-Methyl 3-(methoxycarbonylamino)-2-methyl-3-phenyl propanoate (a*nti*-75)



56

anti-**75**
(2*R*,3*S*)-methyl 3-(methoxycarbonylamino)
-2-methyl-3-phenylpropanoate

(*2R,3S*)-Methyl 3-(methoxycarbonylamino)-2-methyl-3-phenylpropanoate (anti-**75**) was prepared by using Tris HCl (pH 8, 30mL, 50mM), $NADP^+$ (200 µL, 100 mM stock solution), glucose-6-phosphate (4mL, 100 mM stock solution), glucose-6-phosphate dehydrogenease (48 µL), YqjM CCE (10mL, 15mg/mL) and methyl 2-((methoxycarbonylamino)(phenyl)methyl)acrylate (**77**) (100 mg, 0.40 mmol) (0.5M solution (100 mg) in EtOH:H_2O (2:1)) at 30°C. The product (*anti*-**75**) was separated by column chromatography using 20-40% ethyl acetate petrol ether mixture.

Product (*anti*-**75**): 66 mg, 0.26 mmol, Yield: 65 %

Chemical Formula: $C_{13}H_{17}NO_4$

Molecular Weight: 251.28

Appearance: colorless solid

m.p: 85-88°C (lit 88 - 89°C[177])

Optical rotation: $[\alpha]_D^{25}$ = - 37.08 (c = 0.25 EtOH) (lit[161] $[\alpha]_D^{25}$ = - 39.5 (c = 0.55 EtOH)

---

[177] Takagi, M.; Yamamoto, K. *Tetrahedron.* **1991,** *41*, 42, 8869-8882.

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 1.23 (d, J = 7.2Hz, 3H, $\underline{CH_3}CH$), 2.86-3.00 (m, 1H, $CH_3\underline{CH}$), 3.58 (s, 3H, $COO\underline{CH_3}$), 3.65 (s, 3H, $COO\underline{CH_3}$), 4.85 (dd, J = 6.6, J = 8.2 Hz, 1H, $\underline{CH}OH$), 6.04 (bd, J = 7.0Hz, 1H, NH), 7.21-7.28 (m, 5H, ArH).

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 15.6 (q, $\underline{CH_3}CH$), 45.0 (d, $CH_3\underline{CH}$), 51.8 (q, $COO\underline{CH_3}$), 52.2 (q, $COO\underline{CH_3}$), 57.3 (d, $\underline{CH}NH$), 126.2 (d, C4), 127.5 (d, C3/ C3'), 128.5 (d, C2/ C2'), 140.6 (s, C1), 156.6 (s, $NH\underline{C}OOMe$), 175.2 (s, $\underline{C}OOMe$).

GC-MS

m/z 251 (M^+, 2), 164 (100), 121 (31).

3.5 Redox Cascade Reactions of Carvones

3.5.1 Typical Procedures for Redox Cascade Reaction

3.5.1.1 Redox Screening Experiments

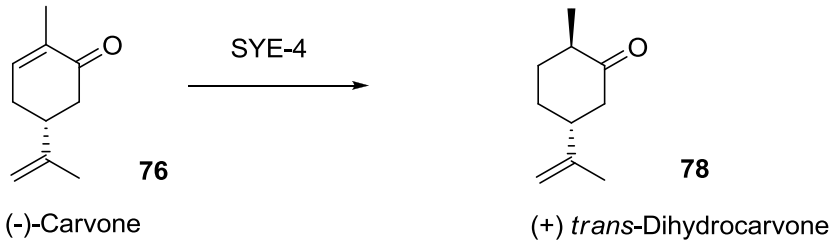
Cell lysate was isolated from recombinat cells expressing EREDs (SYE-3, SYE-4, OPR1, OPR3, YqjM, W116I) and BVMOs ($CHMO_{Acenito}$, $CHMO_{Brevi\ I}$, $CHMO_{Brevi\ II}$, $CPMO_{Coma}$). An Erlenmeyer flask containing sterile TB medium (200mL) supplemented with antibiotic was incubated with 2% vol of overnight culture grown on LB medium up to O.D. of 1 within 3h at 28°C. Production of enzyme was induced by IPTG (0.5mM) (160µL) and the flask was shaken (120 rpm) at 28°C for 24h. The cells pellet collected by centrifugation (6000 rpm for 15min) was suspended in PBS (30mL), centrifuged (6000 rpm for 4 min) and pellet was resuspended in PBS (5mL). Protease inhibitor (phenyl methane sulfonyl fluoride) was added as 1µL/mL. Cells were sonicated using a Bandelin Sonoplus HD3200 (KE76 probe,50% amplitude, 10 sec on and 60 sec off, 6 cycles, 4°C). After sonication, suspension was centrifuged (10,000 rpm for 15 min at 4^{o}C) to obtain cell lysate, which was stored at -20°C. Bradford assay (protocol described above in section 3.3.3) was conducted to calculate the concentration of protein in cell lysate. Biotransformation (200µL) was performed with CCE (Ene-reductases and BVMOs 5mg each, 100µL) in sterile multiwell plates (500µL each well) in the presence of Tris HCl (pH 8, 50mM, 90.5 µL), $NADP^+$ (200µM, 0.4µL), glucose-6-phosphate (4mM, 8µL), glucose-6-phosphate dehydrogenease (1 unit, 0.24µL) and substrate (2mM, 0.8µL (stock solution in EtOH:H_2O(2:1)) at 30°C for 6h. Samples were collected after 1h, 3h and 6h (for W116I 24h time was required). Product was extracted with ethyl acetate containing internal standard (methyl benzoate). Samples were analyzed by chiral GC and GC-MS.

3.5.1.2 Procedure for Redox Biotransformation of Carvones on Preparative Scale

Redox biotransformation of carvones with proteins (EREDs and BVMOs) in sterile baffled Erlenmeyer flask was performed in the presence of Tris HCl (pH 8, 50mM), $NADP^+$ (100mM stock solution), glucose-6-phosphate (100 mM stock solution), glucose-6-phosphate dehydrogenease, EREDs and BVMOs proteins and substrate (0.5M solution (30-50 mg) in EtOH:H_2O (2:1)) at 30°C. Product was extracted with diethyl ether and purified through column chromatography. Samples were analyzed by chiral GC and GC-MS.

3.5.2 Scale up Bioreduction and Redox Reactions of Carvones

3.5.2.1 Synthesis of (+)-(*2R,5R*)-2-Methyl-5-(prop-1-en-2-yl)cyclohexanone (78)



(−)-Carvone (**76**) (10 mg, 0.066 mmol, in ethanol: water (2:1)) was reduced by Tris HCl (pH 8, 18mL, 50mM), $NADP^+$ (80 µL, 100mM stock solution), glucose-6-phosphate (1.6 mL, 100 mM stock solution), glucose-6-phosphate dehydrogenease (68 µL) and SYE-4 CCE (5.6mL, 35.5mg/mL) at 28°C for 24h using the general protocol for scale up bioraduction of carvones to (+)-*trans*-dihydrocarvone (**78**). The product (**78**) was extracted with diethyl ether and purified with column chromatography (silica gel, PE: Et_2O, 6:1).

Product (**78**): 6mg, 0.039 mmol

Yield: 85%

Chemical Formula: $C_{10}H_{16}O$

Molecular Weight: 152.23

Optical rotation: $[\alpha]_D^{22}$ = +14.2 (c = 0.8, $CHCl_3$) (lit[178] $[\alpha]_D^{20}$ = +16.2 (neat)

Appearance: Colorless oil

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 1.02 (d, J = 6.8Hz, 3H, CH_3), 1.46-1.84 (m, 4H, H3/H4), 1.66 (s, 3H, $=CCH_3$), 2.28-2.56 (m, 4H, H2/H5/H6), 4.62-4.76 (m, 2H, $=CH_2$).

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 15.6(q, CH_3), 21.5(q, $=C\underline{C}H_3$), 26.3(t, C3), 30.6(t, C4), 43.9(d, C5), 44.1(t, 6C), 44.6(t, C2), 111.5(t, $=\underline{C}H_2$), 146.8(s $=\underline{C}CH_3$), 214.0(s, C=O).

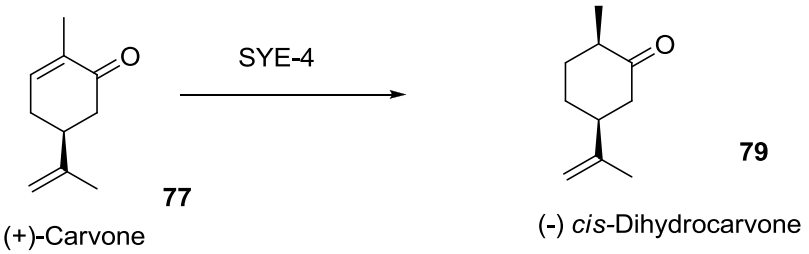
GC- MS

m/z =152 (M^+, 27), 137 (12), 95 (81), 82(42), 67 (100).

---

[178] Chen, Y. W.; Shao, S.; Li. T.; Li. Y. *Synthesis*, **1992,** 1061-1062.

3.5.2.2 Synthesis of (-)-(*2R,5S*)-2-Methyl-5-(prop-1-en-2-yl)cyclohexanone (79)



(+)-Carvone (**77**) (10 mg, 0.066 mmol, in ethanol: water (2:1)) was reduced by using Tris HCl (pH 8, 18mL, 50mM), $NADP^+$ (80 µL, 100mM stock solution), glucose-6-phosphate (1.6 mL, 100 mM stock solution), glucose-6-phosphate dehydrogenease (68 µL) and SYE-4 CCE (5.6mL, 35.5mg/mL) at 28°C for 24h using the general protocol for scale up bioraduction of carvones to (-)-*cis*-dihydrocarvone (**79**). The product (**79**) was extracted with diethyl ether and purified with column chromatography (silica gel, PE: Et_2O, 6:1).

Product (**79**): 5.4 mg, 0.035 mmol, Yield: 83%

Chemical Formula: $C_{10}H_{16}O$, Molecular Weight: 152.23

Optical rotation: $[\alpha]_D^{22} = -16.9$ (c = 1.2, $CHCl_3$) (lit[179] $[\alpha]_D^{22} = -19.5$ (c = 0.34, $CHCl_3$)

Appearance: Colorless oil

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 0.98 (d, J = 6.5Hz, 3H, CH_3), 1.28-1.90 (m, 4H, H3/H4), 1.46 (s, 3H, $=CCH_3$), 2.01-2.18 (m, 4H, H2/H5/H6), 4.45-4.48 (m, 2H, $=CH_2$).

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

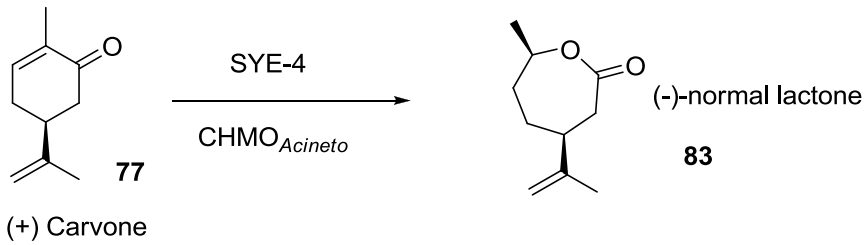
δ 14.3 (q, CH_3), 20.4 (q, $=C\underline{C}H_3$), 30.7 (t, C3), 34.8 (t, C4), 44.6 (d, C5), 46.8 (d, 6C), 46.9 (t, C2), 109.5 (t, $=\underline{C}H_2$), 147.5 (s, $=\underline{C}CH_3$), 212.4 (s, C=O).

GC-MS m/z =152 (M^+, 15), 137 (11), 95 (66), 81(43), 67 (100).

---

[179] Kei, S.; Naoji, K.; Hiroki, H.; Shin-ya, Y.; Toshifumi, H. *Bull. Chem. Soc. Jpn.* **2004,** *77*, 2269–2272.

3.5.2.3 Synthesis of (-)-(*4S,7R*)-7-Methyl-4-(prop-1-en-2-yl)oxepan-2-one (83)



(+)-Carvone (**77**) (30 mg, 0.2 mmol, in ethanol: water (2:1)) was converted to (-)-(*4S,7R*)-7-methyl-4-(prop-1-en-2-yl)oxepan-2-one (**83**) by using Tris HCl (pH 8, 16mL, 50mM), $NADP^+$ (28 µL, 100mM stock solution), glucose-6-phosphate (1.12 mL, 100 mM stock solution), glucose-6-phosphate dehydrogenease (41.3 µL), SYE-4 CCE (1.34mL, 35.5mg/mL) and $CHMO_{Acenito}$ (2mL, 17.5mg/mL) at 30°C for 24h using the general protocol for scale up bioraduction of carvones. The product (**83**) was extracted with diethyl ether and purified with column chromatography (silica gel, PE: Et_2O, 3:1).

Product (**83**): 23 mg, 0.13 mmol, Yield: 69%

Chemical Formula: $C_{10}H_{16}O_2$

Molecular Weight: 168.23

Optical rotation: $[\alpha]_D^{22} = -5.7$ (c = 0.19, $CHCl_3$) (lit[141] $[\alpha]_D^{22}$ −3.7 (*c* = 3.78, CHCl3)

Appearance: Colorless oil

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 1.30 (d, J = 6.4 Hz, 3H, CH_3), 1.64–1.90 (m, 4H, H4/H5/H6), 1.72 (s, 3H, $=CCH_3$), 2.41–2.52 (m, 1H, H3), 2.68–2.98 (m, 2H, H3/H4), 4.38–4.47 (m, 1H, H7), 4.76–4.79 (m, 2H, $=CH_2$).

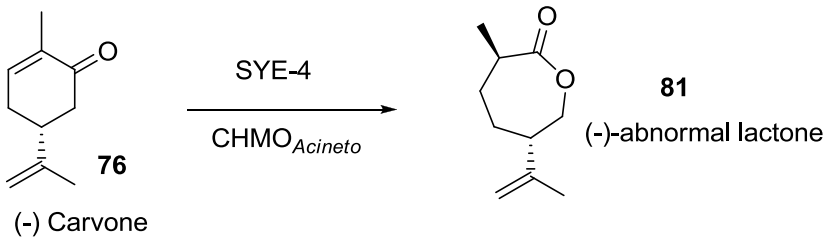
^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 21.3 (q, CH_3), 21.8 (q, $=C\underline{C}H_3$), 29.7 (t, C5), 33.2 (t, C6), 38.4 (t, C3), 38.6 (d, C4), 75.4(d, C7), 111.3 (t, $=\underline{C}H_2$), 146.3 (s, $=\underline{C}CH_3$), 173.7 (s, C=O)

GC-MS

m/z 168 (M+, 2%), 138 (23),110 (98), 95 (51), 81 (19), 68 (100).

3.5.2.4 Synthesis of (-)-(*3R,6S*)-3-Methyl-6-(prop-1-en-2-yl)oxepan-2-one (81)



(-)-Carvone (**76**) (30 mg, 0.2 mmol, in ethanol: water (2:1)) was converted to (-)-(*3R,6S*)-3-methyl-6-(prop-1-en-2-yl)oxepan-2-one (**81**) by using Tris HCl (pH 8, 16mL, 50mM), $NADP^+$ (28 µL, 100mM stock solution), glucose-6-phosphate (1.12 mL, 100 mM stock solution), glucose-6-phosphate dehydrogenease (41.3 µL), SYE-4 CCE (1.34mL, 35.5mg/mL) and $CHMO_{Acenito}$ (2mL, 17.5mg/mL) at 30°C for 24h using the general protocol for scale up bioraduction of carvones. The product (**81**) was extracted with diethyl ether and purified with column chromatography (PE: Et_2O, 3:1).

Product (**81**): 24 mg, 0.14 mmol, Yield: 71%

Chemical Formula: $C_{10}H_{16}O_2$

Molecular Weight: 168.23

Optical rotation: $[\alpha]_D^{22} = -31.4$ (c = 0.70, $CHCl_3$) (lit[141] $[\alpha]_D^{22}$ −34.6 (*c* = 0.9, CHCl3)

Appearance: colorless oil

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 1.21 (d, J = 6.6Hz, 3H, CH_3), 1.55–1.83 (m, 4H, H4/H5), 1.76 (s, 3H, $=CCH_3$), 2.27–2.35 (m, 1H, H3), 2.72–2.80 (m, 2H, H6), 4.14–4.20 (m, 1H, H7), 4.81–4.83 (m, 2H, $=CH_2$).

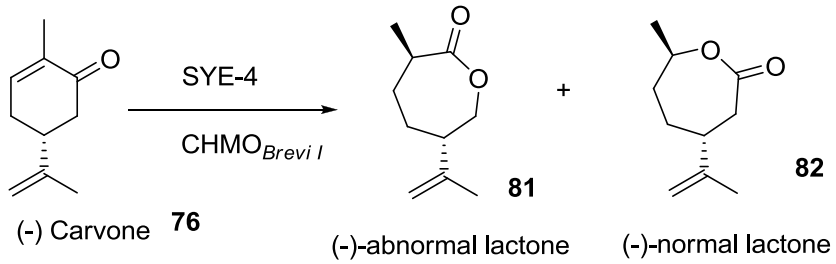
^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 18.4 (q, CH_3), 21.8 (q, $=C\underline{C}H_3$), 31.8 (t, C4), 34.2 (t, C5), 37.2 (d, C3), 46.4 (d, C6), 71.6 (t, C7), 111.1 (t, $=\underline{C}H_2$), 145.6 (s, $=\underline{C}CH_3$) 177.8 (s, C=O).

GC-MS

m/z 168 (M+, 7%), 125 (34),108 (40), 81 (30), 68 (100).

3.5.2.5 Synthesis of (-)-(*4R,7R*)-7-Methyl-4-(prop-1-en-2-yl)oxepan-2-one (82) and (-)-(*3R,6S*)-3-methyl-6-(prop-1-en-2-yl)oxepan-2-one (81)



(-)-Carvone (**76**) (50 mg, 0.33 mmol, in ethanol: water (2:1)) was converted to (-)-(*4R,7R*)-7-methyl-4-(prop-1-en-2-yl)oxepan-2-one (**82**) and (-)-(*3R,6S*)-3-methyl-6-(prop-1-en-2-yl)oxepan-2-one (**81**) using Tris HCl (pH 8, 29mL, 50mM), $NADP^+$ (80 µL, 100mM stock solution), glucose-6-phosphate (3.2 mL, 100 mM stock solution), glucose-6-phosphate dehydrogenease (24 µL), SYE-4 CCE (8mL, 35.5mg/mL), $CHMO_{Brevi1}$ (4.78mL, 41.8 mg/mL) and substrate (0.5M solution (30-50 mg) in EtOH:H_2O (2:1)) at 30°C for 24h using the general protocol for scale up bioraduction of carvones. The mixture of normal and abnormal lactone obtained was extracted with diethyl ether and purified by chromatography using (PE: Et_2O, 6:1)

3.5.2.6 Synthesis of (-)-(*4R,7R*)-7-Methyl-4-(prop-1-en-2-yl)oxepan-2-one (normal lactone) (82)

Product (**82**): 15 mg, 0.08 mmol, Yield: 30%

Chemical Formula: $C_{10}H_{16}O_2$

Molecular Weight: 168.23

Optical rotation: $[\alpha]_D^{22} = -39.6$ (c = 0.96, $CHCl_3$)(lit[141] $[\alpha]_D^{22}$ −42.1 (*c = 1.12*, CHCl3)

Appearance: Colorless oil

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 1.40 (d, J = 8.4Hz, 3H, CH_3), 1.62–1.97 (m, 4H, H5/H6), 1.73 (s, 3H, $=CCH_3$), 2.24–2.35 (m, 1H, H3), 2.59–2.78 (m, 2H, H3/H4), 4.39–4.53 (m, 1H, H7), 4.73–4.76 (m, 2H, $=CH_2$).

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 20.1 (q, CH_3), 22.6 (q, =C<u>C</u>H_3), 34.3 (t, C5), 35.8 (t, C6), 40.1 (t, C3), 41.7 (d, C4), 76.3 (d, C7), 110.1 (t, =<u>C</u>H_2), 148.4 (s, =<u>C</u>CH_3), 174.6 (s, C=O).

GC-MS

m/*z* 168 (M+, 8%), 139 (15),108 (63), 81 (39), 67 (100).

3.5.2.7 Synthesis of (-)-(*3R,6S*)-3-Methyl-6-(prop-1-en-2-yl)oxepan-2-one (abnormal lactone) (81)

Yield (**81**): 11 mg, 0.06 mmol

Product: 21%

Chemical Formula: $C_{10}H_{16}O_2$

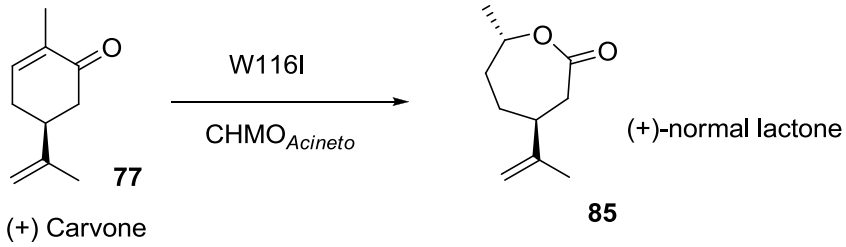
Molecular Weight: 168.23

Optical rotation: $[\alpha]_D^{22}$ = −35.2.6 (c = 1.13, $CHCl_3$) (lit[141] $[\alpha]_D^{22}$ −34.6 (*c* = 0.9, CHCl3)

Appearance: Colorless oil

Spectroscopic data for compound (**81**) is given above.

3.5.2.8 Synthesis of (-)-(*4S,7S*)-7-Methyl-4-(prop-1-en-2-yl)oxepan-2-one (85)



(+)-Carvone (**77**) (30 mg, 0.2 mmol, in ethanol: water (2:1)) was converted (-)-(*4S,7S*)-7-methyl-4-(prop-1-en-2-yl)oxepan-2-one (**85**) by using Tris HCl (pH 8, 16mL, 50mM), $NADP^+$ (28 µL, 100mM stock solution), glucose-6-phosphate (1.12 mL, 100 mM stock solution), glucose-6-phosphate dehydrogenease (41.3 µL), W116I CCE (1.78mL, 47.2mg/mL) and $CHMO_{Acenito}$ (2mL, 17.5mg/mL) at 30°C for 24h using the general protocol for scale up bioraduction of carvones. The product (**85**) was extracted with diethyl ether and purified with column chromatography (PE: Et_2O, 3:1).

Product (**85**): 25 mg, 0.14 mmol, Yield: 76%

Chemical Formula: $C_{10}H_{16}O_2$, Molecular Weight: 168.23

Optical rotation: $[\alpha]_D^{22}$ = +42.3 (c = 1.13, $CHCl_3$) (lit[141] $[\alpha]_D^{20}$ = +45.5 (*c* = 1.48, CHCl3)

Appearance: Colorless oil

^{1}H NMR (200 MHz; $CDCl_3$; Me_4Si)

δ 1.29 (d, J 6.2Hz, 3H, CH_3), 1.63–1.93 (m, 4H, H5/H6), 1.75 (s, 3H, $=CCH_3$), 2.41–2.52 (m, 1H, H3), 2.68–2.96 (m, 2H, H3/H4), 4.38–4.49 (m, 1H, H7), 4.77–4.79 (m, 2H, $=CH_2$).

^{13}C-NMR (50 MHz; $CDCl_3$; Me_4Si)

δ 21.3 (q, CH_3), 21.8 (q, $=C\underline{C}H_3$), 29.7 (t, C5),), 33.2 (t, C6), 38.4 (t, C3), 38.6 (d, C4), 75.4 (d, C7), 111.3 (t, $=\underline{C}H_2$), 146.3 (s, $=\underline{C}CH_3$), 173.7 (s, C=O)

GC-MS

m/z 168 (M+, 10%), 139 (9),108 (51), 67 (100).

References

1) Anthonsen, T. In *Applied Biocatalysis*; Adlercreutz. P.; Straathof, A. J. J. Eds.; Harwood Academic Publishers: UK, **1999**, 18-53.

2) Faber, K. *Biotransformations in Organic Chemistry*, 4th ed., Springer-Verlag, Berlin, **2000**.

3) Jayasinghe L. Y.; Smallridge A. J.; Trewhella M. A. *Tetrahedron Letters*, **1993**, *34*, 24, 3949-3950.

4) Crossley, R. *Chirality and the Biological Activity of Drugs*, CRC Press, Boca Raton, **1995.**

5) Andreas, S. B.; Bettina, R. R. *Biocatalysis*, **2004** WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

6) Straathof, J. J.; Panke, S.; Schmid, A. *Curr. Opin. Biotechnol*. **2002**, *13*, 548–556.

7) a) *Biocatalysis in the Pharmaceutical and Biotechnology Industries*, Patle, R.N. (ed.) CRC Press, Boca Raton, **2007**. b) *Enzyme Catalysis in Organic Synthesis*, Drauz, K.; Waldmann, H. (eds.) Wiley-VCH, Weinheim **2002**.

8) Frost. Sullivan. *Business assessment, Chem. Eng. News* **2003**, *81*, 18, 45-55.

9) Faber, K. *Biotransformations in Organic Chemistry*, Springer, Berlin, **1997**.

10) Garcia-Urdiales, E.; Alfonso, I.; Gotor, V. *Chem. Rev.* **2005**, *105*, 313-354.

11) a) Wichmann, R.; Wandrey, C.; Bückmann, A.F.; Kula, M.R. *Biotechnol. Bioeng.* **1981**, *23*, 2789-2802. b) Wong, C.H.; Whitesides, G.M. *J. Am. Chem. Soc.* **1981**, *103*, 4890-4899. c) Tishkov, V.I.; Galkin, A.G.; Marchenko, G.N.; Tsyganokov, Y.D.; Egorov, H.M. *Biotechnol. Appl. Biochem.* **1993**, *18*, 201-207. d) Kataoka, M. *Appl. Microbiol. Biotechnol.* **1999**, *51*, 486-490. e) Vrtis, J.M.; White, A.K.; Metcalf, W.W.; vam der Donk, W.A. *Angew. Chem. Int. Ed.* **2002**, *41*, 3257-3259.

12) a) Arrigo, D. P.; Pedrocchi-Fantoni, G.; Servi, S. *Stereoselect. Biocatal.* **2000**, 365-396. b) Stewart, J.D. *Curr. Opin. Biotechnol.* **2000**, *11*, 363-368. c) Stewart, J.D. *Biotechnol. Genet. Engin. Rev.* **1997**, *14*, 67-143.

13) a) Dömling, A.; Ugi, I. *Angew. Chem.* **2000**, *112*, 3300-3344. b) Tietze, L.F.; Modi, A. *Med. Res. Rev.* **2000**, *20*, 304322. c) Zhu, J. *Eur. J. Org. Chem.* **2003**, 1133-1144. d) Wangelin, A.J.; Neumann, H.; Gördes, D.; Klaus, S.; Strübing, D.; Beller, M. *Chem.*

Eur. J. **2003**, *9*, 4286-4294. e) Tempest, P.A. *Curr. Opin. Drug Discovery Dev.* **2005**, *8*, 776-788.

14) Fogg, D.E.; dos Santos, E.N. *Coord. Chem. Rev.* **2004**, *248*, 2365-2379.

15) Frost, J. *Chem. Engineer* **1996**, *611*, 32-33.

16) a) Mayer, S.F.; Kroutil, W.; Faber, K. *Chem. Soc. Rev.* **2001**, *30*, 332-339. b) Bruggink, A.; Schoevaart, R.; Kieboom, T. *Org. Proc. Res. Develop.* **2003**, *7*, 622-640. c) Pellissier, H. *Tetrahedron* **2006**, *62*, 2143-2173.

17) Simons, C.; Hanefeld, U.; Arends, I.W.C.E.; Maschmeyer, T.; Sheldon, R.A. *Topics Catal.* **2006**, *40*, 35-44.

18) a) Mihovilovic, M.D. *Curr. Org. Chem.* **2006**, *10*, 1265-1287. b) Kamerbeek, N.M.; Janssen, D.B.; van Berkel, W.J.H.; Fraaije, M.W. *Adv. Synth. Catal.* **2003**, *345*, 667-678. c) Mihovilovic, M.D.; Müller, B.; Stanetty, P. *Eur. J. Org. Chem.* **2002**, 3711-3730.

19) Yang, J. W.; Hechavarria, M. T.; Vignola, N.; List, B. *Angew Chem Int Ed Engl.* **2005**, 44, 108-110.

20) Stuermer, R., Hauer, B., Hall, M., Faber, K., *Curr. Opin. Chem. Biol.* **2007,** *11*, 203–213.

21) a) Khan, H.; Harris, R. J., Barna, T.; Craig, D. H.; Bruce, N. C.; Munro, A. W.; Moody, P. C.;. Scrutton, N. S. *J. Biol. Chem.* **2002**, *277*, 21906 – 21912. b) Karplus, P. A.;. Fox, K. M.; Massey, V. *FASEB J.* **1995,** *9*, 1518 –1526. c) Khan, H.; Barna, T.; Bruce, N. C.; Munro, A. W.; Leys, D.; Scrutton, N. S. *FEBSJ.* **2005**, *272*, 4660 –4671. d) Kohli, R. M.; Massey, V. *J. Biol. Chem*. **1998,** *273*, 32763 – 32770. e) Messiha, H. L.; Munro, A. W.; Bruce, N. C.; Barsukov, I.; Scrutton, N. S. *J. Biol. Chem.* **2005,** *280*, 10695 –10709. f) Nakamura, T.; Yoshimura, J.; Ogura, Y. *J. Biochem.* **1965**, *57*, 554 –564.

22) Breithaupt, C.; Strassner, J.; Breitinger, U.; Huber, R.; Macheroux, P.; Schaller, A.; Clausen, T. *Structure* **2001**, *9*, 419– 429.

23) Kohli, R. M.; Massey, V. *J. Biol. Chem.* **1998**, *273*, 32763 – 32770.

24) Toogood, H. S.; Fryszkowska, A.; Hare, V.; Fisher, K.; Roujeinikova, A.; Leys, D.; Gardiner, J. M.; Stephens, G. M.; Scrutton, N. S. *Adv. Synth. Catal.* **2008,** *350*, 2789 – 2803.

25) Brown, B. J.; Hyun, J. W.; Duvvuri, S.; Karplus, P. A.; Massey, V. *J. Biol. Chem.* **2002,** *277*, 2138 –2145.

26) Kohli, R. M.; Massey, V. *J. Biol. Chem.* **1998**, *273*, 32763– 32770.

27) a) Barna, T. M.; Khan, H.; Bruce, N. C.; Barsukov, I.; Scrutton, N. S.; Moody, P. C. *J. Mol. Biol.* **2001,** *310*, 433–447. b) Khan, H.; Barna, T.; Bruce, N. C.; Munro, A. W.; Leys, D.; Scrutton, N. S. *FEBS J.* **2005,** *272*, 4660 –4671.

28) Toogood, H. S.; Gardiner, J. M.; Scrutton, N. S. *ChemCatChem* **2010**, *2*, 892 – 914.

29) Barna, T. M.; Khan, H.; Bruce, N. C.; Barsukov, I.; Scrutton, N. S.; Moody, P. C. *J. Mol. Biol.* **2001,** *310*, 433–447.

30) Breithaupt, C.; Strassner, J.; Breitinger, U.; Huber, R.; Macheroux, P.; Schaller, A.; Clausen, T. *Structure* **2001,** *9*, 419– 429.

31) Kitzing, K.; Fitzpatrick, T. B.; Wilken, C.; Sawa, J.; Bourenkov, G. P.; Macheroux, P.; Clausen, T. *J. Biol. Chem.* **2005,** *280*, 27904 – 27913.

32) Khan, H., Harris, R. J.; Barna, T.; Craig, D. H.; Bruce, N. C.; Munro, A.W.; Moody, P. C.; Scrutton, N. S. *J. Biol. Chem.* **2002,** *277*, 21906 – 21912.

33) a) Corpet, F. *Nucleic Acids Res.* **1988,** *16*, 1088–10890. b) Gramatica, P.; Manitto, P.; Poli, L. *J. Org. Chem.* **1985,** *50*, 4625–4628.

34) Fryszkowska, A.; Toogood, H. S.; Sakuma, M.; Gardiner, J. M.; Stephens, G. M.; S crutton, N. S. *Adv. Synth. Catal.* **2009,** *351*, 2976–2990.

35)a) Hall, M.; Stueckler, C.; Kroutil, W.; Macheroux, P.; Faber, K. *Angew. Chem.* **2007**, *119*, 4008–4011. b) *Angew. Chem. Int. Ed.* **2007,** *46*, 3934 –3937. c) Mueller, N. J.; Stueckler, C.; Hauer, B.; Baudendistel, N.; Housden, H.; Bruce, N. C.; Faber, K. *Adv. Synth. Catal.* **2010,** *352*, 387– 394.

36) Fryszkowska, A.; Fisher, K.; Gardiner, J. M.; Stephens, G. M. *J. Org. Chem.* **2008,** *73*, 4295–4298.

37) Stueckler, C.; Hall, M.; Ehammer, H.; Pointner, E.; Kroutil, W.; Macheroux, P.; Faber K. *Org. Lett*. **2007,** *9*, 5409– 5411.

38) Fryszkowska, A.; Toogood, H. S.; Sakuma, M.; Gardiner, J. M.; Stephens, G. M.; Scrutton, N. S. *Adv. Synth. Catal.* **2009**, *351*, 2976 –2990.

39) a) Fronza, G.; Fuganti, C.; Mendozza, M.; Rigoni, R.; Servia, S.; Zucchi, G. *Pure Appl. Chem.* **1996,** *68*, 2065–2071. b) Takabe, K.; Hiyoshi, H.; Sawada, H.; Tanaka, M.; Miyazaki, A.; Yamada, T.; Katagiri, T.; Yoda, H. *Tetrahedron: Asymmetry* **1992,** *3*, 1399 – 1400. c) Gramatica, P.; Manitto, P.; Monti, D.; Speranza, G. *Tetrahedron* **1987,** *43*, 4481–4486.

40) Hall, M.; Hauer, B.; Stuermer, R.; Kroutil, W.; Faber, K. *Tetrahedron: Asymmetry* **2006,** *17,* 3058–3062.

41) a) Fuganti, C.; Grasselli, P.; Servi, S.; Hoegberg, H. E. *J. Chem. Soc., Perkin Trans. 1* **1988,** *12,* 3061–3065. b) Leuenberger, H. G. W.; Boguth, W.; Barner, R.; Schmid, M.; Z ell, R. *Helv. Chim. Acta* **1979,** *62,* 455–463.

42) Hirata, T.; Shimoda, K.; Gondai, T. *Chem. Lett.* **2000,** *29,* 850–851.

43) Fryszkowska, A.; Toogood, H. S., Sakuma, M.; Gardiner, J. M.; Stephens, G. M.; Scrutton, N. S. *Adv. Synth. Catal.* **2009**, *351,* 2976–2990.

44) Mueller, N. J.; Stueckler, C.; Hauer, B.; Baudendistel, N.; Housden, H.; Bruce, N. C.; Faber, K. *Adv. Synth. Catal.* **2010,** *352,* 387–394.

45) Fryszkowska, A.; Toogood, H. S.; Sakuma, M.; Gardiner, J. M.; Stephens, G. M.; Scrutton, N. S. *Adv. Synth. Catal.* **2009**, *351,* 2976–2990.

46) a) Hall, M.; Stueckler, C.; Kroutil, W.; Macheroux, P.; Faber, K. *Angew. Chem.* **2007,** *119,* 4008–4011. b) *Angew. Chem. Int. Ed.* **2007,** *46,* 3934–3937.

47) a) Bader, J.; Simon, H. *Microbiol.* **1980,** *127,* 279–287. b) Simon, H.; Bader, J.; Gunther, H., Neumann, S., Thanos, J. *Angew. Chem.* **1985**, *97,* 541–555. c) *Angew. Chem. Int. Ed. Engl.* **1985,** *24,* 539–553.

48) a) Stueckler, C.; Reiter, T. C.; Baudendistel, N.; Faber, K. *Tetrahedron,* **2010,** *66,* 663–667. b) Vaz, A. D.; Chakraborty, S.; Massey, V. *Biochemistry*, **1995,** *34,* 4246–4256.

49) Buckman, J.; Miller, S. M. *Biochemistry*, **1998,** *37,* 14326–14336.

50) Stueckler, C.; Reiter, T. C.; Baudendistel, N.; Faber, K. *Tetrahedron,* **2010,** *66,* 663–667.

51) Taglieber, A.; Schulz, F.; Hollmann, F.; Rusek, M.; Reetz, M. T. *ChemBio- Chem,* **2008,** *9,* 565–572.

52) Warburg, O.; Christian, W. *Naturwissenschaften,* **1932**, *20*, 688-696.

53) Haas, E. *Biochem*, **1938**, *298,* 378-390.

54) Theorell, H. **1955.** Nobel Prize Lecture (http://nobelprize.org/nobel_prizes/medicine/laureates/1955/theorell-lecture.pdf).

55) Massey, V.; Strickland, S.; Mayhew, S. G.; Howell, L. G.; Engel, P. C.; Matthews, R. G.; Schuman, M.; Sullivan, P. A**.** *Biochem Biophys Res Commun* **, 1969**. *36,* 891-897.

56) a) Schopfer, L. M.; Massey, V. Old Yellow Enzyme. In *A Study of Enzymes*, **1991.** 247-269. Edited by S. A. Kuby. Cleveland, OH: CRC Press. **b)** Vaz, A. N.; Chakraborty, S.; Massey, V. *Biochemistry,* **1995,** *34,* 4246-4256.

57) Warburg, O.; Christian, W. *Biochem. Z.* **1933,** *263*, 228–229.

58) Madani, N. D.; Malloy, P. J.; Rodriguez-Pombo, P.; Krishnan, A. V.;Feldman, D. *Proc. Natl. Acad. Sci. U.S.A.* **1994,** *91*, 922–926.

59) Terez, B.; Hanan, L. M.; Carlo, P.; Neil, C. B.; Nigel, S. S.; Peter, C.; Moody, E. *The Journal of Biological Chemistry*. ***2002,****277*, 30976-30983.

60) Lim, L.W.; Shamala, N.; Mathews, F.S.; Steenkamp, D.J.; Hamlin, R.; Xuong, N.H. *J. Biol. Chem.***1986**, *261*, 15140-15146.

61) Stott, K.; Saito, K.; Thiele, D. J.; Massey, V. *J. Biol. Chem*. **1993,** *268*, 6097–6106.

62) French, C. E.; Nicklin, S.; Bruce, N. C. *J. Bacteriol.* **1996,** *178*, 6623–6627.

63) Snape, J. R.; Walkley, N. A.; Morby, A. P.; Nicklin, S.; White, G. F. *J. Bacteriol.* **1997,** *179*, 7796–7802.

64) French, C. E.; Bruce, N. C. *Biochem. J.* **1995,** *312*, 671–678.

65) Rohde, B. H.; Schmid, R.; Ullrich, M. S. *J. Bacteriol.* **1999,** *181*, 814–822.

66) Blehert, D. S.; Fox, B. G.; Chambliss, G. H. *J. Bacteriol.* **1999,** *181*, 6254–6263.

67) Miura, K.; Tomioka, Y.; Suzuki, H.; Yonezawa, M.; Hishinuma, T.; Mizugaki, M. *Biol. Pharm. Bull.* **1997,** *20*, 110–112.

68) Fitzpatrick, T. B.; Amrhein, N.; Macheroux, P. *J. Biol. Chem.* **2003** *278*, 19891–19897.

69) a) Toogood, H. S.; Fryszkowska, A.; Hare, V.; Fisher, K.; Roujeinikova, A.; Leys, D.; Gardiner, J. M.; Stephens, G. M.; Scrutton, N. S. *Adv. Synth. Catal.* **2008**, *350*, 2789 – 2803. b) Chaparro-Riggers, J. F. Rogers, T. A.; Vazquez-Figueroa, E.; Polizzi, K. M.; Bommarius, A. S. *Adv. Synth. Catal.* **2007**, 349, 1521 –1531. c) Swiderska, M. A.; Stewart, J. D. *Org. Lett*. **2006**, *8*, 6131 – 6133. d) Hall, M.; Stueckler, C.; Kroutil, W.; Macheroux, P.; Faber, K. *Angew. Chem*. **2007**, *119*, 4008 –4011; *Angew. Chem. Int. Ed*. **2007**, *46*, 3934 –3937.

70) Khan, H.; Harris, R. J.; Barna, T.; Craig, D. H.; Bruce, N. C.; Munro, A. W.; Moody, P. C.; Scrutton, N. S. *J. Biol. Chem.* **2002,** *277*, 21906 – 21912.

71) a) Muller, A.; Hauer, B.; Rosche, B. *J. Mol. Catal. B,* **2006,** *38*, 126 –130. b) Muller, A.; Hauer, B.; Rosche, B. *Biotechnol. Bioeng*. **2007,** *98*, 22 –29.

72) Leuenberger, H. G. W.; Boguth, W.; Widmer, E.; Zell, R. *Helv. Chim. Acta* **1976,** *59*, 1832 –1849.

73) Craig, D. H.; Moody, P. C.; Bruce, N. C.; Scrutton, N. S. *Biochemistry* **1998,** *37*, 7598–7607.

74) Buckman, J.; Miller, S. M. *Biochemistry.* **1998,** *6*, 37, 14326-36.

75) Blehert, D. S.; Fox, B. G.; Chambliss, G. H. *J. Bacteriol*. **1999,** *181*, 6254–6263.

76) a) Miura, K.; Tomioka, Y.; Suzuki, H.; Yonezawa, M.; Hishinuma, T.; Mizugaki, M. *Biol. Pharm. Bull.* **1997,** *20*, 110–112. b) Mizugaki, M.; Unuma, T.; Yamanaka, H. *Chem. Pharm. Bull.* **1979,** *27*, 2334 – 2337. Vaz, A. D.;Chakraborty, S.; Massey, V. *Biochemistry* **1995,** *34*, 4246 –4256.

77) a) Opperman, D. J.: Piater, L. A.; Heerden, E. *J. Bacteriol.* **2008,** *190*, 3076 –3082. b) Opperman, D. J.; Sewell, B. T.; Litthauer, D.; Isupov, M. N.; Littlechild, J. A.; van Heerden, E. *Biochem. Biophys. Res. Commun*. **2010,** *393*, 426 –431.

78) Meah, Y.;Massey, V. *Proc. Natl. Acad. Sci. USA*, **2000**, *97*, 10733 – 10738.

79) Williams, R. E.; Rathbone, D. A.; Scrutton, N. S.; Bruce, N. C. *Appl. Environ. Microbiol.* **2004**, *70*, 3566 –3574.

80) Wada, M.; Yoshizumi, A.; Noda, Y.; Kataoka, M.; Shimizu, S.; Takagi, H.; Nakamori, S. *Appl. Environ. Microbiol.* **2003**, *69*, 933 –937.

81) a) Muller, R.; Sturmer, B.; Hauer, B.; Rosche. *Angew. Chem*. **2007,** *119*, 3380–3382. b) *Angew. Chem. Int. Ed.* **2007,** *46*, 3316 –3318. c) Adalbjçrnsson, B. V.; Toogood, H. S.; Fryszkowska, A.; Pudney, C. R.; Jowitt, T. A.; Leys, D.; Scrutton, N. S. *ChemBioChem.* **2010,** *11*, 197–207.

82) Niino, Y. S.; Chakraborty, S.; Brown, B. J.; Massey, V. *J. Biol. Chem*. **1995**, *270*, 1983 – 1991.

83) Mueller, N. J.; Stueckler, C.; Hall, M.; Macheroux, P.; Faber, K. *Org. Biomol. Chem.* **2009**, *7*, 1115– 1119.

84) Strassner, J.; Furholz, A.; Macheroux, P.; Amrhein, N.; Schaller, A.; *J. Biol. Chem.* **1999,** *274*, 35067–35073.

85) Khan, H.; Barna, T.; Harris, R. J.; Bruce, N. C.; Barsukov, I.; Munro, A. W.; Moody, P. C.; Scrutton, N. S. *J. Biol. Chem*. **2004**, *279*, 30563 – 30572.

86) a) French, C. E.; Nicklin, S.; Bruce, N. C. *J. Bacteriol.* **1996,** *178*, 6623–6627. b) Miseviciene, L.; Anusevicius, Z.; Sarlauskas, J.; Harris, R. J.; Scrutton, N. S.; Cenas, N. *Acta Biochim. Pol.* **2007**, *54*, 379 –385. c) Nivinskas, H.; Sarlauskas, J.; Anusevicius, Z.; Toogood, H. S.; Scrutton, N. S.; Cenas, N.; *FEBS J.* **2008**, *275*, 6192– 6203.d) Dillewijn, P. V.; Wittich, R. M.; Caballero, A.; Ramos, J. L. *Appl. Environ. Microbiol.* **2008**, *74*, 6820 – 6823.

87) Hall, M.; Stueckler, C.; Ehammer, H.; Pointner, E.; Oberdorfer, G.; Gruber, K.; Hauer, B.; Stuermer, R.; Kroutil, W.; Macheroux, P.; Faber, K. *Adv. Synth. Catal.* **2008,** *350*, 411–418.

88) a) Messiha, H. L.; Bruce, N. C.; Sattelle, B. M.; Sutcliffe, M. J.; Munro, A. W.; Scrutton, N. S. *J. Biol. Chem*. **2005**, *280*, 27103– 27110. b) Bougioukou, D. J.; Kille, S.; Taglieber, A.; Reetz, M. T. *Adv. Synth. Catal*. **2009**, *351*, 3287–3305.

89) a) Schaller, F.; Weiler, E. W. *J. Biol. Chem*. **1997**, *272*, 28066 –28072. b) Costa, C. L.; Arruda, P.; Benedetti, C. E. *Plant Mol. Biol.* **2000**, *44*, 61– 71.

90) a) Schweiger, P.; Gross, H.; Wesener, S.; Deppenmeier, U. *Appl. Microbiol. Biotechnol.* **2008**, *80*, 995– 1006. b) Yin, B.; Yang, X.; Wei, G.; Ma, Y.; Wei, D. *Mol. Biotechnol*. **2008**, *38*, 241 –245.

91) a) Brige, A.; van den Hemel, D.; Carpentier, W.; de Smet, L.; van Beeumen, J.J. *Biochem. J.* **2006**, *394*, 335-344. b) van den Hemel, D., Brige, A.; Savvides, S. N.; Beeumen, J. V. *J. Biol.Chem*. **2006**, *281*, 28152–28161.

92) Kosjek, B.; Fleitz, F. J.; Dormer, P. G.; Kuethe, J. T.; Devine, P. N. *Tetrahedron:Asymmetry*, **2008**, *19*, 1403- 1406.

93) a) Swiderska, M.A.; Stewart, J.D. *J. Mol. Catal. B: Enzym.* **2006**, *42*, 52-54. b) Swiderska, M.A.; Stewart, J.D. *Org. Lett.* **2006**, *8*, 6131-6133.

94) Heidelberg, J. F.; Paulsen, I. T.; Nelson, K. E.; Gaidos, E. J.; Nelson, W. C.; Read, T. D.; Eisen, J. A.; Seshadri, R.; Ward, N.; Methe, B. et al. *Nature Biotech*. **2002,** *20*, 1118–1123.

95) The PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC.

96) Breithaupt, C.; Kurzbauer, R.; Schaller, F.; Stintzi, A.; Schaller, A, Huber, R.; Macheroux, P.; Clausen,T. *J Mol. Biol.* **2009,** *392*, 1266–1277.

97) a) Chaparro-Riggers, J. F.; Rogers, T. A.; Vazquez-Figueroa, E.; Polizzi, K. M.; Bommarius, A. S. *Adv. Synth. Catal.* **2007**, *349*, 1521–1531. b) Fryszkowska, A.; Toogood, H. S.; Sakuma, M.; Gardiner, J. M.; Stephens, G. M. N.; Scrutton, S. *Adv. Synth. Catal.* **2009,** *351*, 2976 –2990. C) Vaz, A. D.; Chakraborty, S.; Massey, V.; *Biochemistry.* **1995,** *34*, 4246–4256.

98) Hall, M.; Hauer, B.; Stuermer, R.; Kroutil, W.; Faber, K. *Tetrahedron: Asymmetry.* **2006,** *17*, 3058– 3062.

99) a) Fardelone, L. C.; Augusto, J.; Rodrigues, R.; Moran, P. J. S. *J. Mol. Catal. B.* **2004**, *29*, 41–45. b) Fronza, G. C. Fuganti, S. Serra, *Eur. J. Org. Chem.* **2009,** 6160 – 6171.

100) Kirk, R. E.; Othmer, D. F. *Encyclopedia of Chemical Technology, 14*, John Wiley & Sons, NY, **2005**.

101) Leuenberger, H. G. W.; Boguth, W.; Widmer, E.; Zell, R.; *Helv. Chim. Acta* **1976,** *59*, 1832–1849.

102) a) Kataoka, M.; Kotaka, A.; Thiwthong, R.; Wada, M.; Nakamori, S.; Shimizu, S. *J. Biotechnol.* **2004,** *114*, 1–9. b) Kataoka, M.; Kotaka, A.; Hasegawa, A.; Wada, M.; Yoshizumi, A.; Nakamori, S.; Shimizu, S. *Biosci. Biotechnol. Biochem*. **2002,** *66*, 2651–2657.

103) Bougioukou, D. J.; Kille, S.; Taglieber, A.; Reetz, M. T. *Adv. Synth. Catal.* **2009**, *351*, 3287–3305.

104) Chaparro-Riggers, J. F.; Rogers, T. A.; Vazquez-Figueroa, E.; Polizzi, K. M.; Bommarius, A. S. *Adv. Synth. Catal.* **2007,** *349*, 1521–1531.

105) a) Vaz, A. D.; Chakraborty, S.; Massey, V. *Biochemistry.* **1995,** *34*, 4246 –4256. b) Kataoka, M.; Kotaka, A.; Hasegawa, A.; Wada, M.; Yoshizumi, A.; Nakamori, S.; S himizu, S.; *Biosci. Biotechnol. Biochem*. **2002,** *66*, 2651–2657.

106) Hirata, T.; Takarada, A.; Matsushima, A.; Konodo, Y.; Hamada, H.; *Tetrahedron: Asymmetry*. **2004**, *15*, 15–16.

107) Stuermer, R.; Hauer, B.; Hall, M.; Faber, K. *Curr. Opin. Chem. Biol*. **2007,** *11*, 203–213.

108) a) Hall, M.; Stueckler, C.; Kroutil, W.; Macheroux, P.; Faber, K. *Angew. Chem.* **2007,** *119*, 4008–4011. b) *Angew. Chem. Int. Ed.* **2007,** 46, 3934 –3937.

109) Mueller, N. J.; Stueckler, C.; Hauer, B.; Baudendistel, N.; Housden, H.; Bruce, N. C.; Faber, K. *Adv. Synth. Catal*. **2010,** *352*, 387–394.

110) Eich, E. Solanaceae and convolvulaceae: *Secondary Metabolites Biosynthesis, Chemotaxonomy, Biological and Economic Significance*. Springer, Berlin, **2008**.

111) Takabe, K.; Hiyoshi, H.; Sawada, H.; Tanaka, M.; Miyazaki, A.; Yamada, T.; Katagiri, T.; Yoda, H. *Tetrahedron: Asymmetry.* **1992,** *3*, 1399–1400.

112) Goretti, M.; Ponzoni, C.; Caselli, E.; Marchigiani, E.; Cramarossa, M. R.; Turchetti, B.; Buzzini, P.; Forti, L.; *Enzyme Microb. Technol.* **2009**, *45*, 463–468.

113) Shimoda, K.; Hirata, T. *J. Mol. Catal.* B. **2000**, *8*, 255–264.

114) Padhi, S. K.; Bougioukou, D. J.; Stewart, J. D. *J. Am. Chem. Soc.* **2009,** *131*, 3271 – 3280.

115) Opperman, D. J.; Sewell, B. T.; Litthauer, D.; Isupov, M. N.; Littlechild, J. A.; Heerden, E. *Biochem. Biophys. Res. Commun*. **2010,** *393*, 426–431.

116) a) Knowles, W. S.; *Acc. Chem. Res.* **1983**, *16*, 106–112. b) Tuttle, J. B.; Ouellet, G. G.; MacMillan, D. W. C. *J. Am. Chem. Soc*. **2006,** *128*, 12662–12663. c) Adalbjçrnsson, B. V.; Toogood, H. S.; Fryszkowska, A.; Pudney, C. R.; Jowitt, T. A.; Leys, D.; Scrutton, N. S. *ChemBioChem.* **2010,** *11*, 197–207.

117) Hall, M.; Stueckler, C.; Hauer, B.; Stuermer, R.; Friedrich, T.; Breuer, M.; Kroutil, W.; Faber, K. *Eur. J. Org. Chem*. **2008,** 1511–1516.

118) Opperman, D. J.; Sewell, B. T.; Litthauer, D.; Isupov, M. N.; Littlechild, J. A.; van Heerden, E. *Biochem. Biophys. Res. Commun.* **2010**, *393*, 426–431.

119) Padhi, K. S.; Bougioukou, D. J.; Stewart, J .D. *J. Am. Chem.Soc*, **2009**, *131*, 3271-3280.

120) Ono. N. *The Nitro Group in Organic Synthesis.* Wiley-VCH**,** Weinheim, **2001**.

121) Ohta, H.; Kobayashi, N.; Ozaki, K. *J. Org. Chem.* **1989,** *54*, 1802–1804.

122) Kawai, Y.; Inaba, Y.; Hayashi, M.; Tokitoh, N. *Tetrahedron Lett.* **2001**, *42*, 3367–3368.

123) Mori, A.; Ishiyama, I.; Akita, H.; Suzuki, K.; Mitsuoka, T.; Oishi, T. *Chem. Pharm. Bull.* **1990**, *38*, 3449-3451.

124) a) Kataoka, M.; Kotaka, A.; Thiwthong, R.; Wada, M.; Nakamori, S.; Shimizu, S. *J. Biotechnol.* **2004**, *114*, 1–9. b) Fryszkowska, A.; Fisher, K.; Gardiner, J. M.; Stephens, G. M. *J. Org. Chem.* **2008,** *73*, 4295–4298.

125) Mueller, N. J.; Stueckler, C.; Hauer, B.; Baudendistel, N.; Housden, H.; Bruce, N. C.; Faber, K. *Adv. Synth. Catal*. **2010,** *352*, 387–394.

126) Baeyer, A.; Villiger, V. *Chem. Ber.* **1899**, *32*, 3625–3633.

127) Noyori, R.; Sato, T.; Kobayashi, H. *Bull. Chem. Soc. Jpn.* **1983**, *56*, 2661–2679.

128) Mislow, K.; Brenner, J. *J. Am. Chem. Soc.* **1953**, *75*, 2318–2322.

129) Mihovilovic, M. D.; Rudroff, F.; Grötzl, B. *Curr.Org.Chem.* **2004**, *8*, 1057–1069.

130) Bolm C. in *Peroxide Chemistry* (Ed.: W. Adam), Wiley-VCH, Weinheim, **2000**, 494–510; Strukul, G. *Angew. Chem.* **1998**, *110*, 1256–1267. *Angew. Chem. Int. Ed.* **1998**, *37*, 1198–1209.

131) a) Li, Z.; Van Beilen, J. B.; Duetz, W. A.; Schmid, A.; De Raadt, A.; Griengl, H.; Witholt, B. *Curr. Opin. Chem. Biol.* **2002**, *6*, 136–144. b) Burton, S. G. *Trends Biotechnol.* **2003**, *21*, 543–549.

132) Donoghue, N. A.; Norris, D. B.; Trudgill, P. W. *Eur. J. Biochem.* **1976**, *63*, 175–192.

133) Ryerson, C. C.; Ballou, D. P.; Walsh, C. *Biochemistry.* **1982**, *21*, 2644–2655.

134) Sheng, D.; Ballou, D. P.; Massey, V. *Biochemistry.* **2001**, *40*, 11156-11167.

135) Chandrasekhar, S.; Roy, C. D. *Tetrahedron Lett.* **1987**, *28*, 6371-6372.

136) Deslongchamps, P. *Organic Chemistry Series*, Vol. 1, Pergamon, Oxford, **1983**.

137) Noyori, R.; Sato, T.; Kobayashi, H. *Bull. Chem. Soc. Jpn.* **1983**, *56*, 2661-2679.

138) Kamerbeek, N.M.; Janssen, D.B.; van Berkel, W.J.H.; Fraaije, M.W. *Adv. Synth. Catal.* **2003**, *345*, 667-678.

139) Mihovilovic, M.D.; Müller, B.; Stanetty, P. *Eur. J. Org. Chem.* **2002**, 3711-3730.

140) Mihovilovic, M.D.; *Curr. Org. Chem*. **2006**, *10*, 1265-1287.

141) Cernuchova, P.; Mihovilovic, M.D. *Org. Biomol. Chem.* **2007**, *5*, 1715–1719.

142) Vatsadze, S. Z.; Sviridenkova, N. V.; Manaenkova, M. A.; Semashko, V. S.; Zyk, N. V. *Russian Chemical Bulletin, International Edition*, **2005**, *54*, 9, 2224-2225.

143) Dragutin, F.; Radivoje, V.; Ana Erceg, K.; Grozdana, B.; Vlasta, P.; Damir, K.; Kreimir, M.; Kristina, W.; Draen, V. *Croat. Chem. Acta*, **2003,** *76*, 1, 69–74.

144) Harsha, N.; Borah, R. C.; Boruah, Jagir, S. S. *J. Chem. Research (S),* **1998**, 272-273.

145) Hall, M., Stueckler, C., Kroutil, W., Macheroux, P., Faber, K., *Angew. Chem.* **2007**, *119*, 4008–4011.

146) Hall, M.; Stuekler, C.; Hauer, B.; Stuermer, R.; Friedrich, T.; Breuer, M.; Kroutil, W.; Faber, K. *Eur. J. Org. Chem.* **2008**, 1511-1516.

147) Holz, J.; Schäffner, B.; Zayas, O.; Spannenberg, A.; Bcrnera, A. *Adv. Synth. Catal.* **2008**, *350*, 2533–2543.

148) Yu, C.; Liu, B.; Hu, L. *J. Org. Chem*. **2001**, *66*, 5413–5418.

149) Cornelia, F.; Eimear, M. F.; Stephen, J. C. *J. Org. Chem.* **2004,** *69*, 6496-6499.

150) Aggarwal, V. K.; Emme, I.; Fulford, S. Y. *J. Org. Chem*, **2003,** *68*, 3, 692-700.

151) Isamir, M.; Alexander, E. A.; Joseph, D. E.; Albert, J. N.; Jun, W.; Amy, R. H. *Org. Lett.,* **2003,** *5*, 399-402.

152) Waldemar, A.; Peter, G.; Hans-Ulrich, H.; Chantu, R. S. *J. Org. Chem.* **2000,** *65*, 4919-4922.

153) Xu, X.; Wang, C.; Zhou, Z.; Tang, X.; He, Z.; Tang, C. *Eur. J. Org. Chem.* **2007**, 4487–4491.

154) a) Hart, D. J.; Kanai, K-I.; Thomas, D. G.; Y, T-K. *J. Org. Chem*. **1983**, *48*, 289-294. b) Vidal, J.; Damaestoy, S.; Guy, L.; Hannachi, J-C.; Aubry, A.; Collet, A. *Chem. Eur. J.* **1997**, *3*, 1691-1709.

155) Vidal, J.; Damaestoy, S.; Guy, L.; Hannachi, J-C.; Aubry, A.; Collet, A. *Chem. Eur. J.* **1997**, *3*, 1691-1709.

156) Kupfer, R.; Meier, S.; Wurthwein, E. U. *Synthesis.* **1984,** 688-690.

157) Yamamoto, K.; Takagi, M.; Tsiji, J. *Bull.Chem. Soc. Jpn.* **1988**, *61*, 319-321.

158) Lee, K. Y.; Kim, T. H.; Kim J. N. *Bull. Korean Chem. Soc*. **2004**, *25*, 12.

159) Buchholz, R.; Martin, H.; Hoffmann, R. *Helv. Chim. Acta.* **1991,** 74, 1213-1220.

160) Gennari, C.; Colombo, L.; Bertolini, G.; Schimperna, G. *J. Org. Chem.* **1987**,52, 2754-2760.

161) Takagi, M.; Yamamoto, K. *Tetrahedron.* **1991,** *41*, 42, 8869-8882.

162) Mihovilovic, M. D.; Rudroff, F.; Grotzl, B.; Kapitan, P.; Snajdrova, R.; Rydz, J.; Mach, R. *Angew. Chem. Int. Ed.* **2004**, *44*, 3609–3613.

163) Alphand, V.; Furstoss, R. *Tetrahedron Asymmetry*. **1992**, *3*, 379–382.

164) Salaun, J.; Karkour, B.; Ollivier, J. *Tetrahedron*, **1989,** 45, 10, 3151-3162.

165) Maximiliano, S.; Susana, A. Z. *Tetrahedron: Asymmetry*, **2010,** *21*, 5, 535-539.

166) Cornelia, F.; Eimear, M. F.; Stephen, J. C. *J. Org. Chem.*, **2004,** *69*, 19, 6496-6499.

167) Jeong, Y.; Ryu, J. *J. Org. Chem.*, **2010**, *75*, 4183–4191.

168) Martnez, I.; Andrews, A. E.; Emch, J. D.; Ndakala, A. J.; Wang, J.; Howell, A. R. *Org. Lett.*, **2003**, *5*, 4, 399-402.

169) Ka, Y. L.; Chang, G. L.; Jae, N. K. *Tetrahedron Letters.* **2003**, *44*, 1231–1234.

170) Shi, M.; Xu, Y.; Shi, Y. *Chem. Eur. J.* **2005,** *11*, 1794–1802.

171) Misael, F.; Luciano, F.; Marcus, M. S. *J. Braz. Chem. Soc.* **2009**, *20*, 3, 564-568.

172) Gennari, C.; Colombo, L.; Bertolini, G.; Schimperna, G. *J. Org. Chem.* **1987**, 52, 2754-2760.

173) Miguel, C.; Juan.; Eva, F.; Florenci, G.; Alberto, J. M. *Tetrahedron: Asymmetry*. **2000**, *11*, 3211-3220.

174) Kusakabe, M.; Sato, F. *J. Org.* Chem. **1989,** *54*, 3486-3487.

175) Koga, K.; Yamada, S. *Chemical & Pharmaceutical Bulletin*, **1972**, *20*, 3, 526-388.

176) Jose, L. V.; Dolores, B.; Esther, D.; Monica, R.; Luisa, C. *J. Org. Chem.* **2000,** *65,* 3754-3760.

177) Takagi, M.; Yamamoto, K. *Tetrahedron.* **1991,** *41,* 42, 8869-8882.

178) Chen, Y. W.; Shao, S.; Li. T.; Li. Y. *Synthesis,* **1992,** 1061-1062.

179) Kei, S.; Naoji, K.; Hiroki, H.; Shin-ya, Y.; Toshifumi, H. *Bull. Chem. Soc. Jpn.* **2004,** *77,* 2269–2272.

List of Publications from this Thesis

Journal Publications

1 **Naseem Iqbal** and Marko D. Mihovilovic, Novel One Pot Redox Reactions of Carvones, ***Chemsus Chem,*** 2010 (Drafted)

2 **Naseem Iqbal** and Marko D. Mihovilovic, A study on asymmetric bioreduction of activated double bond using *Shewanella* Yellow Enzyme (SYE), ***Green Chemistry***, 2010 (Drafted)

3 Dario A. Bianchi, **Naseem Iqbal,** Florian Rudroff, Marko D. Mihovilovic, Enantio complementary Access to Carba-Analogs of Tetrahydrofuran Natural Products and Carba-C-Nucleosides Derivatives by Recombinant Baeyer-Villiger Monooxygenases, ***Synlett***, (Drafted).

4 **Naseem Iqbal** and Marko D. Mihovilovic, *Syn*-Selective Bioreduction of Baylis Hillman Adducts, Approach towards β-Amino acids (In process)

Conference Presentations

1 **Naseem Iqbal,** M. D. Mihovilovic. **"**Novel One Pot Redox Biocatalysis of Carvones using Ene-reductases and BVMOs" ***3rd EuCheMS Chemistry Congress*** *2010,* 29 August-2 September, Nürnberg, Germany.

2 **Naseem Iqbal,** M. D. Mihovilovic. "Redox Cascade Reactions of Carvones using EREDs and BVMOs" ***XXIVth European Colloquium on Heterocyclic Chemistry*** 23-27 August, 2010, Vienna, Austria.

3 M. D. Mihovilovic, **Naseem Iqbal.** "Novel Redox Biocatalysis of Terpene using Ene-reductases and BVMOs", ***Gordon Research Conferences, Biocatalysis*** 11-16 July, 2010, Smithfield, RI, USA.

4 **Naseem Iqbal,** M. D. Mihovilovic. "Biocatalytic Olefin Reduction in Asymmetric Synthesis", ***13th Austrian Chemistry Days*** 24-27 August, 2009, Vienna University of Technology, Vienna, Austria.

5 **Naseem Iqbal,** M. D. Mihovilovic. "Stereoselective Bioreduction of Activated Alkenes with *Shewanella* Yellow Enzyme (SYE)", ***Tenth Tetrahedron Symposium*** 23-26 June 2009, Paris, France.

6 **Naseem Iqbal,** M. D. Mihovilovic. "Olefinic Bioreduction", ***Junior Scientist Conference*** 16-18 November, 2008, Vienna University of Technology, Vienna, Austria.

Curriculum Vitae

M.Phil Naseem IQBAL

Institute of Applied Synthetic Chemistry (IAS),
Vienna University of Technology
Getreidemarkt 9/163 A-1060, Vienna, Austria,.
Tel: Off: +43 158801154, Mobile: +43 6502649329
E mail: niqbal@ioc.tuwien.ac.at, gunjial@gmail.com

PERSONAL DATA

- Date of Birth: 15.05.1981
- Place of Birth: Kushab, Pakistan
- Nationality: Pakistani
- Martital status: Married

EDUCATION

- **PhD Chemistry** **Nov 2007- Jan 2011**

  Institiue of Applied Synthetic Chemistry
  Vienna University of Technology, Austria
  Supervisor: Prof. Marko D. Mihovilovic
  Dissertation: Applications of Ene-Reductases in Chiral Synthesis

- **Master in Philosphy of Chemistry (M.Phil)** **2004- 2006**

  Department of Chemistry
  Quaid-i-Azam University Islamabad, Pakistan.
  Advisor: Prof. Dr. Javed Zahdi
  Dissertation: Asymmetric Induction through Metalation of Chiral Oxathioacetals and Diathioacetals

- **Master of Science (M. Sc Chemistry)** **2001- 2003**

  Department of Chemistry, (Govt. College Sarghoda)
  Punjab University Lahore, Pakistan.
  Oganic Chemistry as been the main field of specialization for Master of Science studies

- **Bachelor of Science (B. Sc)** **1999- 2001**

  Govt. College Sarghoda
  Punjab University Lahore, Pakistan.
  Chemistry, Botany, Zoology were major subjects during Bachelor of Science studies

- **Higher Seconday School Certificate (HSSC)** **1997- 1999**

  Borad of Intermediate and Secondary Education
  Sarghoda, Pakistan.
  Pre-Medical Studies with Chemistry, Physics, Biology and English

- **Seconday School Certificate (SSC)** **1995- 1997**

  Borad of Intermediate and Secondary Education
  Sarghoda, Pakistan.
  General Sciences subjects with Chemistry, Physics, Biology, Mathematics and English

RESEARCH INTRESTS

- Biocatalysis (Green Chemistry)
- Synthetic Chemistry
- Nanobiotechnology
- Analytical Techniques in Chemistry

PROFESSIONAL MEMBERSHIPS

- Member Austrian Chemical Society
- Life Member Pakistan Chemical Society

LANGUAGES

- Urdu (native)
- English (fluent)
- German (basic)

HONOURS AND AWARDS

- **PhD Overseas Scholarship** for PhD Chemistry in Austria, Higher Education Comission, Islamabad, Pakistan (Nov 2007).
- **Merit position and Certificate** M.Phil chemistry, at Quaid-i-Azam University, Islamabad, Pakistan (June 2006).
- Nominated for **Ist meeting of Nobel Laureates with young Pakistani Scientist**, representing Quaid-i-Azam University, Islamabad Pakistan (March 2006).

INTERNATIONAL JOURNAL PUBLICATIONS

1 M. Hucı´k, P. Gemeiner, V. Sˇtefuca, Alica V. **Naseem Iqbal,** Marko Mihovilovic, Encapsulation of recombinant E. coli expressing cyclopentanone monooxygenase in polyelectrolyte complex capsules for Baeyer–Villiger biooxidation of 8-oxabicyclo[3.2.1]oct-6-en-3-one, *Biotechnol Lett*, **2010,** 32, 5, 675-680.

2 Javid H. Zaidi, **Naseem Iqbal**, Khalid. M. Khan, M. Arfan Synthesis of Benzyl chloromethyl ether in situ and its use for the Protection and Deprotection of Bifuctional Hydroxyl Compounds. *Letters in Org.Chem*. **2008**, 5, 125-127.

3 Javid H. Zaidi, **Naseem Iqbal**, Asymmetric Induction through Metalation of Chiral Oxathioacetals and Dithioacetals, *Synn. Comm.* **2007**, 37, (17), 2835-2845.

4 **Naseem Iqbal** and Marko D. Mihovilovic, Novel One Pot Redox Reactions of Carvones, *Chemsus Chem,* **2010** (Drafted)

5 **Naseem Iqbal** and Marko D. Mihovilovic, A study on asymmetric bioreduction of activated double bond using *Shewanella* Yellow Enzyme (SYE), *Green Chemistry*, **2010** (Drafted)

6 Dario A. Bianchi, **Naseem Iqbal,** Florian Rudroff, Marko D. Mihovilovic, Enantio complementary Access to Carba-Analogs of Tetrahydrofuran Natural Products and Carba-C-Nucleosides Derivatives by Recombinant Baeyer-Villiger Monooxygenases, *Synlett*, **2010** (Drafted).

7 **Naseem Iqbal** and Marko D. Mihovilovic, *Syn*-Selective Bioreduction of Baylis Hillman Adducts, Approach towards β-Amino acids **2010** (In process)

INTERNATIONAL CONFERENCES

1 **Naseem Iqbal,** M. D. Mihovilovic. **"**Novel One Pot Redox Biocatalysis of Terpene using Ene-reductases and BVMOs" ***3rd EuCheMS Chemistry Congress*** *2010,* 29 August-2 September, Nürnberg, Germany.

2 **Naseem Iqbal,** M. D. Mihovilovic. "Redox Cascade Reactions of Carvones using EREDs and BVMOs" ***XXIVth European Colloquium on Heterocyclic Chemistry*** 23-27 August, 2010, Vienna, Austria.

3 M. D. Mihovilovic, **Naseem Iqbal.** "Novel Redox Biocatalysis of Terpene using Ene-reductases and BVMOs", ***Gordon Research Conferences, Biocatalysis*** 11-16 July, 2010, Smithfield, RI, USA.

4 **Naseem Iqbal,** M. D. Mihovilovic. "Biocatalytic Olefin Reduction in Asymmetric Synthesis", ***13th Austrian Chemistry Days*** 24-27 August, 2009, Vienna University of Technology, Vienna, Austria.

5 **Naseem Iqbal,** M. D. Mihovilovic. "Stereoselective Bioreduction of Activated Alkenes with *Shewanella* Yellow Enzyme (SYE)", ***Tenth Tetrahedron Symposium*** 23-26 June 2009, Paris, France.

6 **Naseem Iqbal,** M. D. Mihovilovic. "Olefinic Bioreduction", ***Junior Scientist Conference*** 16-18 November, 2008, Vienna University of Technology, Vienna, Austria.

7 **Naseem Iqbal,** J. H. Zaidi. "Synthesis of Dithioacetals and Oxathioacetals with Chiral Auxiliaries and Asymmetric Induction through Metalation of Chiral Oxathioacetals and Dithioacetals" ***International Chemistry Conference*** 1-3 Nov 2007, Lahore College for Women University, Lahore Pakistan.

8 **Naseem Iqbal,** J. H. Zaidi. "Asymmetric Induction through Metalation of Chiral Oxathioacetals and Dithioacetals", 7th ***International &17th National Chemistry Conference*** 26-28 Feburary 2007, Chemistry Department DIK University Pakistan.

9 **Naseem Iqbal,** J. H. Zaidi, **"**Synthesis of Dithioacetals and Oxathioacetals with Chiral Auxiliaries", ***6th International &16th National Chemistry Conference*** 6-8 April 2006, Chemistry Department B.Z.U Multan Pakistan.