D I S S E R T A T I O N

Recombinant Whole-Cell Mediated Baeyer-Villiger Oxidation – From Parallel Mini-Scale Screening to Fermenter Up-Scaling for Natural Product Synthesis

ausgeführt zum Zwecke der Erlangung des akademischen Grades eines Doktors der technischen Wissenschaften unter der Leitung von

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Gewidmet der großen Liebe meines Lebens

Danke mein Wuschl, dass es Dich gibt.

Don’t argue with an idiot,
First they drag you down to their level,
Then they beat you with experience,…

First of all I would like to thank my parents Brigitte and Ernst. They supported me in every part of my life and gave me the love and strength to achieve all my aims. The same is true, since we met for the first time, for the Love of my life Sandra (Wuschl). She is the calming influence in our relationship, she is the most important person in my life and I am grateful to share my greatest moments and hopefully the rest of my life with her. In the final stage of the work she gave me some valuable moments to make me forget the pile of the work.

Then I would like to thank my grandparents Amo and Sweety, who are a very important part of my life. Since my grandfather had to cancel his study in chemistry due to the outbreak of world war II, it was his greatest wish to be alive when I am going to finish my study. He is the part of my family who bears the “right” chemistry genes and let it jump to my DNA. Sweety, this is my present for you. I am proud to be your grandson.

My special thanks belong to my supervisor Prof. Marko Mihovilovic, who almost accompanied me my whole time at the university. He is responsible, that I started to study organic chemistry and therefore I would like to thank him, because this was one of my most important and by far best decisions in my life. He always encouraged me to try new methods, was always there to give valuable advice when it was needed, gave me opportunities to go to several conferences and helped me to gain a foothold on the international parquet of the scientific community. I also enjoyed all our activities beside the science, like our annual skiing trip, the interesting discussions in the “SEM III” or the battles on the tennis court (basketball field, table-tennis table…). It is an honour for me to be your student, and hope that the future will be like the past was. THANK YOU.

In addition I want to thank Prof. Peter Stanetty who showed great interest in the progress of my work all time and was never short of useful suggestions and advise. I also enjoyed his ambitions on the tennis court and I am looking forward to upcoming matches.
A special thank goes to my two best friends, Lena and Kathrin, who let me forget the stress of my work and continue to have a sympathetic ear for my problems. Time is changing fast, but both of you will always keep a special part in my heart.

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I would like to thank Dr. Veronique Alphand for welcoming me in her research group and giving me a good, interesting and productive time.

Legend

All compounds prepared or used as starting materials in this thesis are numbered in bold Arabic numbers. Compounds unknown to the literature are additionally underlined. General structures and compounds presented as literature examples are numbered in bold Roman numbers.
Literature citations are indicated by superscript Arabic numbers.

ABSTRACT

Biocatalysis offers a very cost efficient and environment-friendly process for the preparation of fine chemicals. Especially during the last decades the synthesis of enantiomerically pure compounds increased dramatically. The introduction of enzymes as such catalysts into synthetic organic chemistry made possible many new highly enantioselective transformations.
Baeyer-Villiger oxidation using biocatalysts represents a powerful methodology for the one-step asymmetric synthesis of chiral lactones.

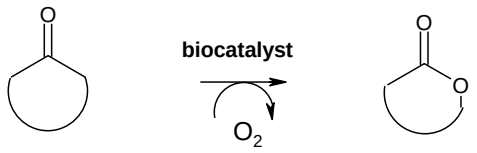


Figure 1 Microbial Baeyer-Villiger oxidation.

During the last years we obtained access to a large number of different Baeyer-Villiger monooxygenases (BVMOs) with so far unknown biocatalytic properties. We also synthesized a large library of structurally diverse ketones (prochiral and racemic) to be investigated in biooxidation reactions. Consequently, the development of an efficient screening methodology based on microscaled whole-cell biotransformations became mandatory. The screening protocol was designed to provide data for conversion and stereoselectivity (determined by chiral GC analysis) in a time efficient manner. This allowed the rapid evaluation and characterization of BVMOs in whole-cell biotransformations with recombinant overexpression systems.

The fermentation optimization of whole-cell mediated Baeyer-Villiger oxidation was the next logical step upon identification of a promising biocatalyst-substrate couple of interest for future exploitation. A “two-in-one” in situ “substrate feeding and product removal” concept (SFPR) using an adsorbent resin and a recombinant *E.coli* strain overexpressing cyclopentanone monooxygenase (CPMO) was elaborated. Due to the limitations of substrate concentration and high toxicity of both starting material and product this SFPR methodology improved prior fermentation results significantly. Several critical parameters of the fermentation process were optimized and multi-gram quantities of chiral lactones became available for subsequent synthetic elaboration.

Application of the biooxidation products obtained with the optimized fermentation protocol was outlined in a short and efficient synthetic approach towards various C-nucleosides. D-(+)-Showdomycin, a natural C-nucleoside, which was first isolated from *Streptomyces showdoensis*, was chosen as a model substrate to demonstrate the applicability of enantioselective biooxidation reactions in natural product synthesis. Within this shortest synthetic sequence to D-(+)-showdomycin reported so far starting from non-chiral precursors, also the absolute configuration for chiral lactones obtained by enzymatic BV-oxidation of prochiral bicycloketones could be established.

In another series a library of BVMOs providing access to enantiocomplementary lactones was utilized in the synthesis of various chiral butyrolactones as precursor for a diversity of lignans. The complete strategy of this thesis is outlined in Figure 2 and the major goals of this project were as follows:

- Development of a rapid screening system for substrate profiling of BVMOs
- Fermentation optimization of BVMO mediated whole-cell biotransformations
- Application of BVMOs in novel synthetic approached to bioactive compounds

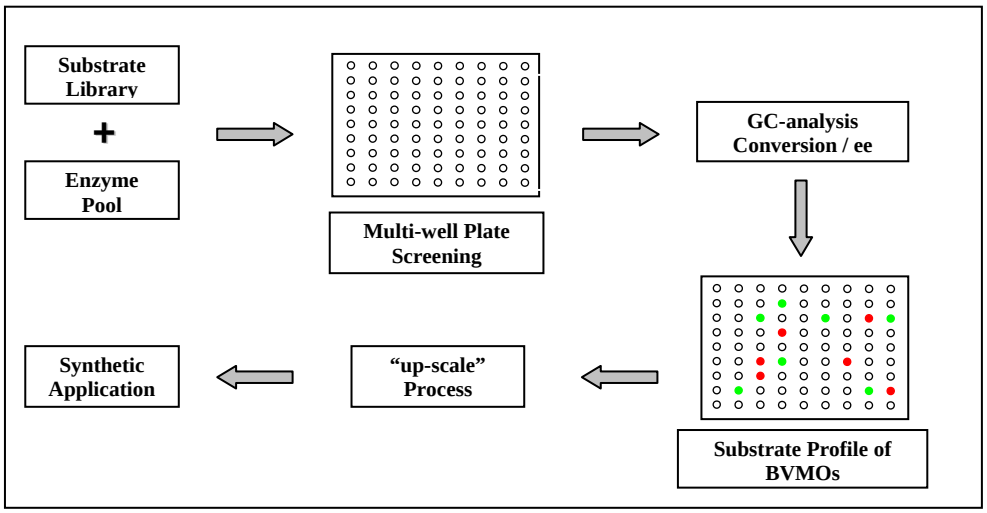
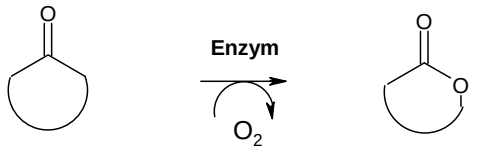


Figure 2 Complete strategy of this thesis.

Consequently, this thesis spans from biocatalyst assignment within screening protocols to up-scaling of the process to preparative quantities to, ultimately, applying this biotransformation in the synthesis of natural compounds.

DEUTSCHE KURZFASSUNG

In den letzten Jahren hat die Synthese von enantiomeren reinen Verbindungen stark zugenommen. Die Einführung von Enzymen als Katalysatoren in der organischen Synthese, hat es ermöglicht unterschiedliche, aber sehr effiziente und neue enantioselektive Transformationen durch zu führen. Die Baeyer-Villiger Oxidation, benannt nach Adolf von Baeyer und Victor Villiger, repräsentiert eine Reaktion, bei der Ketone durch Insertion von molekularem Sauerstoff in die entsprechenden Ester umgewandelt werden. Die Möglichkeit eines C-C Bindungsbruches einer organischen Verbindung und Einführung einer neuen und vielseitig modifizierbaren funktionellen Gruppe macht diese Reaktion zu einem sehr wertvollen Werkzeug für die organische Synthese.



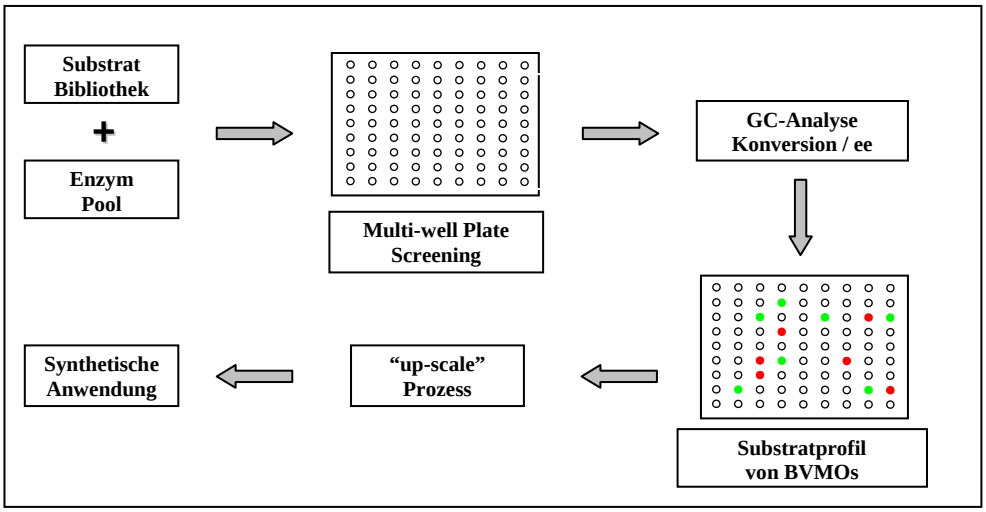
Schema 1 Mikrobielle Baeyer-Villiger Oxidation

In den letzten Jahren haben wir Zugang zu einer Reihe unterschiedlicher Baeyer-Villiger Monooxygenasen (BVMOs) mit bis dato unbekannten biokatalytischen Eigenschaften bekommen. Des Weiteren haben wir eine Substanzbibliothek von strukturell unterschiedlichen Ketonen (prochiral und racemisch) synthetisiert um diese in den bereits beschriebenen Biooxidationen zu untersuchen. Daher wurde die Entwicklung eines effizienten Screening-Verfahrens, basierend auf micro-scaled „ganz-Zell“ Biotransformationen immer wichtiger. Das Screening Verfahren war konzipiert für die Generierung von Daten basierend auf der Konversion und Stereoselektivität (gemessen mittels GC-Analyse) in einem möglichst kurzen Zeitraum. Das ermöglichte uns die rasche Evaluierung und Charakterisierung von BVMOs in „ganz-Zell“ Biotransformationen mit rekombinanten Überexpressionssystemen.

Nach der Entwicklung dieses Screening-Verfahrens, war die Optimierung des Fermentationsprozesses für „ganz-Zell“ unterstützte Baeyer-Villiger Oxidation der nächste logische Schritt. Hierbei wurde ein spezielles Verfahren, das so genannte „SFPR“ (2 in 1 in situ „Substrate Feeding and Product Removal“) Konzept, welches auf der Verwendung von einem Adsorbent und einem rekombinanten *E.coli* Stamm, der cyclopentanone monooxygenase überexpremiert basiert. Mittels dieses Verfahrens konnten schwerwiegende Probleme der „ganz-Zell“ Fermentation, wie die Limitierung durch die Konzentration des Substrates oder die Toxizität der Reaktionspartner, im Gegensatz zu früheren Ergebnissen deutlich entschärft werden, was zu deutlich besseren Ergebnissen führte. Somit konnten wir die Synthese von chiralen Lactonen im Gramm-Maßstab realisieren und diese zugänglich für weitere synthetische Applikationen machen.

Durch die deutliche Effizienzsteigerung des Fermentationsprozesses konnten wir die Anwendbarkeit der mikrobiellen BV-Oxidation an Hand eines konkreten Beispieles zeigen. Zu diesem Zwecke wurde eine neue, und bis dato kürzeste Synthesesequenz für die Herstellung von D-(+)-Showdomycin etabliert. Showdomycin ist ein sogenanntes C-Nucleosid, welches das erste Mal in *Streptomyces showdoensis* gefunden wurde, und zeichnet sich durch seine Bedeutung in der Krebs und HIV-Forschung aus. Durch die Entwicklung dieser Synthesesequenz konnte auch die Absolutkonfiguration des enzymatischen Bizyclischen BV-Oxidations Produktes bestimmt werden.
Ein weiterer Teil der Arbeit befasste sich mit einer Bibliothek von BVMOs welche Zugang zu enantiokomplementären Lactonen ermöglichte. Diese wurden für die Synthese verschiedenster Butyrolactone verwendet, welche Vorstufen zu einer Anzahl von Lignanen repräsentieren.
Das vollständige Konzept (Schema 2) dieser Arbeit und deren Hauptziele sind folgend zusammengefasst:

- Entwicklung eines schnellen und effizienten Screening – Verfahrens
- Fermentationsoptimierung von Baeyer-Villiger unterstützten „ganz-Zell“ Biotransformationen
- Anwendung von BVMOs in neuen Synthesestrategien für die Herstellung von bioaktiven Verbindungen.



Schema 2 Das komplette Konzept dieser Arbeit.

Mit dieser umfassenden Arbeit konnte der Bogen von der Charakterisierung und Bestimmung von Biokatalysatoren mittels eines Screening-Verfahrens, bis hin zur Optimierung des Fermentationsprozesses für industrielle Anwendungen gespannt werden. Weiters konnten wir die Bedeutung der mikrobiellen Baeyer-Villiger Oxidation für die organische Chemie anhand der Entwicklung neuer Synthesestrategien für die Herstellung von Naturstoffen zeigen.

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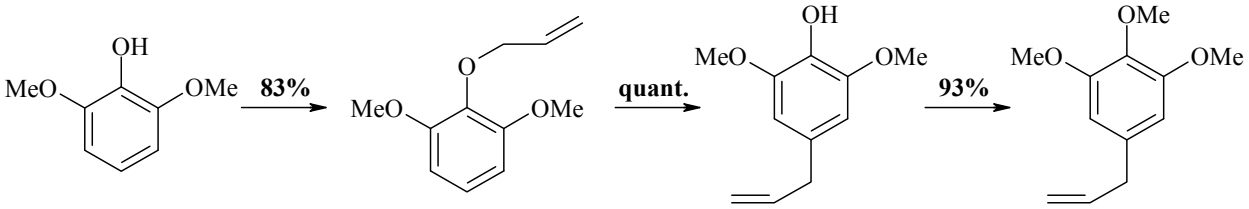
General Schemes and Substrate Library



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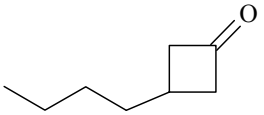

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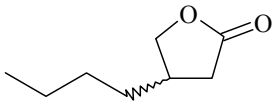

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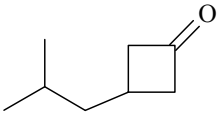

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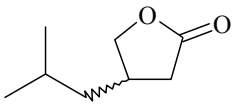

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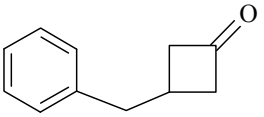

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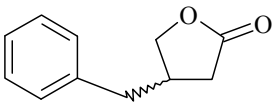

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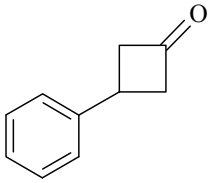

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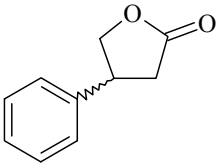

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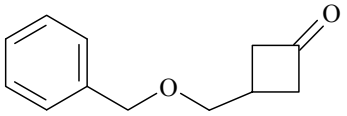

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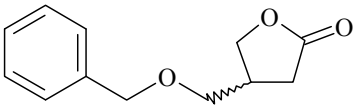

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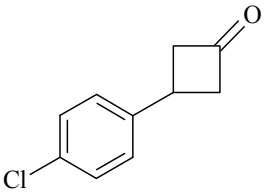

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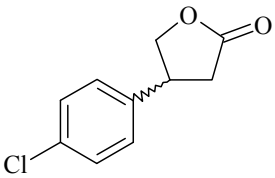

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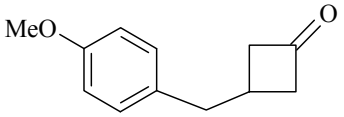

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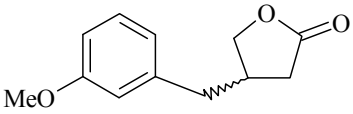

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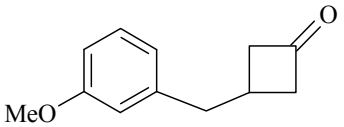

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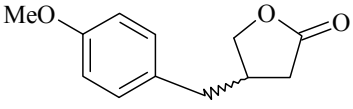

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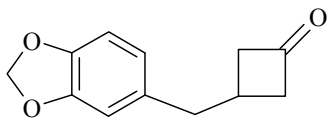

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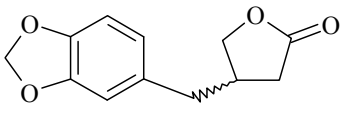

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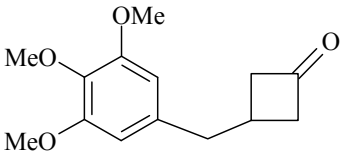

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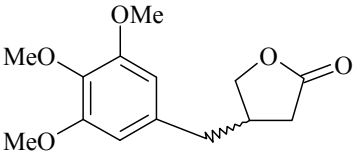

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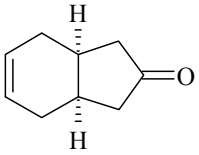

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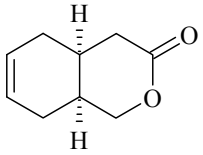

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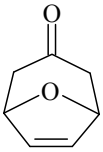

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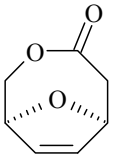

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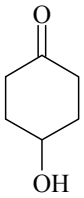

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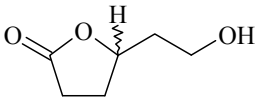

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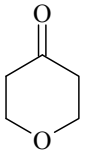

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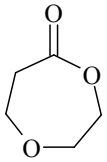

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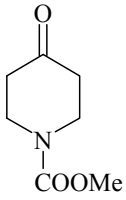

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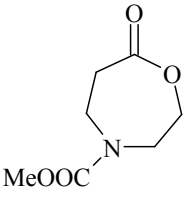

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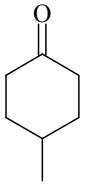

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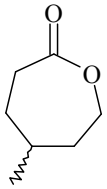

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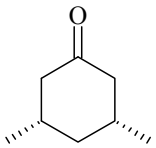

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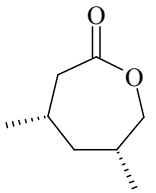

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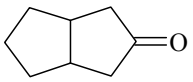

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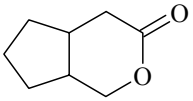

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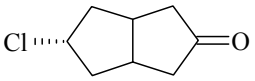

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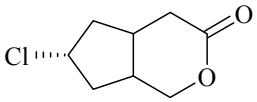

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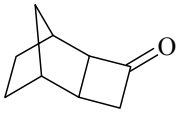

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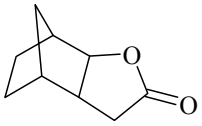

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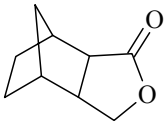
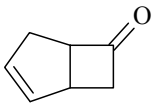


24b "an"



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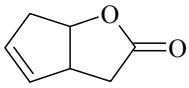
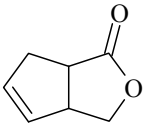


25b "n"



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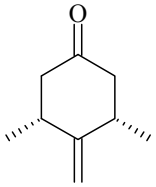

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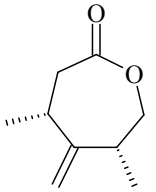
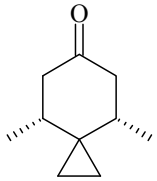


26b



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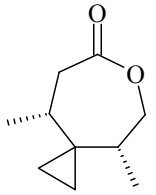

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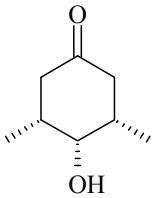

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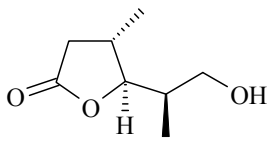

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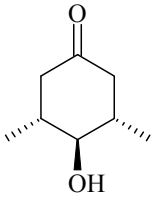

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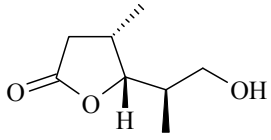

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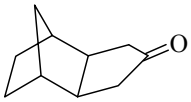

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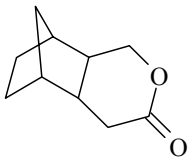

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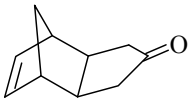

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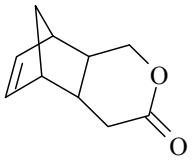

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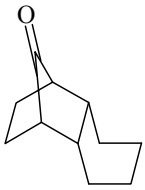

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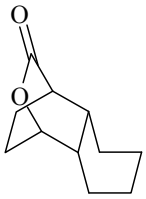

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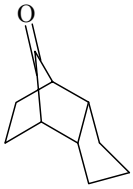

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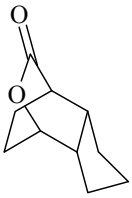

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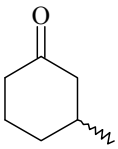
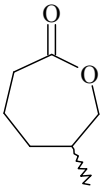
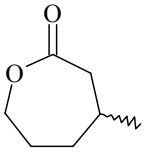


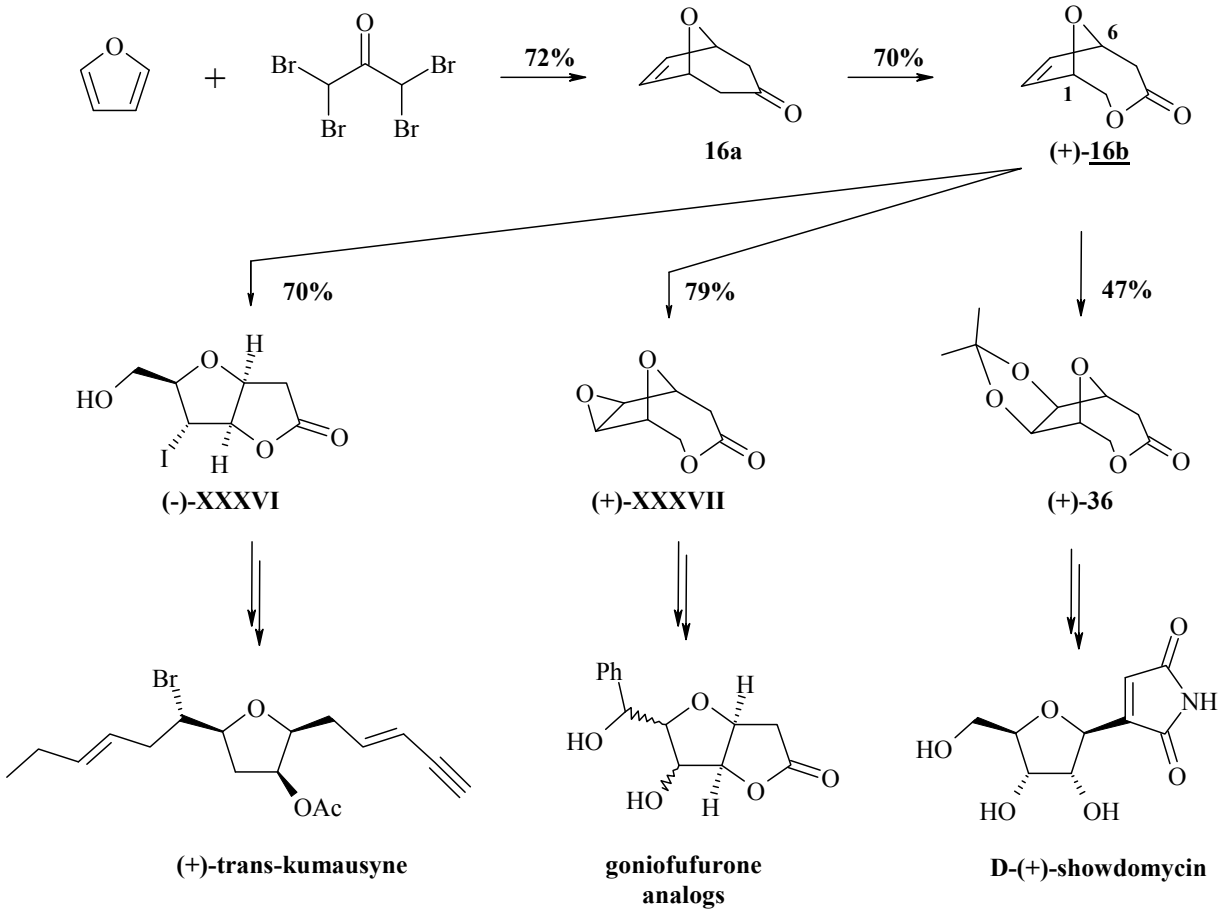
34a

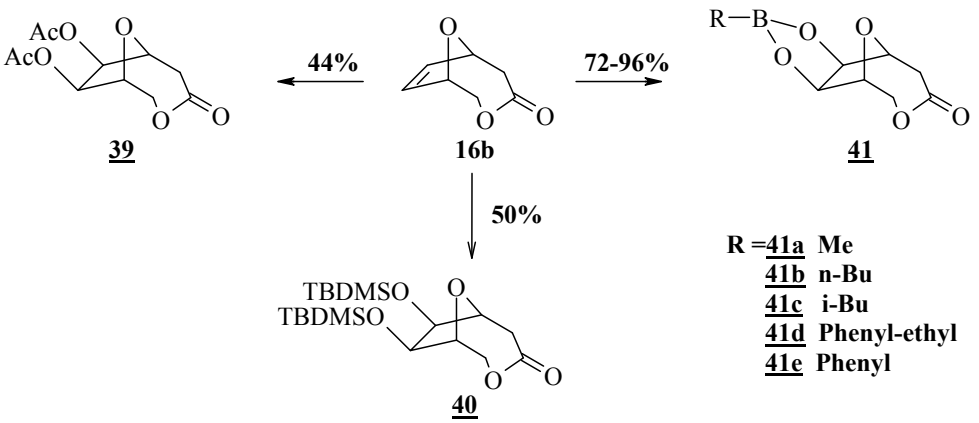


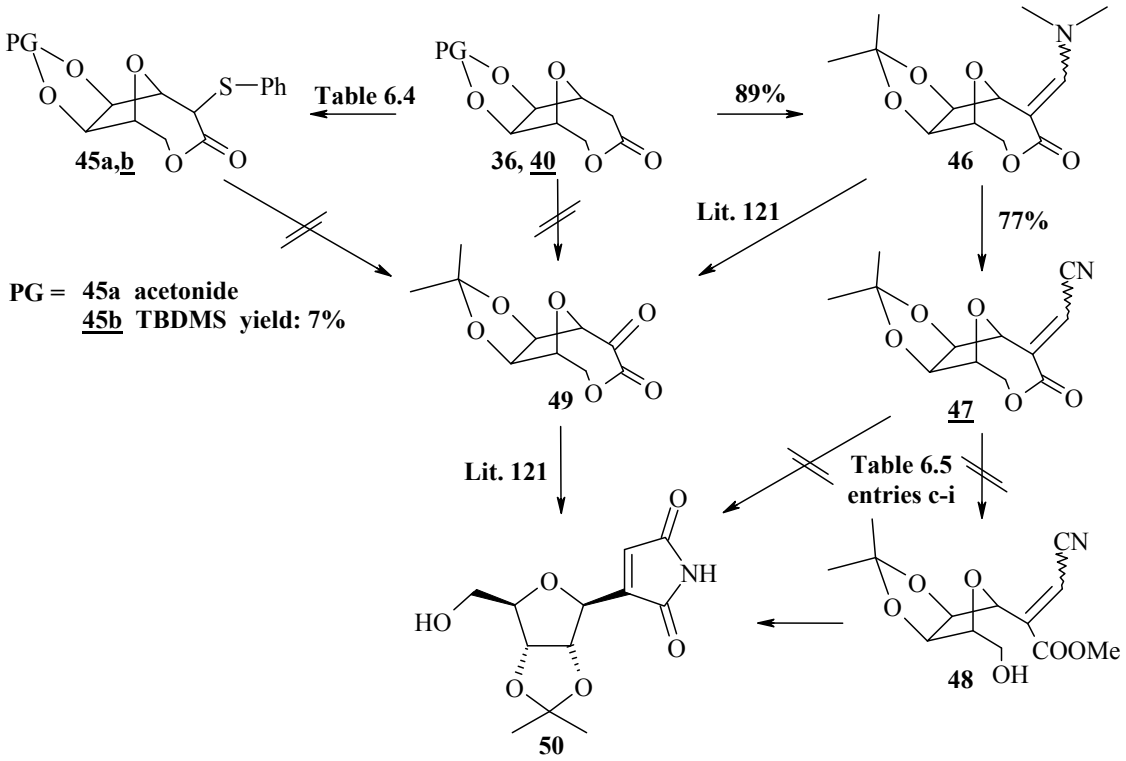
34b "proximal"



34b "distal"


72%
70%
16a
(+)-16b
70%
79%
47%
(-)-XXXVI
(+)-XXXVII
(+)-36
(+)-trans-kumausyne
goniofufurone analogs
D-(+)-showdomycin


44%
72-96%
39
16b
41
50%
40
R = 41a Me
41b n-Bu
41c i-Bu
41d Phenyl-ethyl
41e Phenyl


PG
S—Ph
45a,b
Table 6.4
36, 40
89%
46
Lit. 121
77%
PG = 45a acetonide
45b TBDMS yield: 7%
49
47
Lit. 121
Table 6.5
entries c-i
NH
HO
50
CN
COOMe
OH
48

Introduction

1.1 General Aspects

Efficient, selective and specific insertion of oxygen, especially of a single atom, into an organic, compound is a reaction that is difficult to perform by chemical means. Although some reagents have been developed that are able to catalyze specific oxygenation reactions. One of the most appealing oxygen-insertion reactions with respect to synthetic application is the Baeyer-Villiger oxidation, which was established in 1899 and was named by its inventors Adolf Baeyer and Viktor Villiger. This reaction represents a powerful methodology in the synthesis of esters and lactones by breaking carbon-carbon bonds in an oxygen insertion process.[1] Within their initial publication, stereochemical features of the reaction were described in details. In the following decades, a comprehensive understanding of the mechanism and prediction of migration has been obtained.[2,3]

The mechanism of this transformation was initially proposed by Criegee[4] (Figure 1.1) and was described *via* a two step process. In the first step a nucleophilic attack of a carbonyl group (**I**) by a peroxo species (**II**) takes place and leads to the formation of the tetrahedral "Criegee-intermediate" (**III**). Subsequently, this intermediate (**IV**) undergoes rearrangement to the corresponding ester or lactone (**V**).

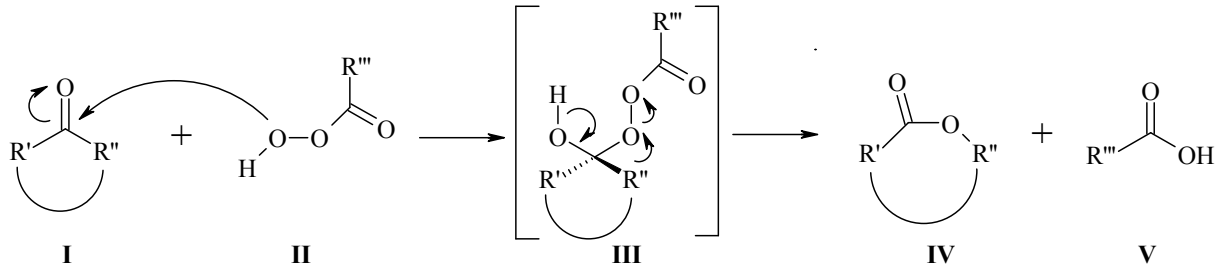


Figure 1.1 Peracid mediated Baeyer-Villiger oxidation.

In case of α-substitution of ketone (**I**), the more nucleophilic residue tends to migrate towards the peroxo oxygen with strict retention of the configuration at the migrating carbon center.[5] During the last century the required properties for a successful and predictable behaviour of the migrating centre within the Baeyer-Villiger oxidation have been particularly investigated.[6] The regiochemistry of the oxygenation is determined by conformational, steric, and electronic

[1] Baeyer, A.; Villiger, V. *Chem. Ber.* **1899**, *32*, 3625-3633.

[2] Krow, G.R. *Org. React.* **1993**, *43*, 251-798.

[3] Renz, M.; Meunier, B. *Eur. J. Org. Chem.* **1999**, 737-750.

[4] Criegee, R. *Liebigs Ann. Chem.* **1948**, *560*, 127-141.

[5] Mislow, K.; Brenner, J. *J. Am. Chem. Soc.* **1953**, *75*, 2318-2322.

[6] Noyori, R.; Sato, T.; Kobayashi, H. *Bull. Chem. Soc. Jpn.* **1983**, *56*, 2661-2679.

properties of the molecule. Subsuming, these cognitions implement the reaction as a valuable tool in the regio- and stereoselective synthesis.

Due to the widespread application of optically pure lactones as key intermediates in the synthesis of natural products and bioactive compounds, the area of enantioselective Baeyer-Villiger oxidations became a highly active field in recent research in asymmetric chemistry.[7] Up to now, two completely different approaches are currently elaborated, that implement the concept of "green chemistry" towards sustainable, environment friendly, and atom efficient processes by using molecular oxygen as oxidant. Over recent years organometal based *de novo* designed chiral catalysts have been continuously improved and represents a promising approach for future process developments and become interesting candidates for industrial scale applications.[8] Alternatively, flavin containing Baeyer-Villiger monooxygenases (BVMOs) as nature's equivalent to peracids are enzymes that display a remarkably broad acceptance profile for non-natural substrates as outlined by recent review articles. Therefore, they represent by far the most interesting and versatile alternative to the classical chemical Baeyer-Villiger transformation.[9,10],[11]

Conventional chemical Baeyer-Villiger oxidation is performed in the presence of oxidizing agents like hydrogen peroxide or peracids. Due to safety reasons, the industrial application of such oxidation processes is rather limited. The limited compatibility with functional groups like double bonds or nitrogen functionalities within the molecule further complicates the commercial application.

1.2 Enzymatic Baeyer-Villiger Oxidation

The unrivalled specificity of enzymes performing monooxygenations is still unmatched. Oxygenation reactions represent one of the most important subclasses in organic chemistry. The insertion of oxygen performed by oxygenases commences generally in very high chemo-, regio-, and stereoselectivity.[12] Due to the applicability and importance of such enzymes for industrial purposes research in this area became more and more intensive over the last decade. Several enzymes are currently used or are on the verge for applications in industry.

A multitude of new monooxygenases have been discovered containing a wide range of different catalytic properties and chemical selectivities. Hence this class of monooxygenases (EC 1.13 and

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[7] Mihovilovic, M.D.; Rudroff, F.; Grötzl, B. *Curr. Org. Chem.* **2004**, *8*, 1057-1069.

[8] a) Bolm, C. in *Peroxide Chemistry* (ed. W. Adam), Wiley-VCH: Weinheim, **2000**, 494-510; b) Strukul, G. *Angew. Chem.* **1998**, *110*, 1256-1267; *Angew. Chem. Int. Ed.* **1998**, *37*, 1198-1209.

[9] Mihovilovic, M.D. *Curr. Org. Chem*. **2006**, *10*, 1265-1287.

[10] a) Walsh, C.T.; Chen, Y.-C.J. *Angew. Chem.* **1988**, *100*, 342-352; b) Willetts, A. *Trends Biotechnol.* **1997**, *15*, 55-62; c) Roberts, S.M.; Wan, P.W.H. *J. Mol. Catal. B: Enzym.* **1998**, *4*, 111-136; d) Kelly, D. R.; *Chim. Oggi* **2000**, *18*, 33-37; e) Kelly, D. R. *Chim. Oggi* **2000**, *18*, 52-56.

[11] a) Taschner, M.J.; Peddada, L.; Cyr, P.; Cjem, Q.-Z.; Black, D.J.; in *Microbial Reagents in Organic Synthesis*, Serve; S. ed.; Kluwer Academic, **1992**, 347-360; b) Flitsch, S.; Grogan, G.; in *Enzyme Catalysis in Organic Synthesis*; Drauz, K.; Waldmann, H. eds.; Wiley-VCH **2002**, 1202-1245.

[12] Burton, S.G. *Trends Biotechnol.* **2003**, *21*, 543-549.

EC 1.14) became a main target of research for synthetic purposes over the last decades. Nevertheless, only a few of them has been explored and applied for their synthetic value. Due to limited availability of enzymes, problems of stability, difficulties in gene isolation and protein expression of such biocatalysts a wide application as a synthetic tool still remains very difficult. Another important challenge or problem to overcome for the use of monooxygenases is associated with the fact that most of these enzymes depend on expensive coenzymes or cofactors for their activities. Recent discovery and characterization of enzymes have indicated that a number of different types of proteins have evolved in nature to catalyze monooxygenation reactions. Such enzymes catalyze a variety of different oxygenation reactions and the type of the oxidation is dependend on the prosthetic groups present within the active site of the enzyme.
Thus, one of the best known examples is the family of cytochrome P450 monooxygenases, which represents a very big family. These heme-containing enzymes occur in many isoforms and have been shown to catalyze a plenitude of different oxygenations. Among the most important catalytic features of P450 enzymes is their ability to oxidize non-activated carbon atoms. They can be used for epoxidation reactions and enable regioselective hydroxylation of steroids and sterols.[13]

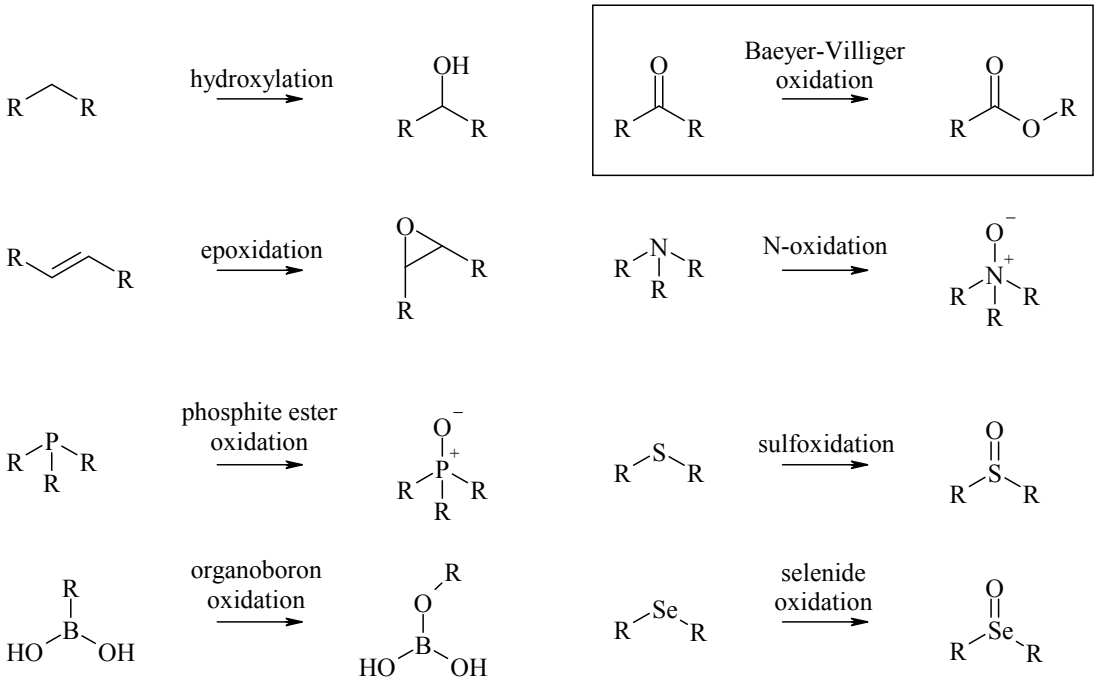

Figure 1.2 Reactions of flavoprotein monooxygenases.

Beside P450 monooxygenases, another widespread classes of such enzymes are non-heme monooxygenases, copper dependent monooxygenases and flavin-dependent monooxygenases. By far the most important and perhaps prevalent group of these enzymes is represented by flavin-dependent monooxygenases.[14,15] This class of enzymes covers a wide range of different

[13] Dawson, J.H.; Sono, M. *Chem. Rev.* **1987**, *87*, 1255-1276.

[14] Van Berkel, W.J.H.; Kamerbeek, N.M.; Fraaije, M.W. *J. Biotechnol*. **2006**, *124*, 670-689.

[15] Walsh, C. *Acc. Chem. Res.* **1980**, *13*, 148-155.

oxygenation reactions while being often highly chemo-, regio- and stereoselective. Oxygenation reactions implemented with such enzymes include epoxidations, phosphite ester oxidations,[16] organoboron oxidations,[17] N-oxidations,[18] sulfoxidations,[19,20,21,22] selenide oxidations,[23] and Baeyer-Villiger oxidations[24,25,26] (Figure 1.2).

The emphasis of preceding research was the discovery and identification of new BVMO genes. Due to several programs for deciphering novel genomes a respectable number of new BVMOs, containing interesting and diverse properties with respect to chemo-, regio-, and stereoselectivity became available for synthetic applications. [26,27,28] Recent progress was made in recognizing trends in substrate acceptance as well as stereopreference. Furthermore first attempts to modify the catalytic activity and influence the performance of BVMOs were successful.
Due to this present development in the field of enzymatic Baeyer-Villiger oxidation emphasis of future research is based on the application of such powerful enzymes in asymmetric organic synthesis as an easy–to-handle and useful tool.

[16] Colonna, S.; Gaggero, N.; Carrea, G.; Ottolina, G.; Pasta, P.; Zambianchi, F. *Tetrahedron Lett.* **2002**, *43*, 1797-1799.

[17] Latham jr., J.A.; Walsh, C. *J. Chem. Soc., Chem. Commun.* **1986**, 527-528.

[18] Ottolina, G.; Bianchi, S.; Belloni, B.; Carrea, G.; Danieli, B. *Tetrahedron Lett.* **1999**, *40*, 8483-8486.

[19] Colonna, S.; Gaggero, N.; Richelmi, C.; Pasta, P. *NATO Sci. Ser. 1* **2000**, *33*, 133-160.

[20] a) Light, D.R.; Waxman, D.J.; Walsh, C. *Biochemistry* **1982**, *21*, 2490-2498; b) Secundo, F.; Carrea, G.; Dallavalle, S.; Franzosi, G. *Tetrahedron Asymm.* **1993**, *4*, 1981-1982; c) Beecher, J.; Richardson, P.; Willetts, A. *Biotechnol. Lett.* **1994**, *16*, 909-912; d) Pasta, P.; Carrea, G.; Holland, H.L.; Dallavalle, S. *Tetrahedron Asymm.* **1995**, *6*, 933-936; e) Kelly, D.R.; Knowles, J.C.; Mahdi, J.G.; Taylor, I.N.; Wright, M. A. *Tetrahedron Asymm.* **1996**, *7*, 365-368; f) Colonna, S.; Gaggero, N.; Pasta, P.; Ottolina, G. *J. Chem. Soc., Chem. Commun.* **1996**, 2303-2307; g) Colonna, S.; Gaggero, N.; Carrea, G.; Pasta, P. *J. Chem. Soc., Chem. Commun.* **1997**, 439-440; h) Holland, H.L.; Gu, J.-X.; Kerridge, A.; Willetts, A. *Biocatal. Biotrans.* **1999**, *17*, 305-317.

[21] a) Colonna, S.; Gaggero, N.; Bertinotti, A.; Carrea, G.; Pasta, P.; Bernardi, A.; *J. Chem. Soc., Chem. Commun.* **1995**, 1123-1124; b) Alphand, V.; Gaggero, N.; Colonna, S.; Furstoss, R. *Tetrahedron Lett.* **1996**, *37*, 6117-6120; c) Alphand, V.; Gaggero, N.; Colonna, S.; Pasta, P.; Furstoss, R. *Tetrahedron* **1997**, *53*, 9695-9706.

[22] Colonna, S.; Gaggero, N.; Carrea, G.; Pasta, P. *J. Chem. Soc., Chem. Commun.* **1998**, 415-416.

[23] Latham jr., J.A.; Branchaud, B.P.Jr.; Chen, Y.-C.J.; Walsh, C. *J. Chem. Soc., Chem. Commun.* **1986**, 528-530.

[24] Bruice, T.C. *Acc. Chem. Res.* **1980**, *13*, 256-262.

[25] Ghisla, S.; Massey, V. *Eur. J. Biochem.* **1989**, *181*, 1-17.

[26] Mihovilovic, M.D.; Müller, B.; Stanetty, P. *Eur. J. Org. Chem.* **2002**, 3711-3730.

[27] Kamerbeek, N.M.; Janssen, D.B.; van Berkel, W.J.H.; Fraaije, M.W. *Adv. Synth. Catal.* **2003**, *345*, 667-678.

[28] Mihovilovic, M.D. *Curr. Org. Chem*. **2006**, *10*, 1265-1287.

1.3 Biochemistry of BVMOs

1.3.1 Mechanism and Classification of BVMOs

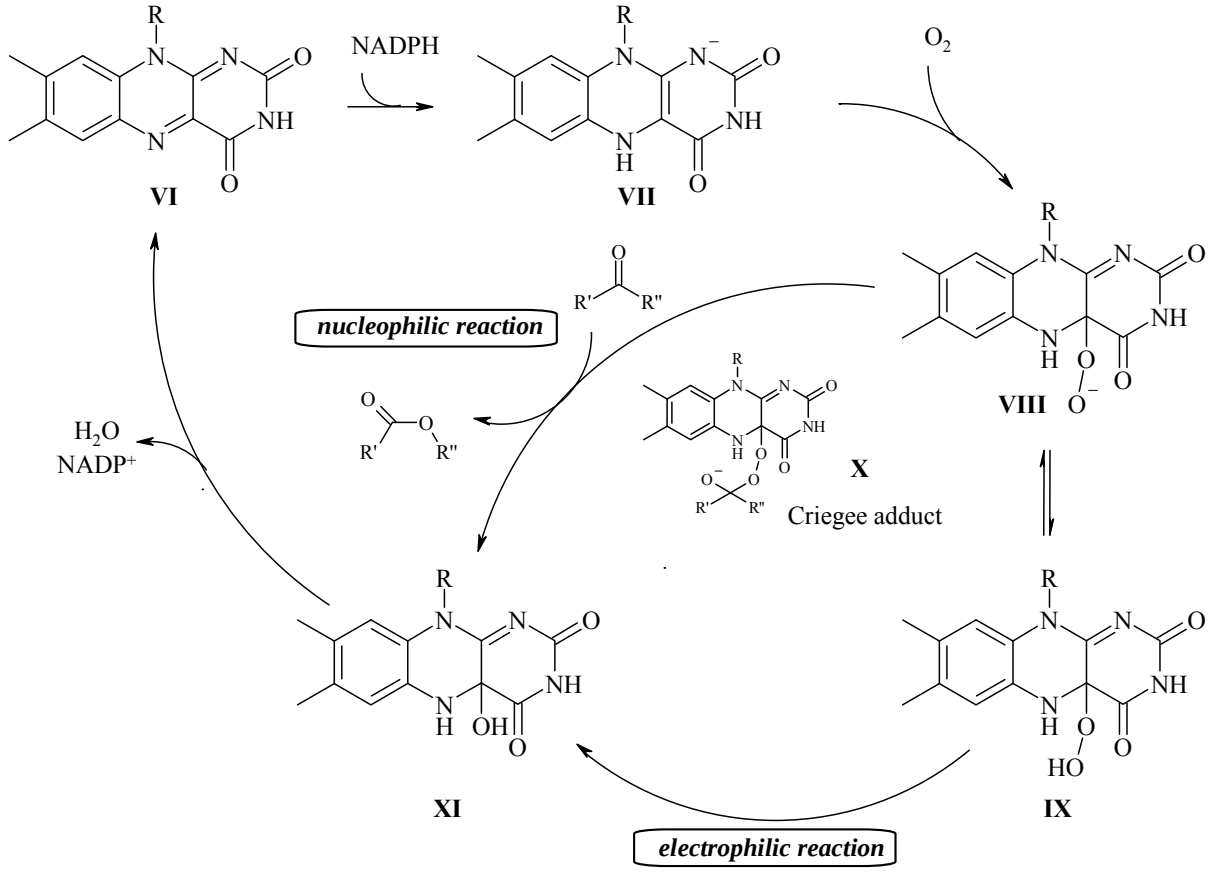

Figure 1.3 General mechanism of enzyme mediated oxygenation reactions of BVMOs.

The concerted reaction mechanism between molecular oxygen and carbon in organic chemistry is spin-forbidden. None the less many enzymes have found a way to circumvent this problem and use molecular oxygen as a substrate to oxygenate organic compounds. Therefore, an activation of molecular oxygen is required. Enzymes often use transition metals, bound or not bound to an organic cofactor like heme in P450 monooxygenases to create an oxygen-transferring species. However, flavin dependent monooxygenases utlilize a purely organic cofactor for such reactions. Based on data obtained from the first isolated cyclohexanone monooxygenase ($CHMO_{Acineto}$) originating from *Acinetobacter calcoaceticus* (NCIB 9871),[29] the first mechanism for BVMO mediated Baeyer-Villiger oxidation was proposed by Walsh *et al* (Figure 1.3).[30] The required activation of the flavin cofactor to react with molecular oxygen was accomplished by reduction of flavin in the presence of NADPH. Only the reduced FAD, intermediate (**VII**), is able to react readily with molecular oxygen and to form the activated 4a-peroxo anion species (**VIII**), which is in equilibrium with the corresponding hydroxyperoxide

[29] Donoghue, N.A.; Norris, D.B.; Trudgill, P.W. *Eur. J. Biochem*. **1976**, *63*, 175-192.

[30] Ryerson, C.C.; Ballou, D.P.; Walsh, C. *Biochemistry* **1982**, *21*, 2644-2655.

(**IX**).[31] Such a peroxyflavin intermediate is unstable and typically fragments into hydrogen peroxide and oxidized flavin. Nevertheless, flavin containing monooxygenases possess the ability to stabilize this species, which can then undergo a subsequent oxidation reaction. The presence of both deprotonated (**VIII**) and protonated (**IX**) intermediates suggests the nucleophilic as well as the electrophilic reactivity of BVMOs. The nucelophilic peroxoanion species (**VIII**) enables the Baeyer-Villiger reaction and the Michael-type addition reactions on activated C=C double bonds, while the electrophilic hydroxyperoxide (**IX**) can undergo heteroatom oxidations.

In the presence of an electrophilic carbonyl group, attack of intermediate (**VIII**) takes place and the tetrahedral Criegee-intermediate (**X**) is generated. Hence, migration of the more preferred carbon takes place in complete analogy to the classical chemical transformation and a single oxygen atom is incorporated into the substrate, while the other oxygen is reduced to water. Due to the geometry of the BVMO and the intermediate within the active site of the enzyme only one carbon-carbon bond can adopt the antiperiplanar configuration which is required for a successful migration. Finally, the catalytic process ends up with the release of the corresponding ester or lactone and the formation of hydroxyflavin (**XI**), which eliminates water. This mechanism has been investigated on $CHMO_{Acineto}$ and represents a general model for other BVMOs with minor modifications.

At present hundreds of flavin containing enzymes have been characterized. A recent classification attempt was published by Fraiije *et al.*[32] whereas classification of flavoproteins was performed based on different criteria, such as type of chemical reaction, the nature of oxidizing or reducing substrates, homology in sequence and finally in topology of 3D structure. The majority contain a non-covalently bound flavin prosthetic group, whereas some others are covalently bound to FAD or FMN. Hence, flavoproteins can be divided into six subclasses A-F. Baeyer-Villiger monooxygenases are members of class B or class C flavoprotein monooxygenases, depending on their cofactor specificity and prosthetic group.

General characteristics of class B monooxygenases are at one hand the tightly bound FAD cofactor, the dependence of NADPH as coenzyme and the fact that they are encoded by a single gene. On the other hand the coenzyme $NADP^+$/NADPH is temporary bound during the catalytic process. Type B enzymes are composed of two dinucleotide binding domains (Rossmann fold), which bind FAD and NADPH. Class C flavoproteins are encoded by multiple genes which encode for one or two monooxygenase components and a reductase part. The reductase can use either NADPH and/or NADH as coenzyme and is responsible for the reduction of FMN which is further used as coenzyme. The structural core of such monooxygenase subunits displays a TIM-barrel fold.

Previously, Willets proposed two different types of BVMOs: *Type 1* BVMOs (= class B), which are homogenous and generally require NADPH and FAD, while *type 2* BVMOs (= class C) are heterogeneous and are NADH and FMN dependent.[33]

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[31] Sheng, D.; Ballou, D. P.; Massey, V. *Biochemistry* **2001**, *40*, 11156-11167.

[32] Van Berkel, W.J.H.; Kamerbeek, N.M.; Fraaile, M.W. *J. Biotechnol*. **2006**, 670-689.

[33] Willets, A. *Trends Biotechnol*. **1997**, *15*, 55-62.

1.3.2 Sequence Motif and Structure of BVMOs

Within the last two decades, several class B BVMOs have been isolated, cloned and characterized. The recent effort to discover new Baeyer-Villiger biocatalysts has resulted in the identification and sequencing of a number of BVMO genes. Based on sequence homology models, sequences belonging to the BVMO family (class B) could be specifically recognized by a strictly conserved sequence motif (FxGxxxHxxxW), which could not be found in other flavoprotein subclasses.[34] This sequence motif represents a linker region of the FAD domain and the NADPH binding domain. Hence, this "fingerprint" sequence can serve as a recognition probe for further investigation in finding new, interesting BVMOs.

The first isolated and intensively investigated BVMO was CHMO from *Acinetobacter sp*. NCIB 9871. Several attempts for the investigation of the three dimensional structure of this enzyme and related proteins were attempted but failed, mainly because of the low stability of such proteins in purified form.

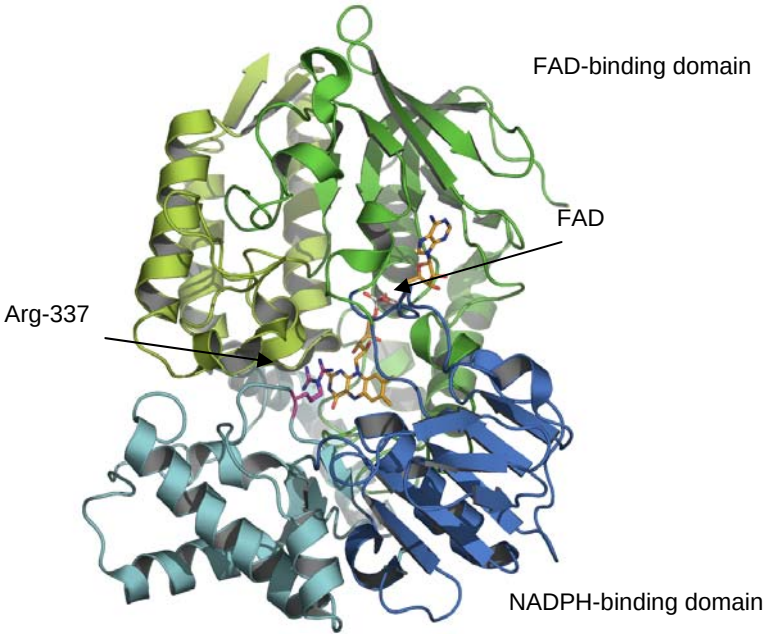

Figure1.4: Crystallographic structure determination of $PAMO_{Thermo}$ as ribbon diagram: FAD-binding domain, NADPH-binding domain, typical domains for BVMOs in pale colors, FAD and Arg-337 in ball-and-stick representation.

A breakthrough was achieved by the recent publication of the first x-ray structure of a class B (type 1) BVMO from moderately thermophilic bacterium *Thermobifida fusca*.[35] This monooxygenase has been overexpressed and characterized revealing the highest activity towards

[34] Fraaije, M.W.; Kamerbeek, N.M.; van Berkel, W.J.H.; Janssen, D.B. *FEBS Lett.* **2002**, *518*, 43-47.

[35] Malito, E.; Alfieri, A.; Fraaije, M.W.; Mattevi, A. *Proc. Nat. Acad. Sci. USA* **2004**, *101*, 13157-13162.

ketones in benzyl position like phenylacetone ($PAMO_{Thermo}$) with very high regioselectivity.[36] In contrast to other BVMOs, so far, $PAMO_{Thermo}$ displayed sufficient stability in purified form to allow crystallographic structure determination (Figure 1.4). As mentioned above, the structure of this protein composes two Rossmann fold domains which bind the FAD and the NADPH coenzyme and the active site is located in the cleft at the interface of both domains.

It is known, that flavoproteins, such as disulfide reductases, contain a similar two domain architecture, however some specific regions appear to be very unique for BVMOs (pale parts of domains). Conclusively, enzymes bearing such kind of a two domain structure seem to be very flexible for conformational changes during the catalytic process. The amino acid arginine close to the active site is strictly conserved in BVMOs and seems to play a key role during the catalytic process.

Arginine at position 337 (Figure 1.5) was located above the tightly bound flavin ring and is in the ideal position to stabilize the peroxoanion species (**VIII**), which is mandatory for a successful transfer of molecular oxygen. Hence, this Arg-337 was found to adopt two different conformations in the protein and represents another indication for a high degree of flexibility of the protein throughout the enzymatic reaction. Moreover, conformational changes are mandatory for the reaction between FAD and NADPH before dioxygenation of the coenzyme.

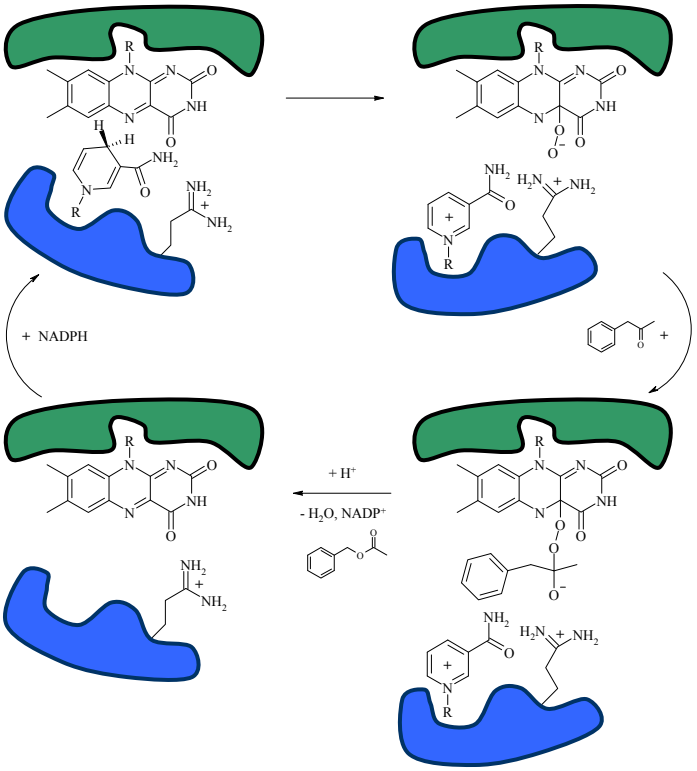

Figure 1.5 Schematic representation of the tentative conformational changes upon biooxidation by $PAMO_{Thermo}$ based on crystallographic structure determination: FAD-binding domain in green, NADPH-binding domain in blue.

[36] Fraaije, M.W.; Kamerbeek, N.M.; Heidekamp, A.J.; Fortin, R.; Janssen, D.B. *J. Biol. Chem.* **2004**, *279*, 3354-3360.

Recent research data suggests that upon reduction of FAD, Arg-337 has to be in an "outer" position to facilitate access of NADPH. Following oxygenation of the FAD moiety implicates the conformational change of Arg-337 to an "inner" position to stabilize the oxygenated cofactor. Finally, after these two initial steps of the catalytic cycle occurred, a desired substrate such as a ketone precursor can be oxidized, but the conformation has to be changed again to enable access of the substrate.
The connective link of the two domains to allow rotation required throughout the whole catalytic process is located in another highly conserved and very flexible region, the so called linker region, which acts as an "elastic band". This part of the fingerprint sequence contains a His at postion 173, which is fully solvent exposed and highly conserved in BVMOs.
Due to the fact that the structure of $PAMO_{Thermo}$ was only solved in the absence of NADPH coenzyme and the substrate, further investigations will be necessary in order to develop a predictive model for biocatalyst performance. However, the understanding of the enzymatic Baeyer-Villiger oxidation on the molecular level enables complete new strategies for ongoing research projects.

1.4 The Role of BVMOs in Nature

BVMOs play an essential role in the degradative metabolic pathways of organisms. As mentioned above, the first isolated BVMO was cyclohexanone monooxygenase from *Acinetobacter sp.* (NCIB 9871, $CHMO_{Acineto}$).[29] It was found, that this enzyme is responsible for the oxygen insertion in the degradation of cyclohexanone to the corresponding lactone. Several organisms, which are found to live in waste water of petrochemical facilities, have the ability to express BVMOs. Due to an excess of alkane precursors in such habitats utilization of an alternative carbon source becomes essential to survive and provides and evolutionary advantage to the organism.

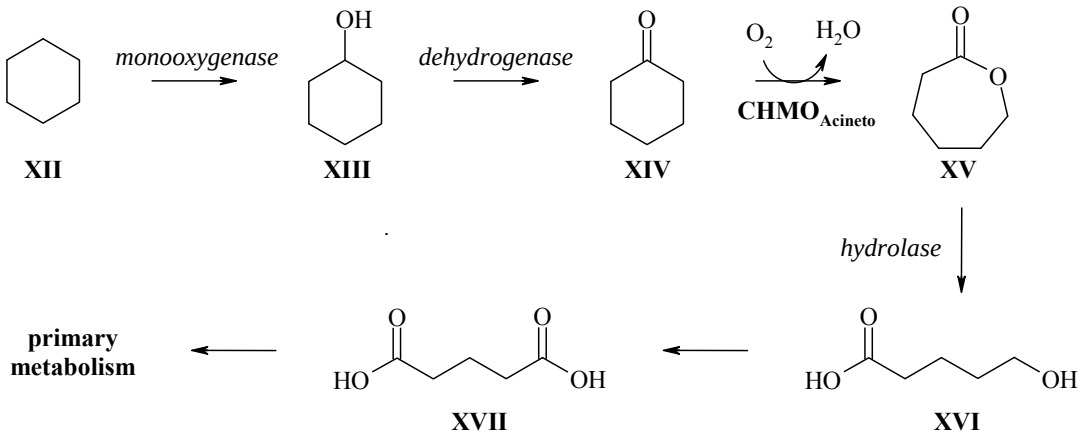

Figure 1.6 Metabolic pathway of cyclohexane in *Acinetobacter sp*.

The metabolism of cyclohexane in native *Acinetobacter sp*. starts with the hydroxylation of (**XII**) by a monooxygenase and continues with oxidation of (**XIII**) to cyclohexanone (**XIV**).

Subsequent degradation by $CHMO_{Acineto}$ to the corresponding lactone (**XV**) represents the conversion of a C-C bond to a polarized lactone functionality and opens access to the ongoing metabolic pathway towards adipate (**XVII**) which ends in the primary metabolism (Figure 1.6).
Since the first discovery and isolation of a Baeyer-Villigerase in nature extensive research was performed to utilize these biocatalysts for chemical transformations. A variety of BVMOs with different microbial origin and properties were identified over the last decades (Table 1.1). Many of these enzymes were investigated in terms of substrate acceptance and stereopreference profiles for non-natural substrates with various structural and electronic diversity. Over the last years enzymatic Baeyer-Villiger oxidation of a variety of cyclic, bi-, and polycyclic ketones was performed, usually displaying excellent chemo-, regio-, and stereoselectivity, and the methodology turned out to be very useful for synthetic chemistry.[37]
Interestingly some BVMOs from the same species were described in the literature, which display remarkable differences in their biocatalytic performances (e.g. $CHMO_{Brevi1}$ and $CHMO_{Brevi2}$). Within this table, references indicates the biochemical identification, characterization and cloning of the proteins.

BVMO	Origin	Year	
		ident.	cloned
Cyclohexanone monooxygenase ($CHMO_{Acineto1}$)	*Acinetobacter* NCIB 9871	1976[29]	1988[38]
Cyclohexanone monooxygenase ($CHMO_{Arthro}$)	*Arthrobacter* BP2	2003[39]	2003[39]
Cyclohexanone monooxygenase ($CHMO_{Brachy}$)	*Brachymonas petroleovorans*	2003[40]	2003[40]
Cyclohexanone monooxygenase ($CHMO_{Brevi1\&2}$)	*Brevibacterium* HCU	2000[41]	2000[41]
Cyclopentanone monooxygenase ($CPMO_{Coma}$)	*Comamonas* NCIB 9872	1976[42]	2002[43]
Cyclohexanone monooxygenase ($CHMO_{Rhodo1\&2}$)	*Rhodococcus* Phi1 & Phi2	2003[39]	2003[39]

Table 1.1 Synthetically relevant BVMOs investigated within this thesis.

[37] Stewart, J.D. *Curr. Org. Chem*. **1998**, *2*, 211-232.

[38] Chen, Y.-C.J.; Peoples, O.P.; Walsh, C.T. *J. Bacteriol.* **1988**, *170*, 781-789.

[39] Brzostowicz, P.; Walters, D.M.; Thomas, S.M.; Nagarajan, V.; Rouviere, P.E. *Appl. Environ. Microbiol.* **2003**, *69*, 334-342.

[40] Bramucci, M.G.; Brzostowicz, P.C.; Kostichka, K.N.; Nagarajan, V.; Rouviere, P.E.; Thomas, S.M. *PCT Int. Appl.* **2003**, WO 2003020890; *Chem. Abstr.* **2003**, *138*, 233997.

[41] Brzostowicz, P.C.; Gibson, K.L.; Thomas, S.M.; Blasko, M.S.; Rouviere, P.E. *J. Bacteriol.* **2000**, *182*, 4241-4248.

[42] a) Griffin, M.; Trudgill, P.W. *Eur. J. Biochem.* **1976**, *63*, 199-209; b) Trudgill, P.W. *Methods Enzymol.* **1990**, *88*, 77-81.

[43] Iwaki, H.; Hasegawa, Y.; Lau, P.C.K.; Wang, S.; Kayser, M.M. *Appl. Environ. Microbiol.* **2002**, *68*, 5681-5684.

Isolation and utilization of described BVMOs in preparative biotransformations varies due to their stability in purified form, their microbial origin and the required cofactors. BVMOs were often identified by selective growth experiments using wild type organisms (whole-cells). With the application of whole-cells in synthetic experiments, obstacles like protein isolation and purification due to low concentrations in wild type organisms as well as cofactor recycling can be circumvented. Nevertheless, unwanted side reactions of such native organisms (e.g. reductase or esterase activities) become problematic from time to time. Hence, for a better understanding of such new biocatalysts, investigations on the performance of the enzyme were determined on the isolated and purified protein.

The recently characterized "fingerprint" sequence motifs for BVMOs enables and facilitates the identification of new and maybe more potent proteins for further investigations. Hence, development of knowledge based approaches in genome mining, based on data obtained by known enzymes shows an enormous number of oxidative biocatalysts from bacterial origin (Figure 1.7). However, so far only a very small number of native BVMO sequences have been identified, isolated and cloned, and even fewer have been investigated for their substrate acceptance and biocatalytic relevance for synthetic applications.

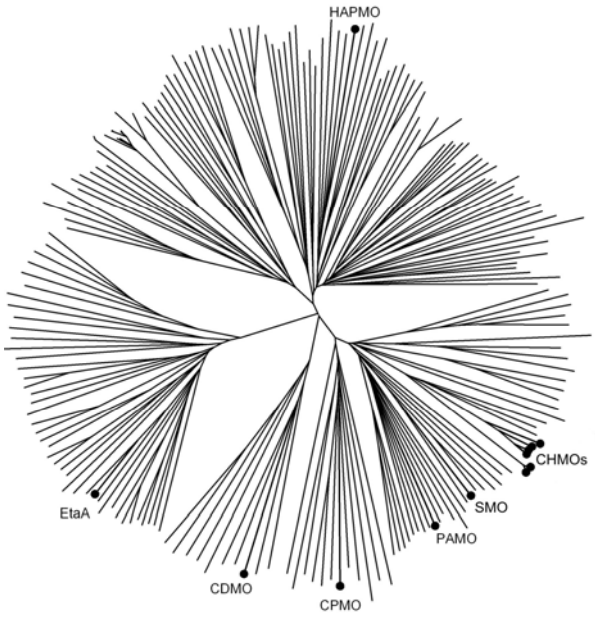

Figure 1.7 Phylogenetic tree analysis of (putative BVMO) sequences. 222 sequences were obtained using the PHI-BLAST option, using the sequence of HAPMO as seed and FXGXXXHXXXW as "fingerprint" sequence motif.

It seems that we have investigated only the top of the ice berg, and many interesting features of these enzymes are yet to be explored. Within this work, we want to demonstrate the versatility of already known, newly cloned, and modified BVMOs.

1.4.1 Isolated Enzymes vs. Whole-cells

The requirement of nicotinamide cofactors like NADPH by BVMO complicates the utilization of such enzymes in the organic synthesis. At least a cofactor regeneration system is required to permit a cost efficient application. Over the last years two principle strategies have been established to overcome this obstacle. On one hand, construction of a two enzyme based system and on the other hand whole-cell mediated fermentation was investigated and both approaches have pro's and con's. While working with the isolated enzyme, cofactor recycling can be achieved by adding a suitable dehydrogenase, which can reduce $NADP^+$ at the expense on an additional substrate. A particularly efficient system for the recovery of $NADP^+$ is based on genetically modified formate dehydrogenase by multipoint site directed evolution of FDH from *Pseudomonas sp*.[44] In this case, formate acts as a cheap auxiliary substrate, which will be oxidized to carbon dioxide as volatile product. Consequently, the equilibrium of the whole process is shifted towards the required direction favoring the linked enzymatic reaction. However, application of this approach is limited by the tolerance of the Baeyer-Villiger products towards the auxiliary substrate (e.g. formate) and the availability of a proper dehydrogenase.
Whole-cell mediated biotransformations offer a different approach for the recovery of the desired cofactor. As long as the cellular machinery is still intact, the living organism provides a natural recycling system for all coenzymes. Many cellular processes from the primary as well as the secondary metabolism are cofactor dependent and have to remain operational to ensure the survival of the whole organism. Hence, the lifetime of intracellular expressed proteins upon protein isolation, purification and storage are minimized. Whole-cells can be stored at frozen state over long periods of time and can be even proliferated again. Nevertheless whole-cell systems are by far more complicated and complex than the above described artificial two enzyme recycling approaches.

Microorganisms containing a low number of genes like *E.coli* seem to be perfect hosts for such cofactor recovery concepts. Unfortunately, even in such "small" organisms the potential of unwanted side reactions by competing enzymes remains tremendous. Substrates useful for specific biotransformations can be accepted by other proteins encoded in the genome of the host and may lead to a significant decrease of overall yield and/or an increase of unwanted by-product(s). Especially ketoreductases present in the host can interfere with subsequent Baeyer-Villiger processes by reduction of the keto-functionality into an alcohol. In addition, lactone products can by hydrolyzed by esterases. Such processes lead to a decrease in isolated yield of a required chiral product. The presence of another biocatalyst of the same type but with opposite enantiopreference can lead to a decrease in stereoselectivity. Both effects are highly unwanted for exploiting whole-cell based biocatalysis for the production of chiral lactones.

[44] Tishkov, V.I.; Galkin, A.G.; Marchenko, G.N.; Tsyganokov, Y.D.; Egorov, H.M. *Biotechnol. Appl. Biochem.* **1993**, *18*, 201-207.

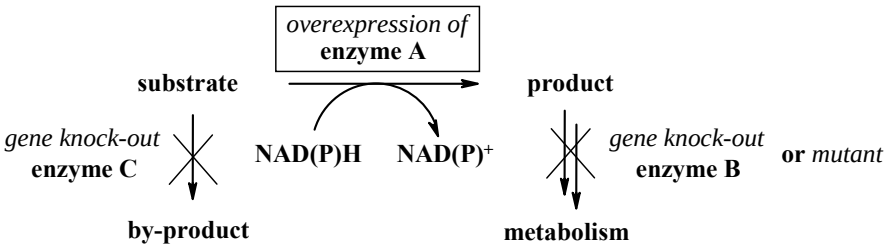

Figure 1.8 Unwanted side reactions while using whole-cell mediated fermentations.

Competitive biotransformations in whole-cell fermentations are displayed in Figure 1.8. Suppression of unwanted side reaction can be performed either by overexpressing the desired protein and increasing the concentration to a large extend or by shutting down the activity of interfering enzymes on the genetic level. The latter strategy can be realized either by gene knock-out of such proteins or utilization of mutant strains deficient in specific enzyme activities.[45] Recent developments in molecular biology permit the specific genetic engineering of microorganisms by creating high performance overexpression systems for the desired biocatalyst. Utilization of strong promoter systems resulted in production of BVMOs in high yield and the enzyme becomes the predominant fraction within the proteome of the cell ultimately minimizing the chance of unwanted side reactions.
Another advantage of such overexpression systems is the possibility to use easy to cultivate hosts such as *E.coli* for investigating enzymes originating from pathogenic organisms. In addition, comparison of fermentation experiments is improved as experiments are carried out in essentially the same host organism. Due to the simple cultivation protocols, recombinant expression systems for BVMOs based on *E.coli* or *S.cerevisiae* are also easy to handle by synthetic chemists with only minor expertise in microbiology.
The protein production is based on the addition of very selective inducer molecules, which can regulate (down or up) the gene transcription, effectively.

The first attempts within the introduction of recombinant overexpression systems in the field of Baeyer-Villiger oxidation were made by the cooperating groups of Stewart and Kayser in the mid nineties upon introduction of modified "designer yeast" (*Saccharomyces cerevisiae*).[46] However, nowadays the host organism of choice usually is *E.coli* due to the availability of stronger and more potent promoter systems, higher protein production, and faster cultivation. A recombinant strain producing $CHMO_{Acineto}$ may serve as a typical example for a two-step protein expression system in *E.coli*. The plasmid map (Figure 1.9) for the $CHMO_{Acineto}$ expression

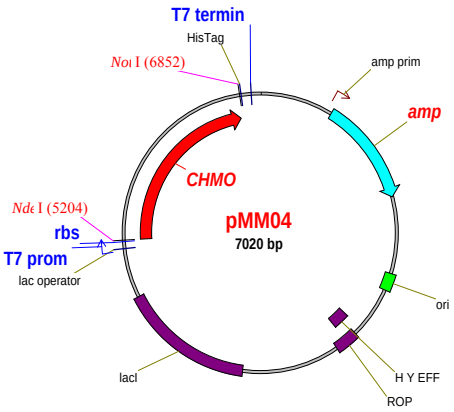

Figure 1.9 Plasmid map for the CHMO expression system (pMM04)

[45] Davey, J.F.; Trudgill, P.W. *Eur. J. Biochem*. **1977**, *74*, 115-127.

[46] Kayser, M.M.; Chen, G.; Stewart, J.D. *Synlett* **1999**, 153-158 and references therein.

system (pMM04) shows the general setup for commonly used T7-promotor based vectors: In the genome of *E.coli* the gene for T7-RNA polymerase was integrated under the control of the lacUV5 promotor. The RNA polymerase originating from bacteriophage T7 is a single polypeptide that recognizes the highly conserved T7 promotor.[47] T7-RNA polymerase initiates and elongates transcripts more efficiently than *E.coli* RNA polymerase.[48] The enzyme produces full length transcripts from DNA templates containing a T7 promotor and from vectors for the high level expression of genes cloned downstream of T7 promoters like the $CHMO_{Acineto}$ gene.[49] The inducing reagent for the highly active polymerase is isopropyl-β-D-galacto pyranoside (IPTG) which is added to the fermentation medium. A T7-promoter sequence is right upstream of the gene for the BVMO and it is selectivity recognized by the polymerase. At once a multiple transcription starts and leads to a very efficient enzyme expression with a large protein concentration (approx. 20% of soluble protein). Expression of the other monooxygenases commences in a similar manner.

1.5 Synthetic Application of BVMOs

1.5.1 Desymmetrization

Introduction of chirality into an organic compound can be performed by two different ways. Starting from a racemic mixture kinetic resolution (Figure 1.10, **A**) can take place by selective reaction of a single enantiomer and optically pure product and starting material, in best cases, can be obtained with a theoretical maximum yield of 50%, each. This conventional approach is only of limited potential for industrial application and is certainly not in accordance with the concept of atom efficiency and "green chemistry".

Recently a very important contribution in the area of kinetic resolution *via* Baeyer-Villiger oxygenation was the development of a dynamic process. In this approach the limitations of 50% maximum yield was overcome by applying *in-situ* racemization conditions of the substrate. The efficiency of such a reaction relies on a fast racemization and a slower biooxidation step. In a proof-of-concept experiment, the dynamic kinetic resolution was optimized for benzyloxy-ketone **XVIIIa** (Figure 1.7, **B**).[50] Classical biooxidation using recombinant whole-cells at pH 7 gave lactone **XVIIIb** in excellent e.e. and almost 50% yield, while the remaining substrate displayed rather low stereoselectivity (43% ee) due to the high acidity of the 2-proton in ketone **XVIIIa**. Changing the pH to 9 resulted in acceleration of substrate racemization and complete conversion to (*R*)-**XVIIIb** was achieved in 85% yield and excellent stereoselectivity within the biooxidation (96% ee).

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[47] a) Chamberlin, M.; McGrath, J.; Waskell, L. *Nature* **1970**, 228,227-231; b) Panayotatos, N.; Truong, K. *Nucleic Acids Res*. **1981**, *9*, 5679-5688; c) Dunn, J. J.; Studier, F. W. *J. Mol. Biol*. **1983**, *166*, 477-535.

[48] a) Chamberlin, M., Ring, J. *J. Biol. CIzem*. **1973**, *248*, 2235-2244; b) Golomb, M.; Chamberlin, M. *J. Biol. Chem*. **1974**, *249*, 2858-2863.

[49] a) Studier, F. W.; Moffatt, B. A. *J. Mol. Biol*. **1986**, *189*, 113-133; b) Mead, D. A.; Skorupa, E. S.; Kemper, B. *Protein Engineering* **1986**, *1*, 67-74.

[50] Berezina, N.; Alphand, V.; Furstoss, R. *Tetrahedron Asymm.* **2002**, *13*, 1953-1955.

A more environmentally friendly and promising application of enzymatic reactions in general can be realized by desymmetrization reactions. In this case, reactions of prochiral substrates are performed with theoretical yield of 100% of enantiomerically pure product in a straight forward transformation (Figure 1.7, **C**).[51] We have recently outlined some formal total syntheses of natural products based on the application of this strategy in microbial Baeyer-Villiger oxidation establishing absolute configurations for up to four stereogenic centers in a single biotransformation.[52]

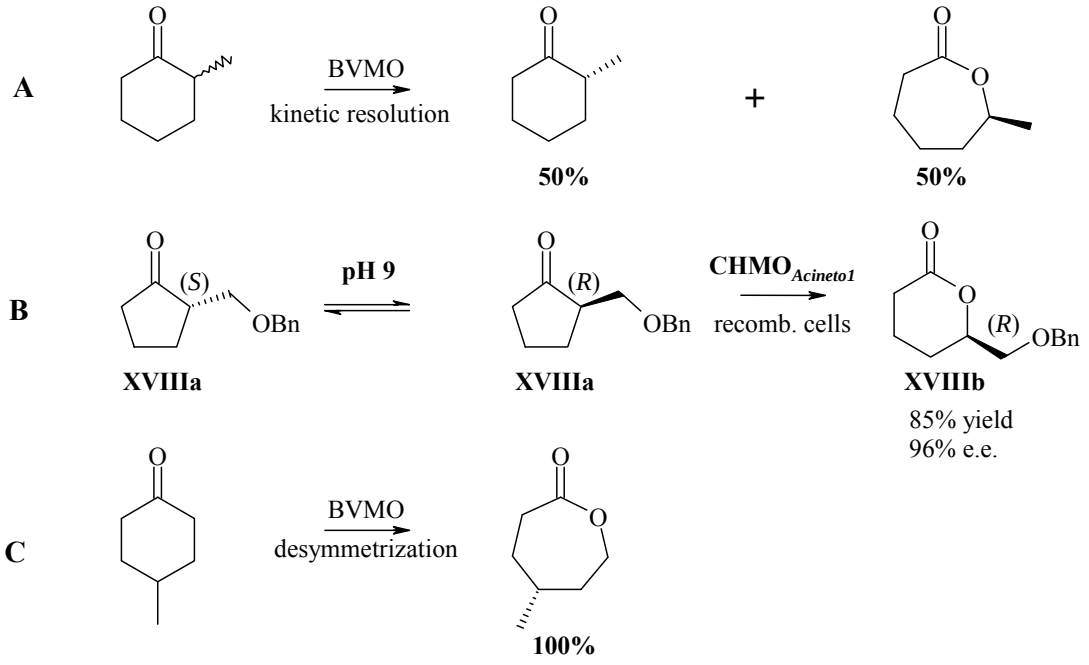


Figure 1.10 Kinetic resolution *vs*. desymmetrization

Enzymes of the BVMO subfamily seem to be rather unstable in isolated form and can be hardly crystallized. Evaluation of the biocatalytic performance of an enzyme can be realized either by rational design together with docking experiments based on the x-ray structure or (and in the case of BVMOs it seems to be by far the fastest and most efficient way) to generate substrate profiles by screening of representative substrates. During the last years of research we focused our studies on the synthesis of diverse prochiral ketones. Hence, within variation of structure, combination of different functional groups and changing electronic properties of such ketones a large number of interesting substrates could be synthesized. Additionally, many new

[51] Garcia-Urdiales, E.; Alfonso, I.; Gotor, V. *Chem. Rev*. **2005**, *105*, 313-354.

[52] a) Braun, I.; Rudroff, F.; Mihovilovic, M.D.; Bach, T.M. *Angew. Chem.* **2006**, *118*, 5667-5670; *Angew. Chem. Int. Ed.* **2006**, *45*, 5541-5543; b) Mihovilovic, M.D.; Bianchi, D.A.; Rudroff, F. *Chem. Commun.* **2006**, 3214-3216; c) Mihovilovic, M.D.; Snajdrova, R.; Winninger, A.; Rudroff, F. *Synlett* **2005**, 2751-2754; d) Mihovilovic, M.D.; Rudroff, F.; Müller, B.; Stanetty, P. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1479-1482; e) Mihovilovic, M.D.; Müller, M.; Kayser, M.M.; Stanetty, P. *Synlett* **2002**, 700-702.

stereoselective routes were investigated for the synthesis of such prochiral ketones.[53] Figure 1.11 displays a collection of substrates, which were used for the screening of newly characterized BVMOs.

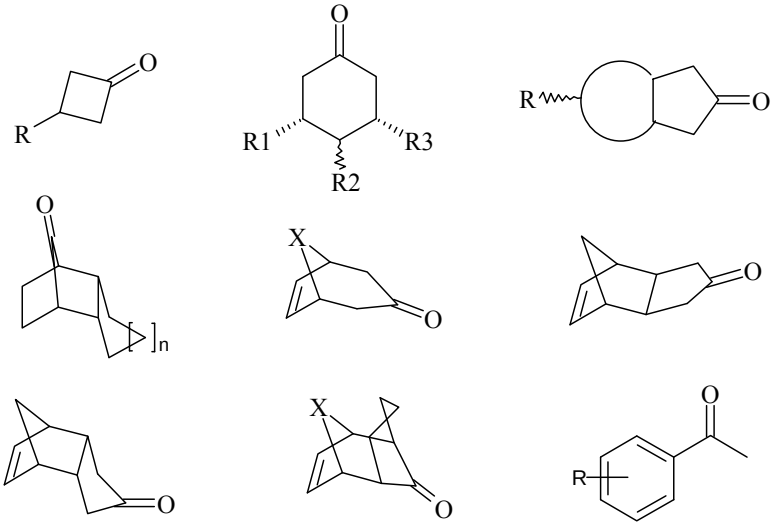


Figure 1.11 Substrate library of various prochiral ketones.

1.5.2 Stereo-and Regioselective Synthesis of Lactones as Precursor for Synthetic Applications

During elaborate studies a number of bacterial and fungal monooxygenases were identified to facilitate the Baeyer-Villiger oxygen insertion reaction accepting various ketones as substrates. A broad spectrum of non natural ketones was tested within the aspect of synthesis of key intermediates for bioactive compounds (Figure 1.8). Enantiocomplementary bioxidations turned out to be a very powerful tool in organic synthesis and opens up a variety of new synthetic approaches towards valuable natural or/and bioactive compounds.

[53] a) Mihovilovic, M.D.; Rudroff, F.; Grötzl, B.; Stanetty, P. *Eur. J. Org. Chem.* **2005**, *5*, 809-816; b) Mihovilovic, M.D.; Müller, B.; Kayser, M.M.; Stanetty, P. *Synlett,* **2002**, 700-702; c) Mihovilovic, M.D.; Müller, B.; Kayser, M.M.; Stewart, J.D.; Stanetty, P. *Synlett,* **2002**, 703-706.

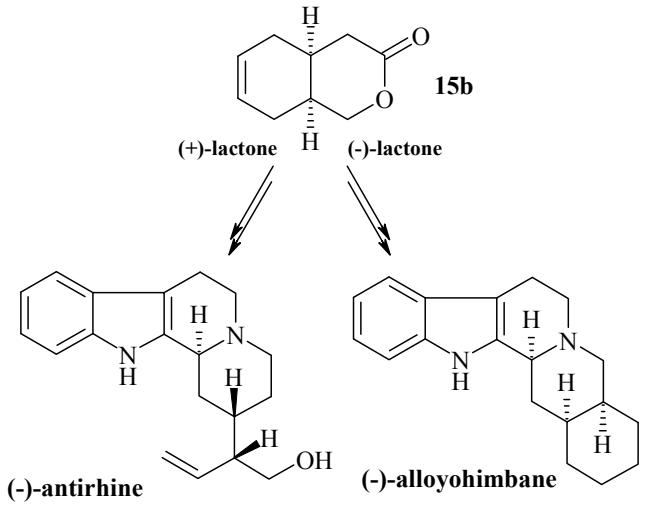


Figure 1.12 Synthetic application of enantiodivergent Baeyer-Villiger products.

Formal total syntheses towards alloyohimbane by using (-)-**15b**, which is accessible *via* biooxidation of the corresponding ketone with $CHMO_{Brachy}$ expressing whole-cells in acceptable optical purity, and to antirhine *via* (+)-**15b**, which is obtained from $CPMO_{Coma}$ mediated biotransformations in excellent stereospecificity were outlined in Figure 1.12.[54]

Another example for the synthetic application of enantiopure Baeyer-Villiger products was outlined in Figure 1.13. Lactone product **28b** was reported by Taschner[55] as a pivotal intermediate in synthetic approaches towards tirandamycin[56] and also served as precursor for calyculin[57].

[54] Hieble, J.P.; Nichols, A.J.; Langer, S.Z.; Ruffolo, R.R.jr. in *Principles of Pharmacology*, Munson, P.L. *(Ed.);* Chapman & Hall, New York: **1995**, 138.

[55] Taschner, M.J.; Black, D.J. *J. Am. Chem. Soc.* **1988**, *110*, 6892-6893.

[56] Taschner, M.J.; Aminbhavi, A.S. *Tetrahedron Lett.* **1989**, *30*, 1029-1032.

[57] a) Yokokawa, F.; Hamada, Y.; Shioiri, T. *Chem. Commun*. **1996**, 871-872; b) Duchamp, D.J.; Branfman, A.R.; Button, A.C.; Rinehart, K.L. *J. Am. Chem. Soc*. **1973**, *95*, 4077-4078.

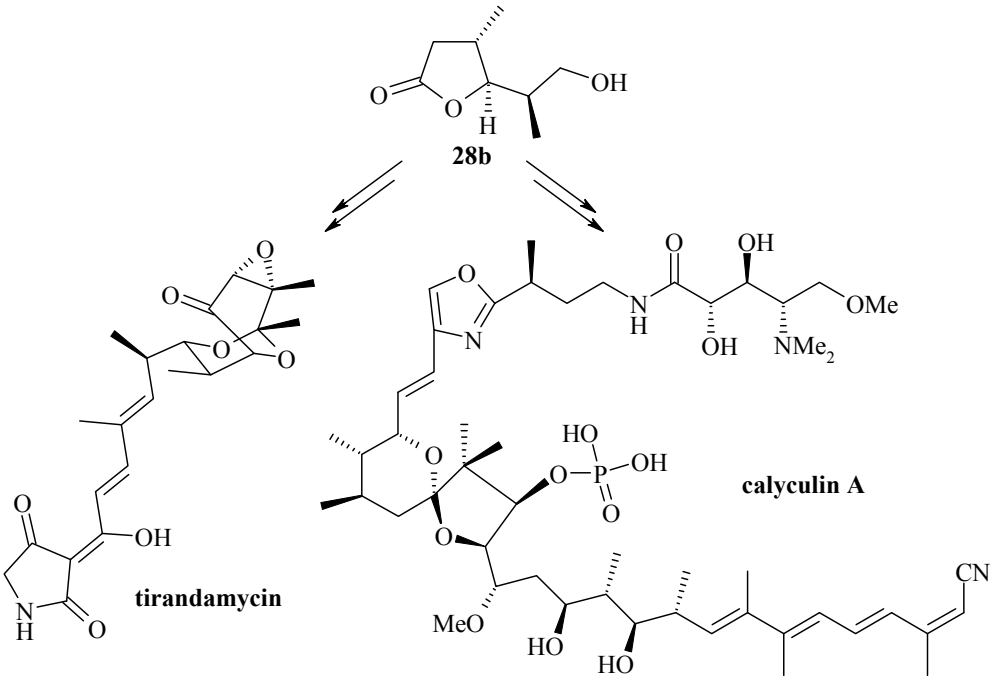


Figure 1.13 Lactone **28b** as key intermediate in natural product synthesis.

Regioselective Baeyer-Villiger oxidation of ketone **25a** (Figure 1.11) to fused bicyclo-lactones opens access to another interesting group of natural products. Both regioisomers were utilized in synthetic approaches to various natural products. The "normal" biooxidation product **25b"n"** represents a key intermediate to access various prostaglandins.[58] "Abnormal" lactone **25b"an"** was utilized in the synthesis of sarkomacin, a potent cytostatic.[59]

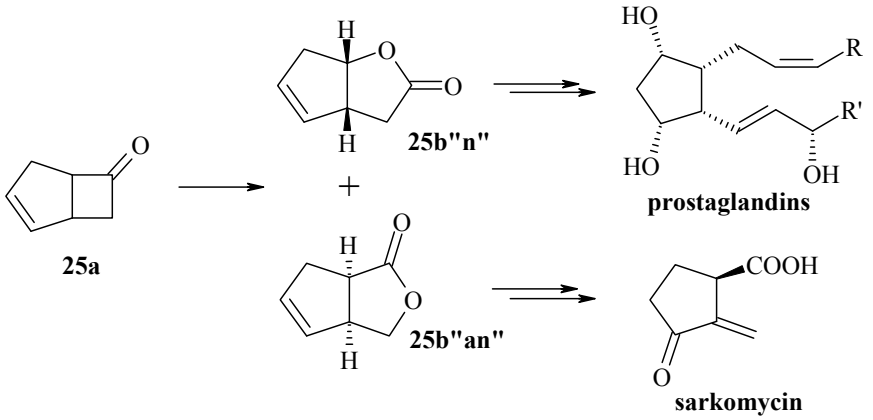


Figure 1.14 "Normal" and "abnormal" fused bicyclo-lactones as intermediates in natural product syntheses.

[58] Alphand, V.; Archelas, A.; Furstoss, R. *Tetrahedron Lett.* **1989**, *30*, 3663-3664.

[59] Andrau, L.; Lebreton, J.; Viazzo, P.; Alphand, V.; Furstoss, R. *Tetrahedron Lett.* **1997**, *38*, 825-826.

1.6 Scope of this Thesis

The group of Prof. Mihovilovic has contributed in the past years significantly to outline the synthetic potential of BVMOs for the preparation of chiral lactones. However, previous approached to determine substrate acceptance profiles for BVMOs (by this group and others) was essentially focusing on shake-flask experiments with (recombinant) whole-cells utilizing up to several mg of substrates in concentrations of 1-5mM. Consequently, the characterization of novel BVMOs was very time and resource consuming. The lack of easy to use and efficient screening techniques complicated testing of both new substrates and newly identified enzymes.
Recent advances in molecular biology open access to a steadily increasing number of new biocatalysts with completely unknown properties. This includes both wild-type enzymes or variant thereof obtained by various strategies of mutagenesis or gene shuffling. On the other hand, due to the lack of three dimensional structures of such enzymes, determination of biocatalytic properties still relies on screening of compound libraries and requires strong input by synthetic organic chemistry. In addition, the microbial asymmetric Baeyer-Villiger reaction represents one of the mildest and functional group independent oxidation methodologies for the cleavage of a carbon-carbon bond. Therefore the application of such a tool in the synthesis of various intermediates for natural and bioactive compounds was increased consequently.
With an increasing number of BVMOs becoming available to our group from various cooperation partners, a rapid screening protocol was required, which provided information on the potential of a certain biocatalyst for application in synthetic chemistry. In this context, the already existing library of ketone precursors was to be expanded for compounds of interest for subsequent synthesis of natural products or bioactive compounds. Within this part of the project, particular emphasis was put on the identification of BVMOs providing access to enantiocomplementary lactone products.
In addition, we identified a clear demand to demonstrate the applicability of such novel BVMOs to provide preparative quantities of chiral building blocks. Hence, the up-scaling of recombinant whole-cell fermentation processes was required in order to prepare gram-quantities of chiral lactones for subsequent synthetic elaboration. Ultimately, the advantage of BVMOs should be documented by developing novel, efficient, and facile synthetic routes to diverse natural products.
Consequently, the major goals of this project were as follows:

- Development of a rapid screening system for substrate profiling of BVMOs
- Fermentation optimization of BVMO mediated whole-cell biotransformations
- Application of BVMOs in novel synthetic approaches to bioactive compounds

The complete strategy of this thesis is outlined in Figure 1.15. During the last years we got access to a large number of different BVMOs with so far unknown biocatalytic properties. We also synthesized a large library of structurally diverse ketones (prochiral and racemic) to be investigated in biooxidation reactions. Consequently, we envisioned the development of an

efficient screening methodology based on microscale whole-cell biotransformations. The screening protocol should be possible in a very time efficient manner with respect to obtaining data for conversion and stereoselectivity (determined by chiral GC analysis).

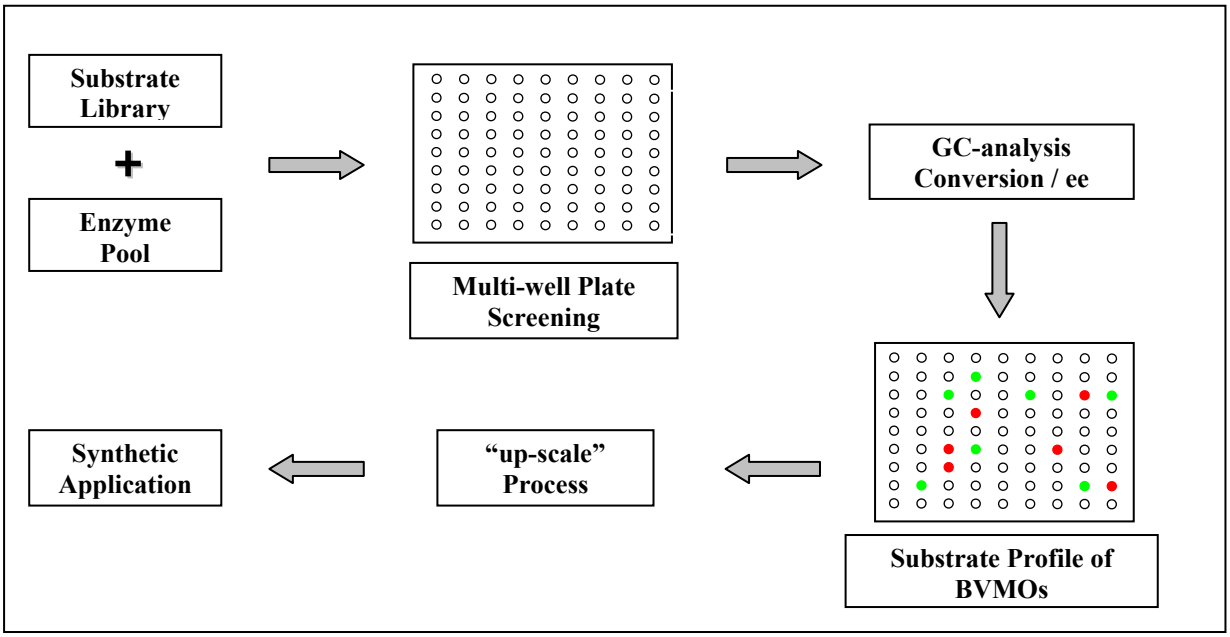


Figure 1.15 From screening to synthetic application – strategy of this work

The fermentation optimization of whole-cell mediated Baeyer-Villiger oxidation was the next logical step upon identification of a promising biocatalyst-substrate couple of interest for future exploitation. One major problem of the fermentation is the comparably low substrate concentration as a result of the known low inhibition levels for BVMOs.[60] In addition, factors such as biocatalyst stability, stability and acceptance of substrates, loss of compound during fermentation etc. were critical in this phase of the project.

Recently, the group of Furstoss *et al* presented a process which is based on a biocatalytic whole-cells strategy - using a recombinant *E. coli* strain overexpressing $CHMO_{Acineto}$ - combined with a "two-in-one" in situ "substrate feeding and product removal" concept (SFPR) using an adsorbent resin.[61]

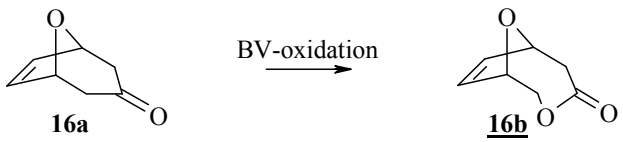
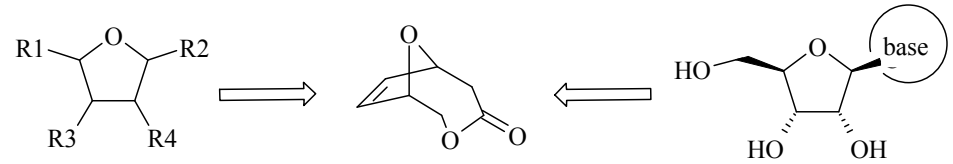


Figure 1.16 Baeyer-Villiger oxidation of bridged bicyclic ketone **16a** to the corresponding key lactone **16b**.

[60] Doig, S.D.; Avenell, P.J.; Bird, P.A.; Gallati, P.; Lander, K.S.; Lye, G.J.; Wohlgemuth, T.; Woodley, J.M. *Biotechnol. Prog.* **2002**, *18*, 1039-1046.

[61] Hilker, I.; Alphand, V.; Wohlgemuth, R.; Furstoss, R. *Adv. Synth. Catal.* **2004**, *346*, 203-214.

Based on this preliminary work we wanted to expand this methodology to new BVMOs, since the concept was outlined only on a single BVMO producing strain and on one substrate. In this context we were particularly interested to optimize the enantioselective microbial Baeyer-Villiger oxidation of bridged bicyclic systems (Figure 1.16), which were envisioned to offer a new entry to various C-nucleosides and poly substituted tetrahydrofurans (Figure 1.17). This class of compounds, which possesses a carbon-carbon linkage between the carbohydrate and the heterocyclic moiety, has become an increasingly interesting area in organic and medicinal chemistry because of their potential utility as therapeutic agents such as antiviral, anticancer and anti-HIV drugs.



poly-substituted furans **C-nucelosides**

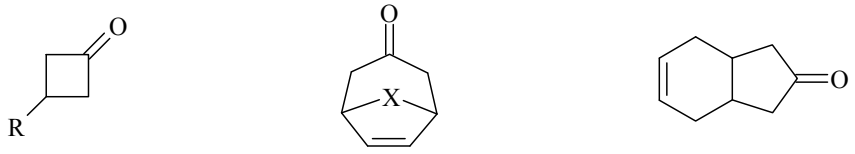
Figure 1.17 Application of microbial Baeyer-Villiger oxidation in the synthesis of bioactive compounds.

Results and Discussion

2 Enzymatic Baeyer-Villiger Oxidation in Natural Product and Bioactive Compound Synthesis

2.1 General Aspects

Very recently we introduced a platform of different recombinant strains of *E.coli* overexpressing Baeyer-Villiger monooxygenases of various microbial origins. This small library of proteins possesses overlapping substrate profile for the biooxidation of various ketones and displays enantiocomplementary and regiodivergent properties.[52,53]
Within this part of the project we wanted to demonstrate the potential of this library of recombinant whole-cell expression systems for bacterial BVMOs to produce chiral (antipodal) lactones with different structural cores.



XIX **XX** **XXI**

Figure 2.1 Valuable prochiral ketone-precursors for synthetic application.

Prochiral ketones (Figure 2.1) represent a readily accepted and very interesting family of substrates with potential as relevant precursors in asymmetric organic synthesis. Since the corresponding lactones are versatile building blocks in bioactive compound and natural product synthesis, considerable attention has focused on this class of biooxidation intermediates. In contrast to kinetic resolutions of racemic starting materials, desymmetrization of prochiral compounds allows the generation of several chiral centers with a theoretical yield of 100%, which makes this approach especially appealing to synthetic chemists.

A brief overview of possible applications of such lactones in synthetic organic chemistry is shown in Figure 2.2. During the last century intensive investigations, especially in the field of asymmetric synthesis were made to solve synthetic problems and increase the accessibility of various natural and bioactive compounds.
Butyrolactones derived from prochiral 3-substituted cyclobutanones (**XIX**) represent one of the most interesting and versatile classes of biooxidation products. Accessibility of antagonistic analgesics like eptazocine,[62] GABA receptor inhibitors,[63] the synthesis of β-

[62] Shiotani, S.; Okada, H.; Yamamoto, T.; Nakamata, K.; Adachi, J.; Nakamoto, H. *Heterocycles* **1996**, *43*, 113-126.

amino acids like β-proline,[64] and structurally related lignans[65] are within their range of application. The corresponding lactone of prochiral bicylco ketone (**XX**) represents another highly valuable platform for the synthesis of various C-nucleosides like (+)-showdomycin, polysubstituted tetrahydrofurans like (+)-*trans*-kumausyne, and the analogs of the cytotoxic metabolite goniofufurone.[52b] Ketones of type (**XXI**) represent interesting intermediates for the synthesis of yohimbine-respirine alkaloids which belong to the class of indole-alkaloids and represent the second largest group of alkaloids.[54] The main interest in this family is based on its pharmacological properties.

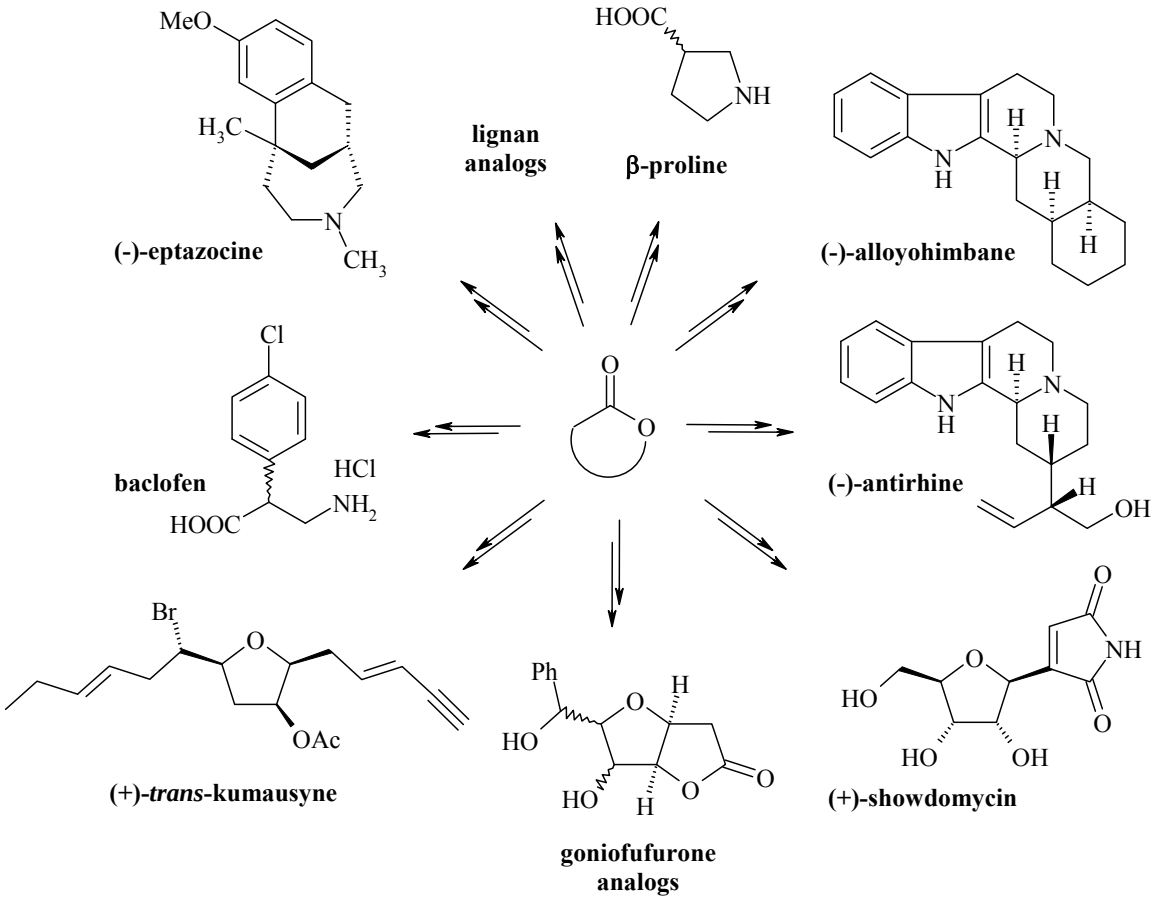


Figure 2.2 The microbial Baeyer-Villiger oxidation in the synthesis of natural and bioactive compounds.

One of the key challenges in asymmetric synthesis and especially in biocatalysis is the aspect of enantiodivergence. Whereas artificial catalytic entities can be easily modified to produce antipodal forms of the required products by inverting the chirality of the inducing ligand field, this strategy cannot be applied in enzyme mediated transformations. There is no efficient process available to yield D-amino acid based proteins. Due to this fact the identification and

[63] Mazzini, C.; Lebreton, J.; Alphand, V.; Furstoss, R. *Tetrahedron Lett*. **1997**, *38*, 1195-1196.

[64] Mazzini, C.; Lebreton, J.; Alphand, V.; Furstoss, R. *J. Org. Chem*. **1997**, *62*, 5215-5218.

[65] Bode, J.W.; Doyle, M.P.; Protopopova, M.N.; Zhou, Q.-L. *J. Org. Chem*. **1996**, *61*, 9146-9155.

characterization of enzymes possessing overlapping substrate specificity and yielding antipodal products is one of the key aspects to establish biocatalytic methods in synthetic chemistry. Desymmetrization reactions are a particularly powerful approach for the *de novo* generation of chirality in biotransformations.[51] We have recently outlined some formal total syntheses of natural products based on the application of this strategy in microbial Baeyer-Villiger oxidation establishing absolute configurations for up to four stereogenic centers in a single biotransformation.[53]

A related phylogenetic tree analysis with biomolecular interpretation has been reported previously for a large set of monooxygenases, in general.[34] Recently, we published the first connection of primary protein sequence with biocatalyst performance for BVMOs.[66] While we observed clustering into two distinct groups on a number of structurally diverse ketones in desymmetrization reactions and regiodivergent biooxidations, this trend seems only applicable to a limited extent for classical kinetic resolutions.[67] We expect the identification and further refinement of trends such as reported in this contribution to further proliferate and simplify substrate screening and profiling.

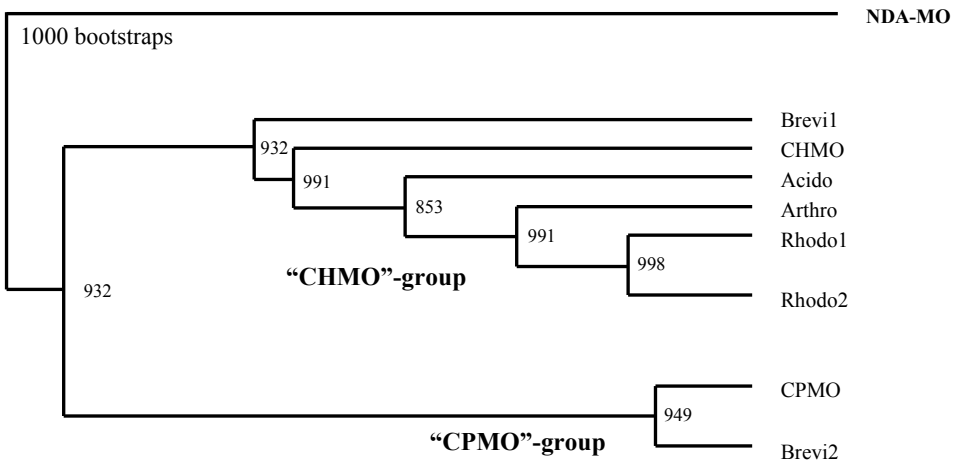


Figure 2.3 Phylogenetic tree analysis of different BVMO's.

Since we and others have observed formation of antipodal lactones by some BVMOs[43,52,53,,68] within this thesis we compare the stereopreference of recombinant monooxygenases originating from *Brachymonas* ($CHMO_{Brachy}$), *Acinetobacter* ($CHMO_{Acineto}$),[29] *Arthrobacter* ($CHMO_{Arthro}$),[39] *Brevibacterium* ($CHMO_{Brevi1}$, $CHMO_{Brevi2}$),[41] *Comamonas* ($CPMO_{Coma}$)[42a] and *Rhodococcus* ($CHMO_{Rhodo1}$, $CHMO_{Rhodo2}$)[34] species in recombinant whole-cell mediated

[66] Mihovilovic, M. D.; Rudroff, F.; Grötzl, B.; Kapitan, P.; Snajdrova, R.; Rydz, J.; Mach, R. *Angew. Chem. Int. Ed.* **2005**, *44*, 3609-3613.

[67] Kyte, B.G.; Rouviere, P.; Cheng, Q.; Stewart, J.D. *J. Org. Chem.* **2004**, *69*, 12-17.

[68] Mihovilovic, M.D.; Müller, B.; Schulze, A.; Stanetty, P.; Kayser, M.M. *Eur. J. Org. Chem.* **2003**, 2243-2249.

Baeyer-Villiger oxidations with respect to enantio- and regiodivergence utilizing *E. coli* as host organism (Figure 2.3).
Hence, we want to demonstrate the potential and the synthetic importance of the microbial Baeyer-Villiger oxidation.

2.2 Synthesis of Prochiral Cyclobutanones

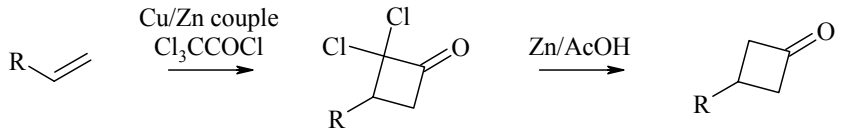


Figure 2.4 Synthesis of prochiral cyclobutanones *via* [2+2] cycloaddition approach.

In this chapter, biooxidation of cyclobutanones to provide chiral butyrolactones is investigated utilizing the before outlined library of bacterial BVMOs within recombinant whole-cell biotransformations. This compound class has been demonstrated to be a highly versatile platform to access natural products and bioactive compounds of great structural diversity, in particular due to the straight-forward access of the substrates *via* a [2+2] cycloaddition reaction (Figure 2.4).[69] Very early, desymmetrizations by BVMOs were recognized as an interesting alternative to more traditional entries to this compound class.[63,64,70] However, previous studies were limited to only very few biocatalysts and antipodal products were not accessible readily. Prochiral cyclobutanones **(XIX)** were synthesized from the corresponding alkenes according to the literature either by Cu/Zn couple mediated [2+2] cyclization under classical thermal[69a] or ultrasonic conditions[69b].

Most of the alkenes were commercially available, except for the starting material of compound **14b** (Figure 2.5). Product **4** was prepared *via* the synthetic route described below. Williams's ether synthesis of **1** with allylbromide in the presence of sodium and methanol led to compound **2**. Subsequent Claisen-rearrangement under neat microwave conditions gave substance **3** after 75min quantitatively compared to conventional thermal heating for 7 days, as reported previously.[71] Methylation of **3** with dimethylsulfate completed this series and led to the desired allyltrimethoxybenzene **4** in 77% yield after three steps.

[69] a) Johnston, B.D.; Czyzewska, E.; Oehlschlager, A.C. *J. Org. Chem.* **1987**, *52*, 3693-3697; b) Mehta, G.; Rao, P.S.H. *Synthetic Commun.* **1985**, *15*, 991-1000.

[70] a) Alphand, V.; Mazzini, C.; Lebreton, J.; Furstoss, R. *J. Mol. Catal. B: Enzym.* **1998**, *5*, 219-221; b) Gagnon, R.; Grogan, G.; Goussain, E.; Pedragosa-Moreau, S.; Richardson, P.F.; Roberts, S.M.; Willets, A.J.; Lebreton, R.; Furstoss, R. *J.Chem. Soc. Perkin Trans 1* **1995**, 2527-2528.

[71] Jing, X.; Gu, W.; Ren, X.; Bie, P.; Pan, X. *J. Chin. Chem. Soc.* **2001**, *48*, 59-63.

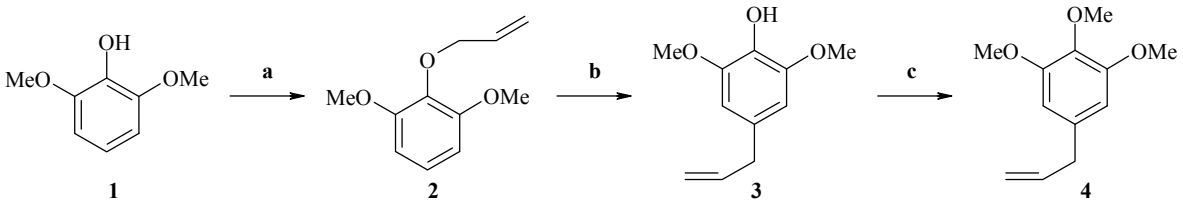


Figure 2.5 a) allylbromide, Na/MeOH, rf, 83%; b) MW, 250W, 180°C, in substance, quant.; c) Me_2SO_4, KOH, 93%;

Conditions and yields for the synthesis of cyclobutanones are summarized in Table 2.1. Following the method of Johnston *et al.*,[69a] 2,2-dichloro-cyclobutanones were prepared by cyclization of an alkene using trichloroacetyl chloride and zinc/copper couple under thermal or ultrasound conditions. About 10 mol% of 1,2-dimethoxyethane were added to the solvent (dry diethyl ether). The trichloroacetyl chloride reacts as dichloroketene ($Cl_2C=C=O$) in a [2+2] cycloaddition with the alkene, resulting in the 2,2-dichloro-cyclobutanones. 1,2-Dimethoxyethane is added to complex the formed $ZnCl_2$-salts.

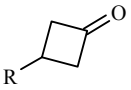
		**Cyclization conditions**	**Reduction conditions**	**Yield (over 2 steps)**
n-Bu	**5a**	thermal	AcOH/Zn; rf	41%
i-Bu	**6a**	thermal	AcOH/Zn; rf	59%
Bn	**7a**	ultrasound	AcOH/Zn; rf	49%
Ph	**8a**	thermal	AcOH/Zn; rf	61%
Bn-O-CH_2	**9a**	thermal	AcOH/Zn; rf	37%
4-MeO-Bn	**10a**	thermal	AcOH/Zn; rf	76%
4-Cl-Ph	**11a**	thermal	AcOH/Zn; rf	59%
3-MeO-Bn	**12a**	thermal	AcOH/Zn; rf	51%
piperonyl	**13a**	thermal	AcOH/Zn; rf	56%
3,4,5-tri-MeO-Bn	**14a**	thermal	AcOH/Zn; rf	48%

Table 2.1 Reaction conditions and yields for the synthesis of prochiral cyclobutanones *via* [2+2] cycloaddition.

A key aspect for a successful [2+2] cycloaddition reaction to the desired cyclobutanone derivatives was the quality of the activated zinc species and all other chemicals. It was mandatory to use always freshly synthesized Cu/Zn couple. The reactivity of such Cu/Zn species was decreasing dramatically upon storage for more than two days under argon

atmosphere. Hence, fresh distillation of trichloro acetylchloride and dry solvents are further required for a successful cyclization. However, Table 2.1 compiles experimental data for the best reaction conditions for the [2+2] cycloaddition.

The synthesis of compound **14a** was more difficult than expected. Unfortunately, the desired dichlorocyclobutanone was very insensitive for MS detection and therefore the reaction control turned out to be more sophisticated. However, the crucial step in this reaction was the preparation of an activate Zn species, which could be performed in different ways. The most common procedure in the literature was the reaction of copper acetate in the presence of glacial acid and zinc dust under reflux conditions. Application of the so generated reagent in the cyclization step with compound **4** did not display formation of the desired product. After intensive literature search, a different method for the activation of zinc was found, whereas zinc dust was suspended in degassed water under N_2-atmosphere and after 30 minutes copper sulfate was added. The black slurry mixture was stirred for another 45 minutes and was then filtered and washed with degassed acetone. Combination of the classical thermal cyclization conditions and the alternative activated zinc gave the desired cyclobutanone **14a** after reduction in 48% yield over 2 steps.

In general, the formed crude 2,2-dichloro-cyclobutanones were then reduced to the respective cyclobutanones by reaction with zinc dust in acetic acid. Overall yields for the synthetic sequence were moderate to good from 37 to 76%.

2.3 Lignans

2.3.1 Introduction and Classification

Lignans constitute a class of natural products with great diversity in structure and biological activity. The term "lignan" represents the woody tissue from which many samples derive and was introduced by Harworth.[72] Classification of lignans can be exemplified according to their carbon skeleton.

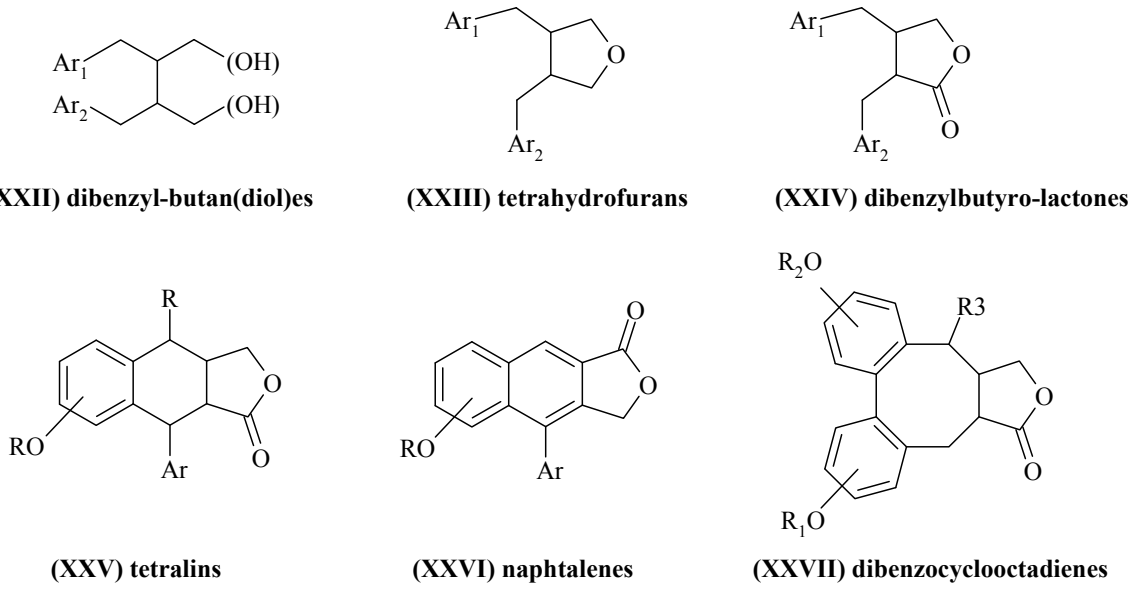


Figure 2.6 Classification of lignans.

The lignans are divided into six subgroups, based on their general structures **XXII-XXVII** (Figure 2.6). Dibenzylbutanes (**XXII**) have C_6C_3 units linked only *via* β-β' (8-8') fashion. Furans (**XXIII**) and lactones (**XXIV**) contain an additional oxygen bridge, whereas tetralins (**XXV**), arylnaphtalenes (**XXVI**) and bridged biphenyls (**XXVII**) possess a second carbon-carbon link.

2.3.2 Biosynthetic Pathway of Lignans

Umezawa *et al.*[73] published the biosynthesis of lignans in *Forsythia* which is initiated by stereoselective coupling of coniferyl alcohol (**XXVIII**) to (+)-pinoresinol (**XXX**) (Figure 2.7). Oxidation of the intermediate to the desired (+)-pinoresinol (**XXX**) is performed by two proteins. One is responsible for defining the proper stereochemistry and does not display any

[72] Harworth, R.D. *J. Chem. Soc*. **1942**, 448-456.

[73] Umezawa, T.; *In Biochemistry and Molecular Biology of Wood*, ed. Higuchi, T.; Springer-Verlag, Berlin and Heidelberg, **1997**, 181-194.

oxidase activity whereas the second protein acts as an oxidase. Sequential stereoselective reduction of (+)-pinoresinol (**XXX**) gives (+)-lariciresinol (**XXXI**) as intermatiate, which is ultimately converted to (-)-secoisolariciresinol (**XXXII**).

Two different isofunctional forms of reductases responsible for this process could be isolated and purified. Both catalyze the sequential, NADPH dependent stereoselective step and show similar kinetic properties and molecular weights.[74] The stereoselectivity of this reaction derives from the inversion of the configuration of the C_2 and C_5 of pinoresinol. The last step for the biosynthesis is the selective dehydrogenation of (-)-secoisolariciresinol (**XXXII**) which leads to the desired (-)-matairesinol (**XXXIII**). Compound (**XXXIII**) represents the branching point to other important groups of lignans such as the tetralin-series.

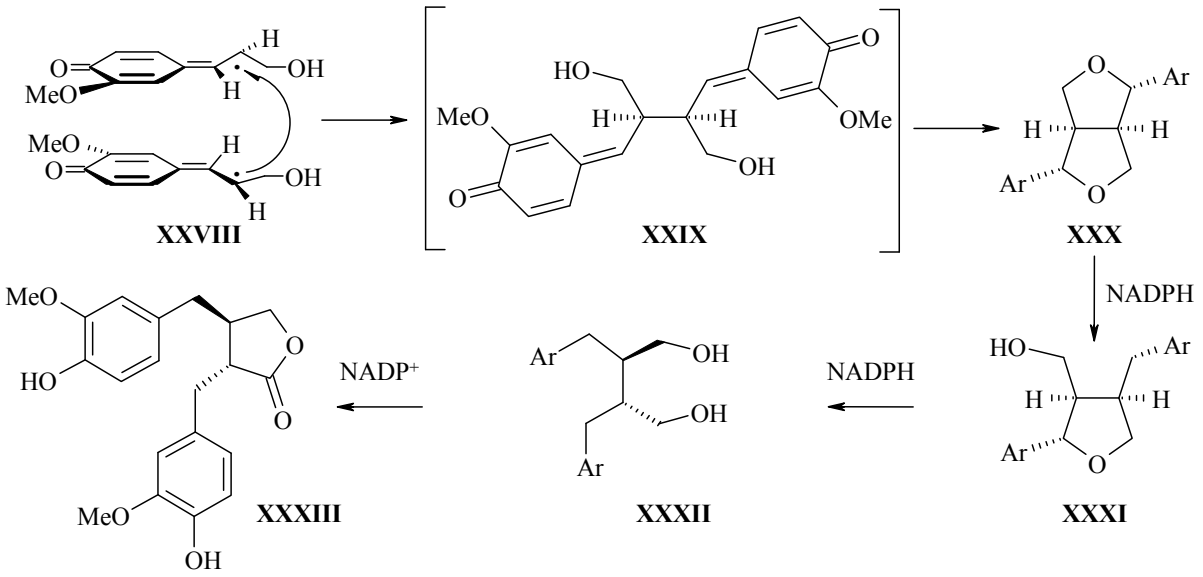


Figure 2.7 Biosynthesis of lignans in *Forsythia*.

2.3.3 Biological Activity of Lignans

Lignans and their derivatives possess significant pharmacological activities. These activities cover a broad range and include in particular antiviral, antitumor, and antifungal properties. Furthermore, they show the ability to specifically inhibit enzymes and possess toxicity to fungi, insects, and invertebrates. Lignans display a biological role at an ecological level, mediating in plant-fungus, plant-plant, and plant-insect relations and last but not least also at the molecular level by interrupting the synthesis of DNA, limiting the transport of nucleotides, and inhibiting enzymes.[75]

Lignans displaying strong activity as potent anti HIV agents include dibenzyl-butyrolactones (**XXIV**), modified aryltetralins (**XXV**) and podophyllotoxin derivatives. Other podophyllotoxin related compounds have been shown to possess immunosuppressive activity

[74] Dinkova-Kostova, A.T.; Gang, D.R.; Davin, L.B.; Bedgar, D.L.; Chu, A.; Norman G. Lewis, N.G. *J. Biol. Chem*. **1996**, 29473-29482.

[75] MacRae, W.D.; Towers, G.H.N. *Phytochemistry* **1984**, *23*, 1207-1220.

and are considered as promising candidates for applications in organ transplantation.[76] Most of the interest in podophyllotoxin derivatives focuses on their role in cancer therapy. More than 200 natural and semisynthetic podophyllotoxin derivatives are known.[77] Due to their pharmacological potential lignans have been the targets of extensive synthetic research over the last two decades.

2.3.4 Asymmetric Microbial Baeyer-Villiger Oxidation in the Synthesis of Lignans

Lignans have been long recognized as challenging targets for organic synthesis due to their diverse structural features.[78] They represent valuable target molecules for asymmetric synthesis due to the clearly defined relative configuration of the chiral centers present. Additionally, they provide a framework for developing asymmetric synthetic methods applicable to substituted aromatic and saturated heterocyclic compounds.
Within the past decades different synthetic approaches for the synthesis of chiral, antipodal butyrolactones were investigated. Brown *et al*. published a condensation – hydrogenation strategy, whereas the appropriate Stobbe condensation products as shown in Figure 2.8 were hydrogenated and resolved to prepare the desired butyrolactones (**(-)-XXXIVb**, (+)-**XXXIVb**).[79] Indeed, selective asymmetric hydrogenation circumvents the separation step and provides access to the enantiopure lactones, as well.[80]

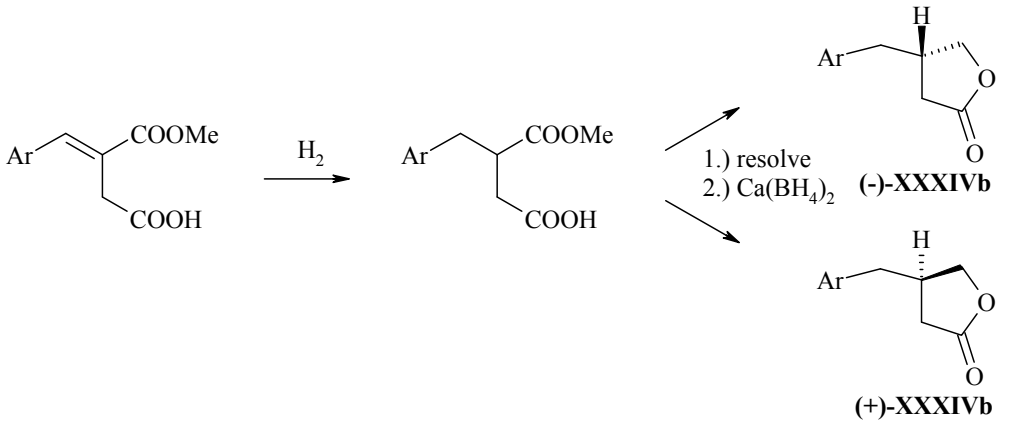


Figure 2.8 Asymmetric approach for the synthesis of butyrolactones by Brown *et al*.

[76] a) Lee, C.T.-L.; Lin, V.C.-K.; Zhang, S.-X.; Zhu, X.-K.; van Vliet, D.; Hu, H.; Beers, S.A.; Wang, Z.-Q.; Cosentino, L.M.; Morris-Natschke, S.L.; Lee, K.-H. *Bioorg. Med. Chem. Lett*. **1997**, *7*, 2897-2902; b) Eich, E.; Pertz, H.; Kaloga, M.; Schulz, J.; Fesen, M.R.; Mazumder, A.; Pommier, Y. *J. Med. Chem*. **1996**, *39*, 86-95; c) Yang, L.-M.; Lin, S.-J.; Yang, T.-H.; Lee, K.-H. *Bioorg. Med. Chem. Lett*. **1996**, *6*, 941-944.

[77] Bohlin, L.; Rosen, B. *Drug Discovery Today* **1996**, *1*, 343-351.

[78] Ward, R.S. *Chem. Soc. Rev.* **1982**, 75-125.

[79] a) Brown, E.; Daugan, A. *Tetrahedron* **1989**, *45*, 141-154; b) Brown, E.; Daugan, A. *Tetrahedron Lett.* **1986**, *27*, 3719-3722; c) Brown, E.; Daugan, A. *Tetrahedron Lett*. **1985**, *26*, 3997-3998.

[80] Kawano, H.; Ishii, Y.; Ikariya, T.; Saburi, M.; Yoshikawa, S.; Uchida, Y.; Kukobayashi, H. *Tetrahedron Lett*. **1987**, *28*, 1905-1908.

Another approach published by Kosugi *et al*. (Figure 2.9) was based on the stereoselective cyclization (Pummerer rearrangement) of alkenyl sulfoxides with dichloroketene which leads to the same precursors (**(-)-XXXIVb**, (+)-**XXXIVb**) in high enantiomeric excess (94% and 96%).[81]

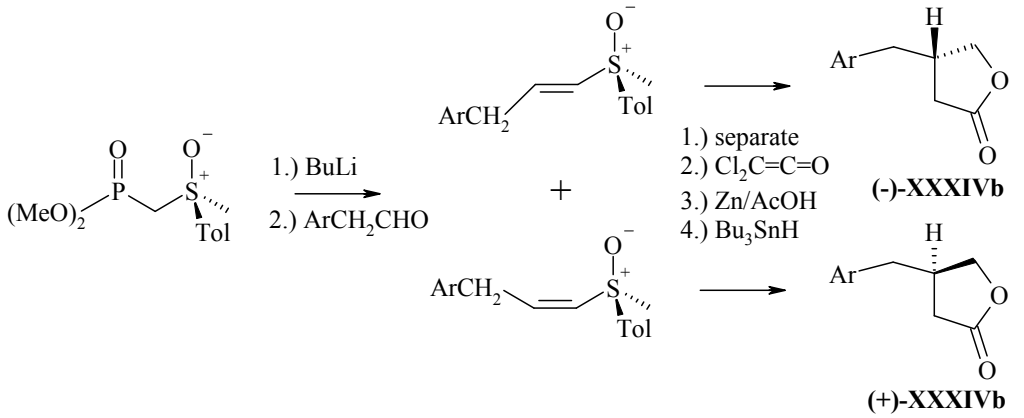


Figure 2.9 Cyclization strategy utilizing alkenyl sulfoxides and dichloroketene by Kosugi *et al*.

Biocatalysis offers another and completely different approach for the synthesis of antipodal butyrolactones. Arylallyl compounds can be cyclized via a [2+2] cycloaddition reaction and subsequent reductive dehalogenation to prochiral cyclobutanones. These compounds can be transformed easily *via* an enantioselective Baeyer-Villiger process into the required chiral butyrolactone intermediates. Due to the easily accessible arylallyl substrates and the well known and established [2+2] cycloaddition reaction a versatile library of useful prochiral cyclobutanones can be synthesized (Figure 2.10).

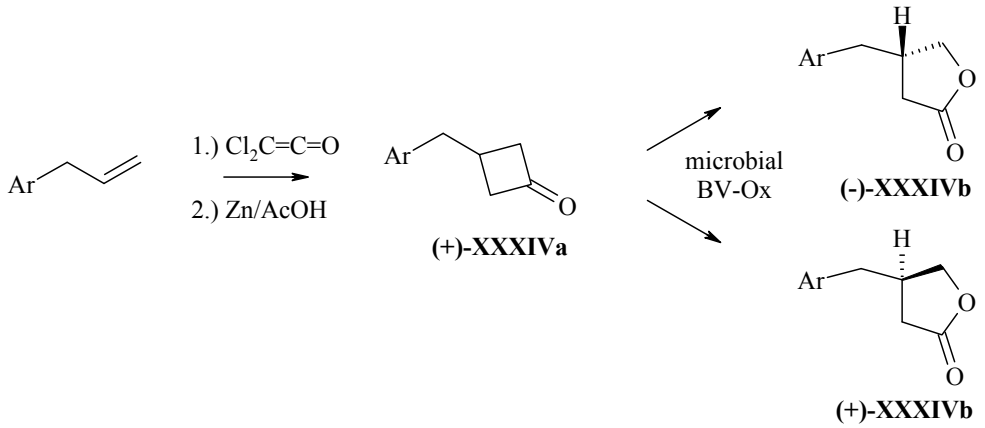
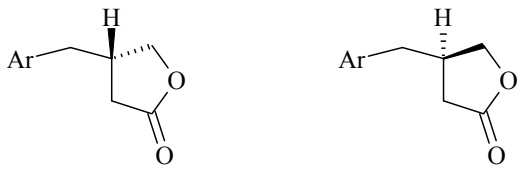


Figure 2.10 Microbial Baeyer-Villiger oxidation approach for the synthesis of antipodal butyrolactones (**(-)-XXXIVb**, (+)-**XXXIVb**)

[81] Kosugi, H.; Tagami, K.; Takahashi, A.; Kanna, H.; Uda, H. *J. Chem. Soc. Perkin 1* **1989**, 935-944.

The diastereoselective alkylation and acylation of chiral benzylbutyrolactones represents one of the best known and earliest approaches to the asymmetric synthesis of lignans.[82]



(-)-XXXIVb **(+)-XXXIVb**

Figure 2.11 Antipodal butyrolactones as key intermediates for asymmetric synthesis of lignans.

Stereoselective alkylation of lactone (-)-**XXXIVb** with LDA and benzyl bromide afforded a variety of lignans depending of the aryl residues: cytostatic (+)-hinokinin (starting from lactone (-)-**13b** by alkylation with 5-bromomethyl-benzo[1,3]dioxole), (+)-enterloactone (accessible by lactone (-)-**12b,** *via* alkylation with 1-bromomethyl-3-methoxy-benzene), and (+)-cordigerine (starting from ((-)-**14b**, alkylation with 5-bromomethyl-1,2,3-trimethoxy-benzene), which has proved to be a feeding deterrent to various insects. Subsequent reduction of enterolactone leads to the corresponding diol (+)-enterodiol (Figure 2.12).

Enterolactone is a lignan produced by fermentation of dietary precursors in the human gut. Lignans consumed in plant foods are metabolized by colonic bacteria to more biologically active metabolites: both the parent compounds and the metabolites are measurable in various body fluids.[83,84,85,86] Thus, circulating and excreted concentrations of enterolactone may be useful biomarkers of exposure to lignan-containing food.

Moreover, enterolactone has been shown to have antitumor and antiangiogenic effects in human cell lines, offering some biological plausibility for the hypothesis that enterolactone exposure reduces the risk of some types of cancer. Epidemiological studies have explored associations between urinary and circulating lignan concentrations and breast cancer.[87,88,89] Therefore, total synthesis of this compound may lead to new paths for the medication of cancer patients.

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[82] Ward, R.S. *Tetrahedron* **1990**, *46*, 5029-5041, and references therein.

[83] Nurmi, T.; Adlercreutz, H. *Anal. Biochem.* **1999**, *274,* 110-117.

[84] Nesbitt, P. D.; Lam, Y.; Thompson, L.U. *Am. J. Clin. Nutr.* **1999**, *69*, 549-555.

[85] Morton, M.S.; Chan, P.S.; Cheng, C.; Blacklock, N.; Matos-Ferreira, A.; Abranches-Monteiro, L.; Correia, R.; Lloyd, S. Griffiths, K. *Prostate* **1997**, *32*, 122-128

[86] Adlercreutz, H.; Fotsis, T.; Kurzer, M.S.; Wähälä, K.; Mäkelä, T.; Hase, T. *Anal. Biochem.* **1995**, *225*, 101-108.

[87] den Tonkelaar, I.; Keinan-Boker, L.; van't Veer, P.; Arts, C.J.M.; Adlercreutz, H.; Thijssen, J.H.H.; Peeters, P.H.M. *Cancer Epidemiol. Biomark. Prev.* **2001**, *10*, 223-228.

[88] Pietinen, P.; Stumpf, K.; Mannisto, S.; Kataja, V.; Uusitupa, M.; Adlercreutz, H. *Cancer Epidemiol. Biomark. Prev.* **2001**, *10*, 339-344.

[89] Ingram, D.; Sanders, K.; Kolybaba, M.; Lopez, D. *Lancet* **1997**, *350*, 990-994.

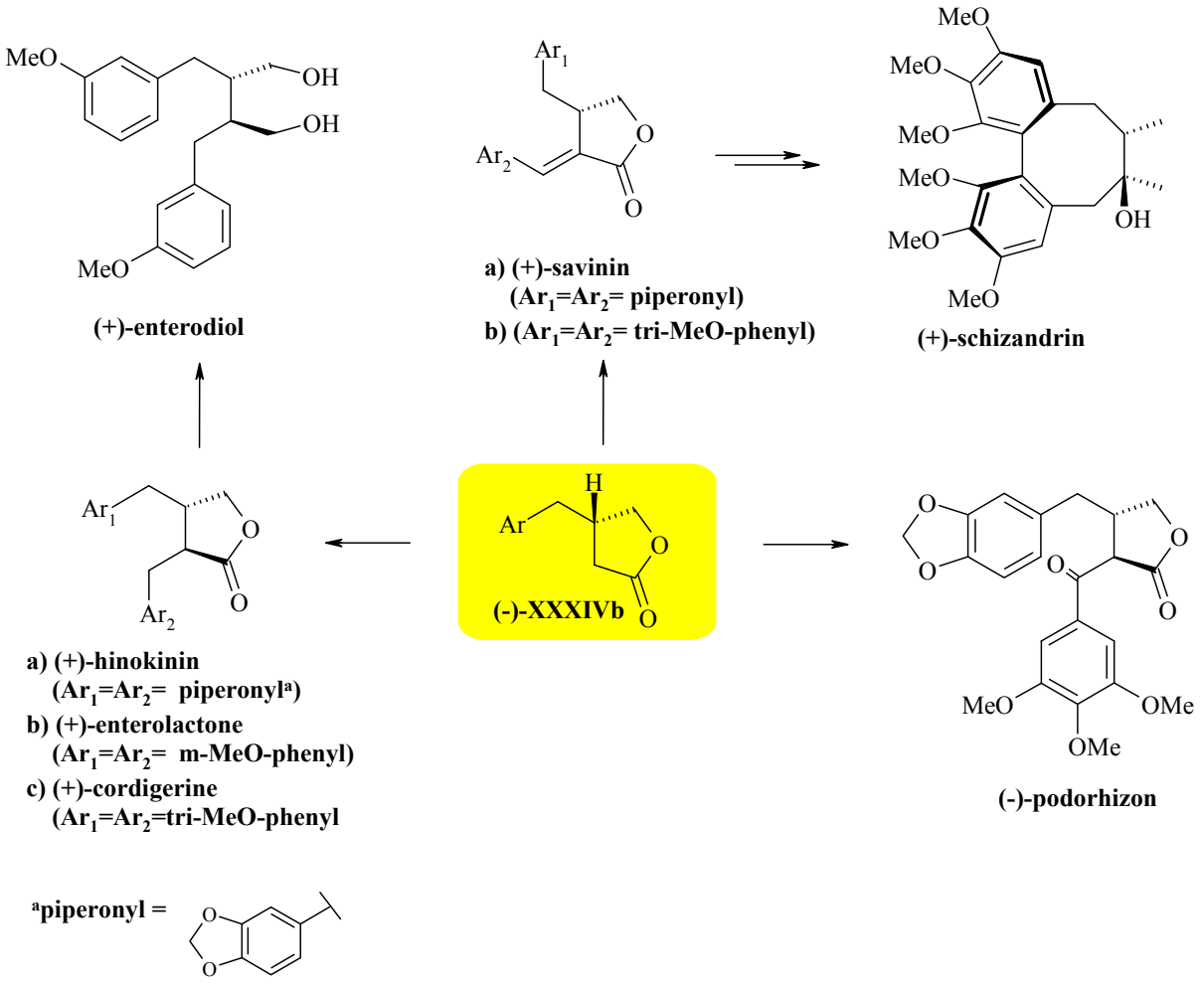


Figure 2.12 Butyrolactone (-)-**XXXIVb** serves as a platform for the synthesis of various lignans.,

Condensation of mono benzyl butyrolactone gave (+)-savinin (starting from (-)-**13b**, benzo[1,3]dioxole-5-carbaldehyde), a valuable precursor for the synthesis of various dibenzocyclooctadiene lignans like (+)-schizandrin (accessible by (-)-**14b**, condensation with 3,4,5-trimethoxy-benzaldehyde). Antioxidant activity and inhibition of cyclic AMP phosphodiesterase of dibenzocyclooctadiene lignans from *Schisandraceae* were reported.[90] Hence, lignans from *Schisandra chinesis* can be used in the treatment of hepatitis, can cause a lowering of levels of serum glutamic-pyruvic transmaniase and can inhibit the central nervous system.[75]

Aldol addition of 3,4,5-trimethoxy-benzaldehyde in the presence of LDA with antipodal butyrolactones (+)-**XXXIVb** and subsequent treatment with trifluoroacetic acid afforded (-)-deoxyisopodophyllotoxin (from (+)-**13b**) (Figure 2.13). The antineoplastic and antiviral

[90] a) Lu, H.; Liu, G.-T. *Planta Med*. **1992**, *58*, 311-313; b) Sakurai, H.; Nikaido, T.; Ohmoto, T.; Ikeya, Y.; Mitsuhashi, H. *Chem. Pharm. Bull*. **1992**, *40*, 1191-1195.

activities of aryltetralin and arylnaphthalene ligans have been evaluated, and deoxypodophyllotoxin represents one of the most potent compounds in all cases.[91]

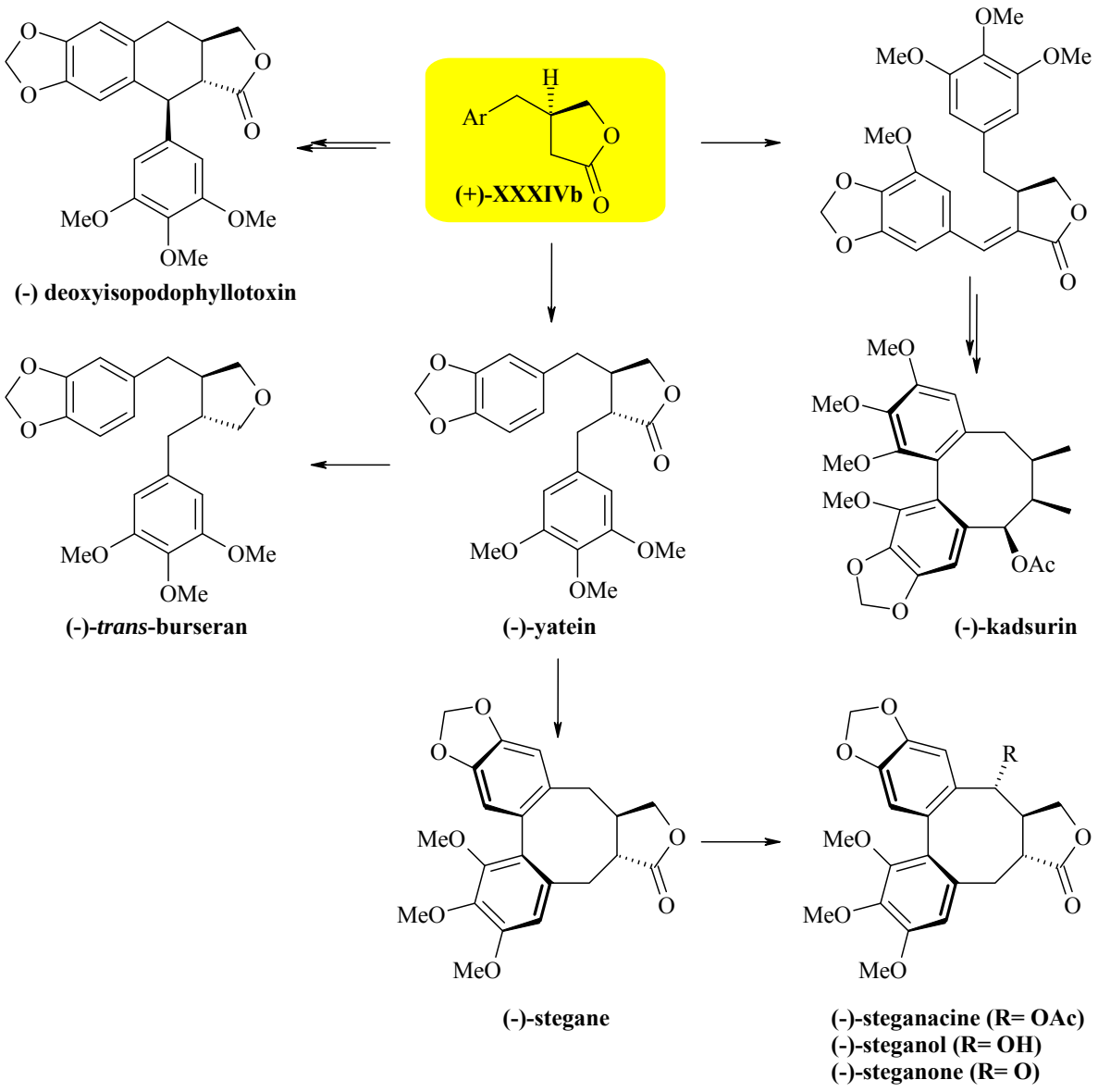


Figure 2.13 Butyrolactone **XXXIVb** serves as a platform for the synthesis of various lignans;

(-)-Yatein (obtained from (+)-**13b** *via* alkylation with 5-bromomethyl-1,2,3-trimethoxy-benzene) can be transformed to other compounds of the lignan series *via* reduction and subsequent cyclization. (-)-*trans*-Burseran (strating from (+)-**13b**) differs from (-)-yatein only in the presence of a furanring rather than a lactone ring but both compounds possess antitumor activity.[75] Intramolecular oxidative coupling of (-)-yatein gave (+)-isostegane which isomerizes under heating immediately to (-)-stegane.

[91] San Feliciano, A.; Gordalizia, M.; Miquel del Corral, J.M.; Castro, M.A.; Garcia-Gravalos, M.D.; Ruiz-Lazaro, P. *Planta Med*. **1993**, *59*, 246-249.

Subsequent acetoxylation of (-)-stegane by benzylic hydroxylation using N-bromo succinimide in aqueous tetrahydrofuran gave (-)-steganol which can be easily oxidized to (-)-steganone. Conventional acetylation of (-)-steganol gave (-)-steganacine.[82] This class of lignans has also been reported to show significant antitumor activity against P388 leukemia in mice and *in vitro* activity against cells derived from human carcinoma.[92] Steganacin, like other spindle poisons, such as podophyllotoxin, exerts its antimitotic activity by affecting the polymerization of spindle microtubules.[92]

2.4 Biotransformations of Prochiral Cyclobutanones with Recombinant Whole-cells Expressing BVMOs

Tables 2.2-2.4 summarize the results for stereoselective biotransformations by whole-cells expressing all BVMOs of the enzyme collection with respect to enantioselectivity in order to provide an overview of the stereopreference of individual biocatalysts.
The assignment of absolute configuration for the obtained butyrolactones is based on previous literature. All biooxidations were carried out on preparative scale. Purification of lactones was generally performed by column chromatography and was analyzed by chiral GC and NMR. Conversion of prochiral cyclobutanones with BVMO producing cells gave good to acceptable yields of the desired butyrolactones.

Full details for selected biotransformations of prochiral cyclobutanones to the corresponding lactones are given in Table 2.5. The selection is based on our recently introduced concept of two families of BVMOs providing access to enantiocompelmentary lactones.[66] Within this study we outlined two distinct clusters based on biocatalyst performance and phylogenetic relationship: CPMO-type biocatalysts include $CPMO_{Coma}$ and $CHMO_{Brevi2}$, the other six enzymes form the CHMO-type group. One representative for each group is included in Table 2.5.

Biooxidation of n-Butylcyclobutanone **5a** gave exclusively the (S) enantiomer in good yields. Interestingly, transformation of **5a** with recombinant cells from $CHMO_{Brevi1}$ gave the desired butyrolactone in highest enantioselectivity (>99% ee, (S)-**5b**), whereas represeantatives of the CPMO-cluster like $CPMO_{Coma}$. (76% ee, (S)-**5b**) and from the CHMO-group such as $CHMO_{Acineto}$ (17% ee, (S)-**5b**) displayed significantly lower stereoselectivity. BV-oxidation of **5a** with $CHMO_{Rhodo1}$ and $CHMO_{Rhodo2}$ gave the desired lactone **5b** only in racemic mixture. Similar results were obtained for the biotransformation of i-butyl cyclobutanone **6a**. Obtained yields were moderate to acceptable and best stereoselectivity was again observed for $CHMO_{Brevi1}$ (>99% ee, (S)-**6b**), superseeding previously reported biooxidation results.

[92] Tomioka, K.; Ishiguro, T.; Mizuguchi, H.; Komeshima, N.; Koga, K.; Tsukagoshi, S.; Tsuruo, T.; Tashiro, T.; Tanida, S.; Kishi, T. *J. Med. Chem.* **1991**, *34*, 54-57.

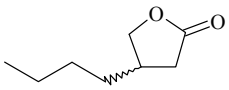
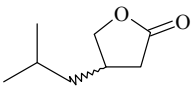
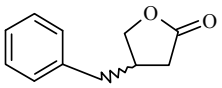
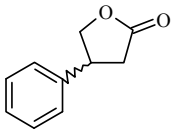
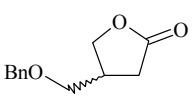
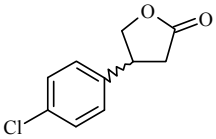
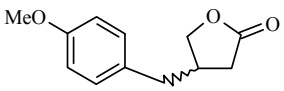
Product				
	5b	**6b**	**7b**	**8b**
$CHMO_{Acineto}$	62% 17% ee, (-)	53% rac.	32% 88% ee, (-)	53% 62% ee, (-)
$CHMO_{Arthro}$	51% 32% ee, (-)	41% 74% ee, (-)	56% 93% ee, (-)	54% 87% ee, (-)
$CHMO_{Brachy}$	55% 14% ee, (-)	24% 77% ee, (-)	38% 84% ee, (-)	45% 93% ee, (-)
$CHMO_{Brevi1}$	65% >99% ee, (-)	30% >99% ee, (-)	30% 93% ee, (-)	73% 98% ee, (-)
$CHMO_{Brevi2}$	74% 69% ee, (-)	43% 22% ee, (-)	27% 59% ee, (-)	50% 39% ee, (+)
$CPMO_{Coma}$	72% 76% ee, (-)	63% 76% ee, (-)	37% 31% ee, (-)	66% 37% ee, (+)
$CHMO_{Rhodo1}$	54% rac.	35% 79% ee, (-)	33% 87% ee, (-)	58% 52% ee, (-)
$CHMO_{Rhodo2}$	58% rac.	40% 45% ee, (-)	31% 87% ee, (-)	63% 50% ee, (-)

Table 2.2 Desymmetrization of prochiral cyclobutanones.

Aryl containing cyclobutanones **7a** and **8a** reflected the classification according to our "two enzyme family" hypothesis to a larger extent. Proteins belonging to the CHMO-family like $CHMO_{Arthro}$ (93% ee, (R)-**7b**) or $CHMO_{Brachy}$ (93% ee, (R)-**8b**) usually gave (-)-lactones in good enantioselectivity. CPMO-type biocatalysts displayed a significantly lower stereoselectivity for lactone **7b** ($CPMO_{Coma}$: 31% ee, (R)-**7b**) and produced antipodal product **8b** ($CHMO_{Brevi2}$: 39% ee, (S)-**8b**). Again, biooxidations with $CHMO_{Brevi1}$ gave very good results with both substrates. During biooxidation of cyclobutanone **8a** formation of a by-product was observed. After isolation and NMR analysis it was characterized as 3-phenyl-cyclobutanol **8c**. It turned out that cyclobutanone **8a** is a rather slow substrate for BVMOs but obviously is readily accepted by (a) reductase(s) originated from the host organism.

Lactones **9b**, **10b** and **11b** represent valuable key intermediates for a variety of natural and bioactive compounds. Therefore we focused our investigations on this specific set of substrates. Substrate **9a** was biooxidized with all protein expressing strains and again both antipodal stereoisomers were obtained. This substrate represents the first in this series where no predictions of enantioselectivity and substrate acceptance could be made based on the hypothesis of two BVMO classes. Within the CHMO-family, results ranged from moderate

enantioselectivity ($CHMO_{Arthro}$, 58% ee, (R)-**9b**) to racemic biooxidation. Transformation of **9a** with $CHMO_{Brevi1}$ gave the antipodal lactone **9b** in moderate optical purity (55% ee, (S)-**9b**).

Product			
	9b	**10b**	**11b**
$CHMO_{Acineto}$	41% 53% ee, (+)	67% 81% ee, (+)	55% 97% ee, (-)
$CHMO_{Arthro}$	18% 58% ee, (-)	50% 87% ee, (+)	89% 97% ee, (-)
$CHMO_{Brachy}$	22% rac.	57% 68% ee, (+)	64% 90% ee, (-)
$CHMO_{Brevi1}$	26% 55% ee, (+)	47% 87% ee, (-)	73% 26% ee, (-)
$CHMO_{Brevi2}$	52% 62% ee, (-)	50% 42% ee, (+)	64% 24% ee, (+)
$CPMO_{Coma}$	53% 63% ee, (-)	78% 44% ee, (+)	56% 24% ee, (+)
$CHMO_{Rhodo1}$	45% 6% ee, (+)	51% 95% ee, (+)	53% 80% ee, (-)
$CHMO_{Rhodo2}$	23% 9% ee, (+)	63% 95% ee, (+)	56% 95% ee, (-)

[a]Biotransformation ceased after 36 hours and did not reach completion

Table 2.4 Desymmetrization of prochiral cyclobutanones.

p-Chloro-phenyl butyrolactone **10b**, a precursor for the synthesis of a $GABA_B$ receptor agonist, was obtained in both antipodal stereoisomers. Whereas $CHMO_{Rhodo1}$ produced the (S)-enantiomer (95% ee, (S)-**10b**), again $CHMO_{Brevi1}$ gave the opposite stereoisomer in good optical purity (87% ee, (R)-**10b**).

Biooxidation of p-methoxyphenyl-cyclobutanone **11a**, a precursor for the synthesis of eptazocine, gave the expected lactone **11b** with all expression strains. Comparison of specific rotation with previously published literature data showed, that $CHMO_{Arthro}$ gave (S)-enantiomer **11b** in excellent optical purity (97% ee, (S)-**11b**), while $CHMO_{Brevi2}$ generated antipodal lactone (R)-**11b** in poor enantioselectivity (24% ee). It is noteworthy, that $CHMO_{Brevi1}$ again gave different results (26% ee, (S)-**11b**) than the other enzymes from the CHMO-familiy.

m-Methoxyphenyl-cyclobutanone **12a** as precursor for enterolactone gave lactone **12b** in excellent enantioselectivity (98% ee, (S)-1**2b**, $CHMO_{Rhodo1}$) with enzymes from the "CHMO-family", whereas CPMO-type enzymes and $CHMO_{Brevi1}$ gave moderate to poor enantioselectivities (35-45% ee, (S)-**12b**). Interestingly, no antipodal m-methoxyphenyl-butyrolactone was obtained within this collection of Baeyer-Villiger monooxygenases and a clear deviation of the behaviour of $CHMO_{Brevi1}$ was observed compared to biooxidations by the other CHMO-type enzymes.

Two other interesting compounds for biooxidations were piperonyl cyclobutanone **13a** and the 3,4,5-trimethoxybenzyl analog **14a**. The corresponding lactones (**13b**, **14b**), obtained in both antipodal forms, are valuable precursors for the synthesis of various lignans (Figure 2.11, 2.12). The CHMO family, like $CHMO_{Rhodo2}$ (with the exception of $CHMO_{Brevi1}$) converted ketone **13a** to (-)-butyrolactone **13b** with excellent stereoselectivity (98% ee, (S)-**13b**). CPMO-type enzymes gave low enantiomeric excess (37-40% ee, (S)-**13b**). Antipodal (+)-butyrolactone **13b** was obtained in acceptable enantioselectivity (75% ee, (R)-**13b**) and moderate yield only upon oxidation with $CHMO_{Brevi1}$.

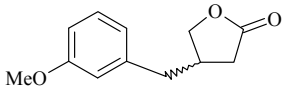
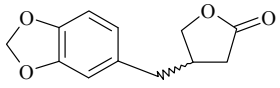
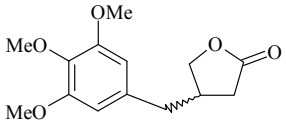
Product			
	12b	**13b**	**14b**
$CHMO_{Acineto}$	50% >99% ee, (-)	35% 97% ee, (-)	86% 90%, ee, (-)
$CHMO_{Arthro}$	43% 93% ee, (-)	35% 98% ee, (-)	72% 94% ee, (-)
$CHMO_{Brachy}$	45% 93% ee, (-)	60% 98% ee, (-)	58% 94% ee, (-)
$CHMO_{Brevi1}$	74% 35% ee, (-)	61% 75% ee, (+)	72% 79% ee, (+)
$CHMO_{Brevi2}$	50% 45% ee, (-)	53% 37% ee, (-)	n.c.
$CPMO_{Coma}$	70% 45% ee, (-)	56% 40% ee, (-)	n.c.
$CHMO_{Rhodo1}$	60% 98% ee, (-)	45% 98% ee, (-)	67% 95% ee, (-)
$CHMO_{Rhodo2}$	72% 98% ee, (-)	52% 98% ee, (-)	55% 92% ee, (-)

Table 2.3 Desymmetrization of prochiral cyclobutanones.

Based on its great potential in natural product synthesis, microbial biooxidations of substrate **14a** was investigated for the first time. The enzymatic Baeyer-Villiger oxidation showed a

similar trend as with other oxygen-functionalized benzylketones: Representatives of the CHMO-cluster accept the substrate readily and the corresponding lactone **14b** was obtained with excellent enantioselectivity ($CHMO_{Arthro}$: 95% ee, (S)-**14b**). Compound **14a** was essentially no substrate for CPMO-type biocatalysts.

Again, an interesting behavior was observed for $CHMO_{Brevi1}$ which provided antipodal lactone **14b** in acceptable enantioselectivity (79% ee, (R)-**14b**) and yield.
In summary, the present study demonstrates the potential of a library of bacterial BVMOs to produce antipodal butyrolactones. The results for oxidation of prochiral cyclobutanones are interesting for the concept of our previous attempts to classify BVMOs by their biocatalytic and stereoselective performance. So far, we had outlined a hypothesis of two BVMO groups, which is based on protein sequence alignment and catalytic performance.[66] This clustering into two groups providing access to antipodal lactones is particularly pronounced on substrates with large energy differences for various conformational forms. Adopting axial or equatorial positions of substitutents at the cyclic core of substrate ketones seems to determine the final stereochemical outcome of the biotransformation.[93] Within six- and five-membered systems, the energy difference of these two conformations seems large enough to be affected by the particular nature of the substituent only to a minor degree. However, a much more pronounced effect by individual functional groups is observed in the cyclobutanone series. Consequently, already minor additional interactions between substrate and parts of the active site of the BVMO can affect the orientation of the ketone within the enzyme, leading to diverse migratory preferences and, ultimately, to different antipodal products. Consequently, subtle differences within the active site of BVMOs can have significant influence on the stereoselectivity of the enzyme, blurring the classification into two clusters.
It is interesting to observe, that within this class of substrates $CHMO_{Brevi1}$ displays enantiocomplementary behavior compared to the other members of the BVMO collection studied. Already within our previous contributions on the clustering of cycloketone accepting BVMOs we noticed the borderline position of this enzyme between the CHMO- and the CPMO-group in the phylogenetic sequence analysis.

Substrate acceptance profiles are in agreement with our two enzyme family theory, while data on enantiopreference gave a somewhat different picture. Biotransformation of compounds **5-8a** with $CHMO_{Brevi1}$ gave the best enantioselectivities. In several cases, no formation of antipodal lactones was observed by the two enzyme clusters, but enantioselectivities for CPMO-type biocatalysts were usually low (**5-7b**, **12b**, **13b**) or no conversion was observed (**14b**). Opposite stereoisomeric lactones were produced by representatives of the two biocatalyst families in the case of substrates **8a** and **10a**, with no clear trend in the case of **9a**. With three substrates, access to antipodal lactones was enabled only by biooxidation using $CHMO_{Brevi1}$ (**11a, 13a**, **14a**).

[93] a) Clouthier, C.M.; Kayser, M.M.; Reetz, M.T. *J. Org. Chem.* **2006**, *71*, 8431-8437; b) Kayser, M.M.; Clouthier, C.M. *J. Org. Chem.* **2006**, *71*, 8424-8430; c) Mihovilovic, M.D.; Chen, G.; Wang, S.; Kyte, B.; Rochon, F.; Kayser, M.M.; Stewart, J.D. *J. Org. Chem.* **2001**, *66*, 733-738.

Product	Enzyme	Yield	*ee*	abs. config.	$[\alpha]^{D}_{20}$
5b	$CHMO_{Acineto}$	62%	17%	(S)[70b]	-2.05 (*c* 2.10, $CHCl_3$)
	$CHMO_{Brevi1}$	65%	>99%	(S)	-5.95 (*c* 2.20, CHCl3)
	$CPMO_{Coma}$	72%	76%	(S)	-4.46 (*c* 2.42, CHCl3)
6b	$CHMO_{Acineto}$	53%	rac	n.d.[70a]	n.a.
	$CHMO_{Brevi1}$	30%	>99%	(S)	-1.47 (*c* 1.16, $CHCl_3$)
	$CPMO_{Coma}$	63%	76%	(S)	-1.02 (*c* 2.46, $CHCl_3$)
7b	$CHMO_{Arthro}$	56%	93%	(S)[70a]	-5.35 (c 1.31, $CHCl_3$)
	$CHMO_{Brevi1}$	30%	93%	(S)	-12.8 (c 0.70, $CHCl_3$)
	$CPMO_{Coma}$	37%	31%	(S)	-2.20 (c 0.86, $CHCl_3$)
8b	$CHMO_{Brachy}$	45%	93%	(R)[70a]	-45.0 (*c* 1.00, MeOH)
	$CHMO_{Brevi1}$	73%	98%	(R)	-47.3 (*c* 1.80, MeOH)
	$CPMO_{Coma}$	66%	37%	(S)	+16.9 (*c* 1.50, MeOH)
9b	$CHMO_{Arthro}$	18%	58%	(R)[70a]	-19.1 (*c* 0.40, $CHCl_3$)
	$CHMO_{Brevi1}$	26%	55%	(S)	+17.1 (*c* 0.66, $CHCl_3$)
	$CPMO_{Coma}$	53%	63%	(R)	-18.9 (*c* 1.34, $CHCl_3$)
10b	$CHMO_{Arthro}$	89%	97%	(S)[64]	-6.11 (c 0.95, $CHCl_3$)
	$CHMO_{Brevi1}$	73%	26%	(S)	-2.10 (c 1.60, $CHCl_3$)
	$CHMO_{Brevi2}$	64%	24%	(R)	+1.08 (c 0.74, $CHCl_3$)
11b	$CHMO_{Rhodo2}$	63%	95%	(S)[63]	+44.2 (*c* 1.02, $CHCl_3$)
	$CHMO_{Brevi1}$	47%	87%	(R)	-37.6 (*c* 0.54, $CHCl_3$)
	$CHMO_{Brevi2}$	50%	42%	(S)	+16.8 (*c* 1.00, $CHCl_3$)
12b	$CHMO_{Rhodo1}$	60%	98%	(S)[70a]	-6.50 (c 1.42, $CHCl_3$)
	$CHMO_{Brevi1}$	74%	35%	(S)	-2.10 (c 1.60, $CHCl_3$)
	$CHMO_{Brevi2}$	50%	45%	(S)	-1.85 (c 0.27, $CHCl_3$)
13b	$CHMO_{Rhodo2}$	52%	98%	(S)[70a]	-4.24 (*c* 0.75, $CHCl_3$)
	$CHMO_{Brevi1}$	61%	75%	(R)	+2.38 (*c* 1.51, $CHCl_3$)
	$CHMO_{Brevi2}$	53%	37%	(S)	-2.15 (*c* 1.30, $CHCl_3$)
14b	$CHMO_{Arthro}$	72%	94%	(S)[94]	-6.10 (*c* 1.00, $CHCl_3$)
	$CHMO_{Brevi1}$	72%	79%	(R)	+4.38 (*c* 1.00, $CHCl_3$)
	$CHMO_{Brevi2}$	n.c.	n.a.	-	n.a.

Table 2.5 Summary of most representative desymmetrizations of prochiral cyclobutanones on preparative scale.

Summarizing, the potential of the above collection of BVMOs in natural product synthesis *via* butyrolactone intermediates was outlined. Almost all whole-cell mediated biotransformations gave lactones in acceptable to good preparative yield.

In context of this study, we only took advantage of the natural diversity of BVMOs and their promiscuity to accept a multitude of non-natural substrates. While not all biooxidations delivered chiral lactones in excellent optical purities, recent contributions in the field successfully outlined strategies to improve the stereoselectivity of such enzymes.[93]

[94] Tanaka, M.; Mitsuhashi, H.; Maruno, M.; Wakamatsu, T. *J. Org. Chem*. **1995**, 4339-4352.

By discovering BVMOs to produce enantiocomplementary butyrolactones as highly valuable intermediates for subsequent elaboration in natural product syntheses, better entry-points for optimization efforts towards a particular chiral product can be provided, ultimately improving the success chance to fine-tune the catalytic performance of a mutant enzyme.

2.5 Biotransformations of Prochiral Bicyclic Ketones with Recombinant Whole-cells Expressing BVMOs

Results for stereoselective biotransformations of ketones **15a** and **16a** by whole-cells expressing BVMOs of the enzyme library are compiled in Table 2.6. Focused on enantioselectivity we want to provide an overview of the stereopreference of individual proteins. All biotransformations were performed on preparative scale and in general the lactones were purified by column chromatography. Conversion of prochiral bicyclic ketones with BVMO expressing cells gave good to acceptable yields of the desired lactones.

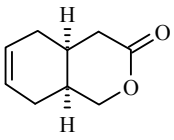
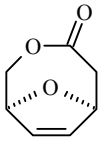
Product		
	15b	**16b**
$CHMO_{Acineto}$	33% 5% ee, (-)	n.c.
$CHMO_{Arthro}$	46% 60% ee, (-)	n.c.
$CHMO_{Brachy}$	56% 85% ee, (-)	n.c.
$CHMO_{Brevi1}$	10% 71% ee, (-)	n.c.
$CHMO_{Brevi2}$	92% 94% ee, (+)	19% 93% ee, (+)
$CPMO_{Coma}$	76% >99% ee, (+)	53%[a] 95% ee, (+)
$CHMO_{Rhodo1}$	47% 73% ee, (-)	n.c.
$CHMO_{Rhodo2}$	51% 73% ee, (-)	n.c.

Table 2.6 Desymmetrization of prochiral bicyclic ketones.

BV-oxidation of ketone **15a** with enzymes from the CHMO-cluster displayed highly diverse results ranging from essentially racemic lactone to good enantioselectivity ($CHMO_{Brachy}$: 85%

ee, (4aS,8aS)-**15b**).[95] Also the yields very only moderate for this group of BVMOs. Again, $CHMO_{Brevi1}$ displays a different biocatalytic behaviour and converted ketone **15a** in very low quantity and moderate enantioselectivity (71% ee, (4aS,8aS)-**15b**). Transformation with enzymes from the CPMO-family yielded the antipodal lactone ($CPMO_{Coma}$: (4aR,8aS)-**15b**) in good yields and excellent optical purity.
Bridged bicyclo ketone **16a** is only oxidized by CPMO-type enzymes to form lactone **16b**. However $CHMO_{Brevi2}$ gave the desired lactone **16b** in very poor yield but very good enantioselectivity (93% ee, (+)-**16b**). Improved yield and optical purity of lactone **16b** was obtained by biotransformation with cells producing $CPMO_{Coma}$ (95% ee, (+)-**16b**).

Conclusively application of microbial Baeyer-Villiger oxidation in the asymmetric synthesis, has reached great potential. The above collection of different BVMOs used in whole-cell mediated biotransformations gave lactones **15b** and **16b** in moderate to good preparative yield. Again, all obtained results were based on the natural diversity of BVMOs and their ability to accept a variety of non-natural substrates. In the case of bicyclo ketone **15a** access to both antipodal lactones were achieved. Hence, not all biooxidations turned out to be highly enantioselective, but recent investigations in the modifaction and optimization of the biocatalytic entity provide improvements of stereoselectivity. [93]
Biocatalytic Baeyer-Villiger oxidations currently offer efficient access to structurally diverse lactones. Biocatalysis represents a practical methodology for the straight forward synthesis of valuable asymmetric building blocks.

[95] Danieli, B.; Lesma, G.; Mauro, M.; Palmisano, G.; Passerella D. *Tetrahedron* **1994**, *50*, 8837-8852.

3 Screening of Mutants

3.1 Stereopreference and Substrate Acceptance of Cyclohexanone Monooxygenase Mutants

Enzymes are increasingly being sought as alternatives to chemical catalysts, particularly as improved recombinant expression systems make them more cost effective. Investigations for enzymes that fit particular applications are now a rapidly expanding research field. However, enzymes isolated from natural sources do not always fulfill the requirements demanded for industrial and biotechnological applications, and the ability of science to modify enzymes to particular specifications based on a rational structure/function approach is still very limited.

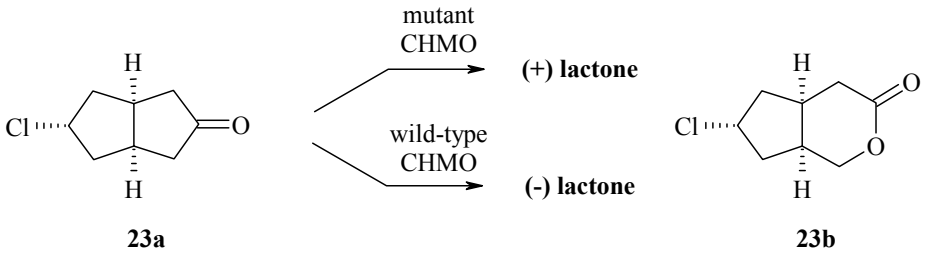


Figure 3.1 General scheme of enantiodivergent Baeyer-Villiger oxidation.

The microbial Baeyer-Villiger oxidation has received substantial attention in recent years as valuable tool for the synthesis of optically pure lactones as attractive intermediates starting from chiral or prochiral ketones.

Until very recently, wide-spread application of such catalytic entities was limited by a general obstacle of biocatalysis: While access to both antipodal (Figure 3.1) forms of a product can be readily achieved with *de novo* designed catalysts (e.g. metal-based), no generally applicable strategy is available to provide enantiocomplementary biocatalysts. Two approaches to overcome this limitation were previously successfully implemented in the area of enzymatic Baeyer-Villiger oxidation by Reetz *et al*[96] and our group. Taking advantage of nature's biodiversity, a platform of recombinant *E. coli* strains overexpressing BVMOs of various microbial origins was introduced.[66] This biooxidation toolbox contains catalysts with overlapping substrate profiles for the conversion of various ketones displaying enantiocomplementary stereospecificity and regiodivergent biotransformations.[97]

Another strategy is based on the modification of a particular biocatalyst of known characteristics. The combination of recent protocols from molecular biology with efficient

[96] Reetz, M.T.; Brunner, B.; Schneider, T.; Schulz, F.; Clouthier, C.M.; Kayser, M.M. *Angew. Chem.Int. Ed.* **2004**, *43*, 4075-4078.

[97] a) Mihovilovic, M.D.; Kapitan, P. *Tetrahedron Lett.* **2004**, *45*, 2751-2754; b) Kelly, D.R.; Knowles, C.J.; Mahdi, J.G.; Taylor, I.N.; Wright, M.A. *J. Chem. Soc., Chem. Commun*. **1995**, 729-730; c) Alphand, V.; Furstoss, R. *J. Org. Chem.* **1992**, *57*, 1306-1309.

screening methods under a directing evolutionary pressure (directed evolution) has been proven a powerful approach[98] towards the design of new asymmetric catalysts.[99]

3.1.1 Directed Enzyme Evolution

Staunch Darwinists attribute all the complexity of living things to an algorithm of mutation and natural selection. The exquisite products of this evolution algorithm are apparent at all levels, from the amazing diversity of life all the way down to individual protein molecules. Scientists and engineers who wish to redesign these same molecules are now implementing their own versions of the algorithm. Directed evolution allows us to explore enzyme functions never required in the natural environment and for which the molecular basis is poorly understood. This bottom-up design approach contrasts with the more conventional, top-down strategy in which proteins are tuned `rationally' using computers and site-directed mutagenesis. Recent advances in the ability to create genetic diversity and to screen or select for improved functions in large libraries of enzyme variants are being combined in a robust approach to solving difficult molecular design problems. With directed evolution we now have the ability to modify individual proteins as well as whole biosynthetic and biodegradation pathways for biotechnology applications.

The major steps in a typical directed enzyme evolution experiment are outlined in Figure 3.2. The genetic diversity for evolution is created by mutagenesis and/or recombination of one or more parent sequences. These altered genes are cloned back into a plasmid for expression in a suitable host organism (bacteria). Clones expressing improved enzymes are identified in a high-throughput screen, or - in some cases - by selection, and the gene(s) encoding those improved enzymes are isolated and reiterated into the next round of directed evolution.

[98] a) Svendsen, A. *Enyzem Functionality – Design, Engineering, and Screening*, Marcel Dekker, New York, **2004**; b) Brakmann, S.; Schwienhorst, A. *Evolutionary Methods in Biotechnology (Clever Tricks for Directed Evolution)*, Wiley-VCH, Weinheim, **2004**; c) Arnold, F.H.; Georgiou, G. *Directed Evolution: Screening and Selection Methods, Vol. 230,* Humana Press, Totowa, NJ, **2003.**

[99] a) Reetz, M. *Tetrahedron* **2002**, *58*, 6595-6602; b) Reetz, M. *Prod. Natl. Acad. Sci. USA* **2004**, *101*, 5716-5722.

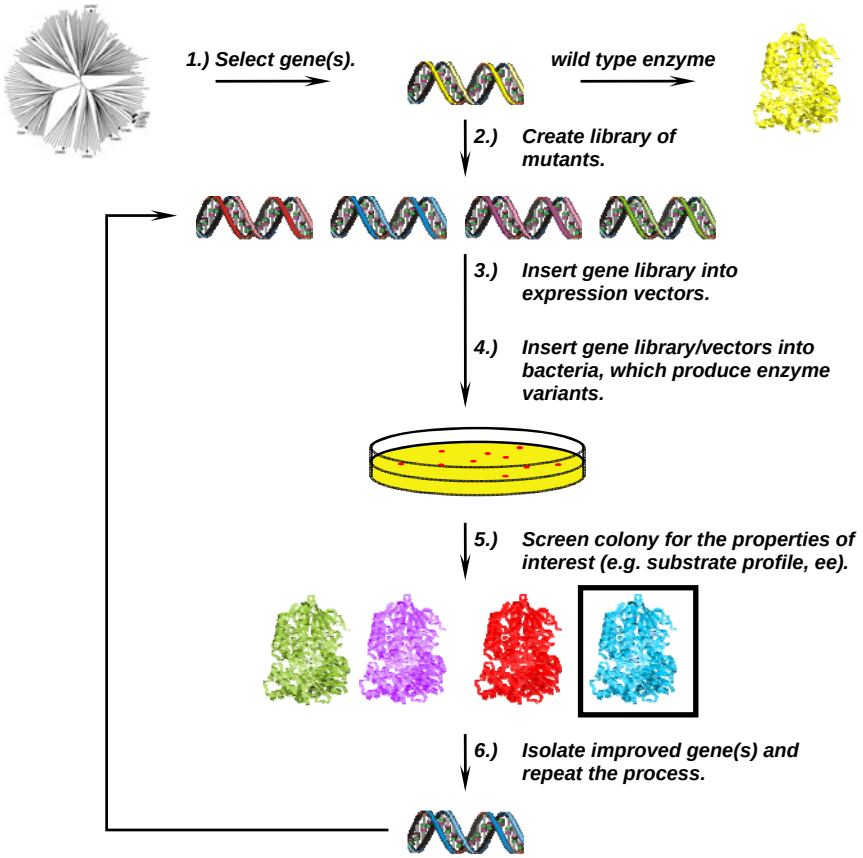


Figure 3.2 Directed enzyme evolution.

Due to this remarkable breakthrough in protein design and engineering, biocatalysis in general became increasingly important in the organic synthesis based on the rapid increase in diversity of catalytic species available. Consequently, the identification of new enzymes is not necessarily a bottle-neck any more but re-modeling of well known proteins by random mutagenesis opens access to completely new research areas as well as applications in asymmetric organic chemistry.

3.1.2 BVMO Mutations

In the field of enzymatic Baeyer-Villiger oxidation random and knowledge-based strategies have been successfully applied to modify both stereopreference and substrate acceptance. In the case of PAMO from moderately thermophilic *Thermobifida fusca* (ZP_57328),[34,100] which converts preferably ketones in benzyl position to the corresponding benzylesters with high regioselectivity, the first 3-dimensional structure of a BVMO[35] was used to modify a region

[100] Fraaije, M.W., Kamerbeek, N.M.; Heidekamp, A.J.; Fortin, R.; Janssen, D.B. *J. Biol. Chem.* **2004**, *279*, 3354.

within the active site. Consequently, the substrate specificity of the enzyme was altered to accept also cycloketone precursors.[101]
The interaction of the FAD and the corresponding amino acids is important for the substrate specificity and seems responsible for the enantiopreference of the protein. Due to the deletion of two amino acids in the stabilizing arginine-interacting loop the substrate profile could be altered by two completely new substrates.

In the case of CHMO from *Acinetobacter* sp. NCIMB 9871[29] enantiodivergent biocatalysts were evolved within two generations using a random approach.[96] In this process, a limited number of amino acids are modified and the most promising catalysts are promoted into a subsequent round of modification. For this study desymmetrization of 4-hydroxy cyclohexanone **17a** was chosen as the model reaction and the CHMO from *Acinetobacter* sp. NCIMB 9871 acted as the "Baeyer-Villigerase". This enzyme provides almost racemic lactone with this particular substrate.
Directed evolution of enantioselective enzymes was performed by error prone polymerase chain reaction (epPCR) as the mutagenesis method. Libraries of $CHMO_{Acineto}$ mutant-genes were produced using different epPCR conditions by changing the $MgCl_2$ concentration and other epPCR parameters to achieve different mutagenesis rates. Figure 3.3 shows the experimental differences of classical polymerase chain reaction and error prone PCR.

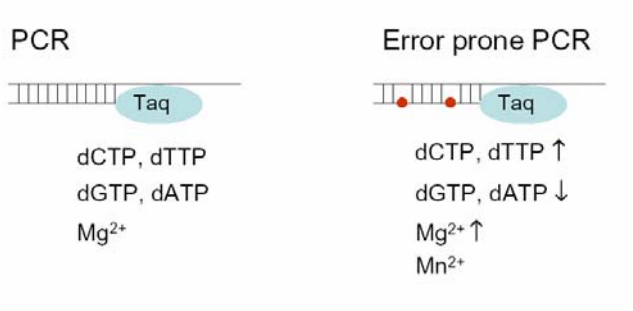
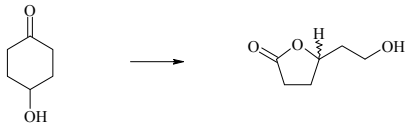


Figure 3.3 Differences in classical and error prone PCR.

After isolation and insertion of the modified genes into an *E.coli* host, they were tested as biocatalysts in the model reaction and were screened against improved enantioselectivity. We selected a number of CHMO mutants that displayed enantiocomplementary biooxidation of 4-hydroxycyclohexanone **17a** (Table 3.1) and tested our library of ketones for effects on the biooxidation of other substrates. This library displays remarkable differences in structure and functionality of ketones. Interestingly, exchange of one amino acid at a specific position (a so called "hot spot") in the protein showed significant differences in the observed enantioselectivity. Wild type $CHMO_{Acineto}$ gave racemic lactone **17b**, but mutants, obtained by directed evolution, yielded lactone **17b** either in (R) or (S) configuration, depending on the amino acid exchanges. Within one round of mutagenesis, starting from a racemic reaction, both antipodal lactones were obtained in moderate to good stereoselectivity.
It should be noted, that lactone **17b** is obtained after rearrangement from the initial 7-ring product to the more stable 5-ring lactone.[55]

[101] Bocola, M.; Schulz, F.; Leca, F.; Vogel, A.; Fraaije, M.W.; Reetz, M.T. *Adv. Synth. Catal.* **2005**, *347*, 979-986.



17b

Mutant	AA-exchanges	17b	ee
wild type	-	(R)	9%
1-C2-B7	F432Y, K500R	(R)	34%
1-F1-F5	L143F	(R)	40%
1-E12-B5	F432I	(R)	49%
1-H7-F4	L426P, A541V	(R)	54%
2-D19-E6	**E292G, T433I, L435Q, T464A, L143F**	**(R)**	**90%**
1-H3-C9	L220Q, P428S, T433A	(S)	18%
1-F4-B9	D41N, F505Y	(S)	46%
1-K6-G2	K78E, F432S	(S)	78%
1-K2-F5	**F432S**	**(S)**	**79%**

Table 3.1 $CHMO_{Acineto}$-mutants and their corresponding AA-exchanges according to reference 96.

Based on these results we wanted to examine the importance and generality of such "hot spots" in cyclohexanone monooxygenase from *Acinetobacter sp*.

3.1.3 Screening Methodology

Since, our previously published biotransformation data were based on preparative shaking flask experiments the importance of an efficient, fast and facile screening methodology[52c] become more and more mandatory for our ongoing research. Therefore an essential part of this thesis was devoted to develop this new methodology in our laboratory. During the last years we established a versatile platform of structurally and functionally diverse substrates and an increasing number of wild type and mutant enzymes in our laboratory (Figure 3.6).
Microscale processing techniques would be a useful tool for the rapid and efficient collection of kinetic and activity data of such enzymes. Woodly *et al.* published in 2002 the use of such microscale processing technologies for the quantification of Biocatalytic Baeyer-Villiger oxidation kinetics.[102] Taking advantage of the preceeding work we aimed at the development of a multi-dish format which reflects fermentation characteristics of shake-flask cultures to a significant extent, hence, allowing also an assessment of biocatalyst efficiency and performance on larger scale. In combination with chiral phase GC analysis, this screening method should allow the investigation of diverse ketones with respect to conversion and stereoselectivity within a relatively short period of time.

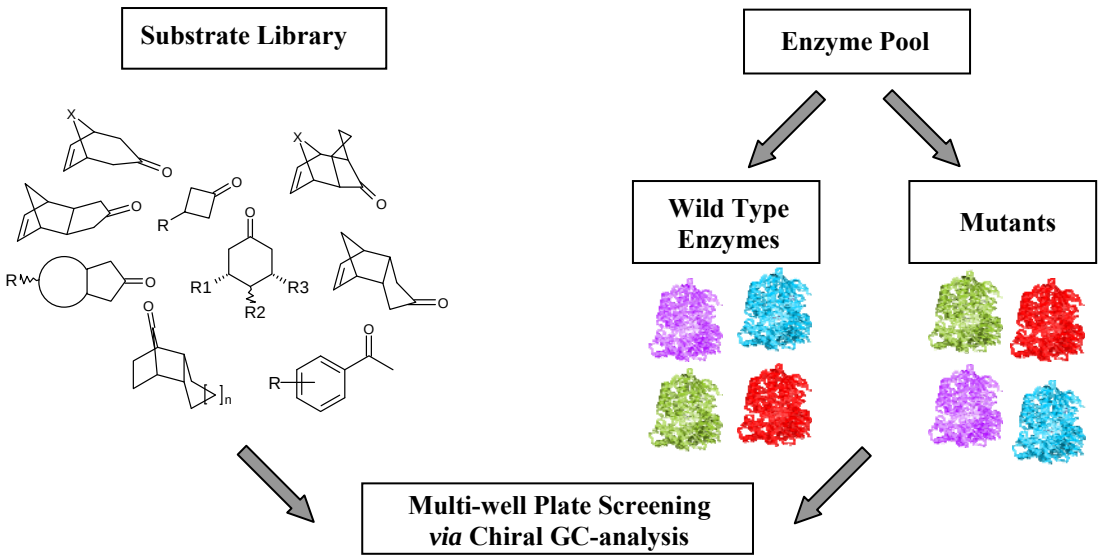


Figure 3.6 Mini-scale multi-well plate screening.

Recombinant *E.coli* based whole-cell expression systems were utilized to implement easy-to-use cofactor recycling (NADPH) and biocatalyst production within the living cells. According to our classical fermentation conditions, fresh LB_{amp} medium was inoculated with 1% of an overnight preculture of recombinant *E. coli* strains in a baffled Erlenmeyer flask. Then the culture was incubated at 37°C on an orbital shaker for 2 hours, and then 50µL IPTG stock solution was added to a final concentration of 0.0025mM. The substrate (100mg/250mL of

[102] Doig, S.D.; Pickering, S.C.R.; Lye, G.J.; Woodley, J.M. *Biotechnol. Bioentg*. **2002**, *80*, 42-49.

medium) was added neat along with β-cyclodextrin (1 equiv.). The culture was incubated overnight at room temperature and subsequently analyzed by GC to determine conversion and stereoselectivity.

Our first attempt for a successful screening protocol was based on this procedure and therefore we decided to perform the screening in a 12-well plate format with a working volume of 2mL.

At the beginning one of the biggest problems was the evaporation of the fermentation media and the volatility of some substrates. After a shaking time of 24hours at 37°C half of the media or more was evaporated. In the case of the 24 well plates with a working volume of 1mL we frequently observed complete evaporation of the fermentation media. Thus, substrate and salt concentrations were changed significantly during the whole fermentation and could influence the performance of the tested BVMO.

We tried to overcome this problem by using a special construction in the orbital shaker whereas the plates were fixed with a piece of foamed material and the lid was fixed by parafilm. Thus, fermentation times of 24 hours at 24°C with a working volume of 1-2mL can be performed without any further problems. For some special reasons the top of the multi-well plate can be also closed with a special adhesive foil to circumvent any further evaporation.

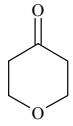
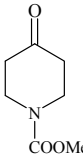
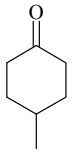
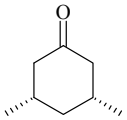
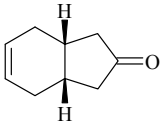
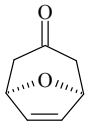
						
Ketone	**18a**	**19a**	**20a**	**21a**	**15a**	**16a**
Strain	**$CHMO_{Acineto}$**	**$CHMO_{Acineto}$**	**$CPMO_{Coma}$**	**$CPMO_{Coma}$**	**$CPMO_{Coma}$**	**$CPMO_{Coma}$**
LB_{amp}	100%	100%	100%	n.a.	90-95%	60-85%
LB_{amp}+ CD	100%	100%	100%	n.a.	90-100%	75-100%
LB_{amp}+ gluc	100%	100%	100%	n.a.	90-100%	75-100%
LB_{amp}+ gluc + CD	n.d.	n.d.	100%	n.a.	90-100%	75-100%
TB_{amp}	n.d.	n.d.	100%	n.a.	90-95%	75-100%
TB_{amp}+ CD	n.d.	n.d.	100%	n.a.	90-100%	75-100%

Table 3.2 Optimization of conditions in parallel mini-scale screening – 12 to 24-well plate formate.

After solving some of the technical problems we investigated the fermentation itself. The media, incubation time, substrate concentration, shaking speed for optimal oxygen transfer and reaction time had to be standardized and optimized. We tested six different substrates and two different recombinant strains for our screening tests (Table 3.2). The first approach was performed with LB_{amp} media without any additives. Pyranone **18a** and piperidone **19a** were converted with cyclohexanone monooxygenase from *Acinetobacter* sp. and gave 100% of the

desired lactones **18b** and **19b** within 24 hours. Subsequent experiments with additional β-cyclodextrin and glucose (4g/L) gave comparable results. Experiments with 4-methylcyclohexanone **20a** and $CPMO_{Coma}$ yielded in 100% of the desired lactone **20b** after 24 hours of fermentation time.
Due to the high volatility of compound **21a** no reproducible results were obtained during several screening efforts. Only traces of the desired product **21b** were detected *via* GC-analysis. Also, the use of adhesive foil, glass inlets, parafilm and lower shaking speed was unsuccessfully.
Another interesting and valuable substrate was compound **15a**. The biotransformation performed in LB_{amp} without any additives yielded successfully in more than 90% of the desired product **15b**.
The most interesting substrate was the oxygen bicyclic ketone **16a**. Due to slow kinetics, instability and the importance in the synthetic application this ketone represents by far the most challenging target. Traditional biotransformation in LB_{amp} gave the desired lactone **16b** in 60-85% yield, determined by GC-analysis. The results were strongly dependent on the condition of the bacteria. It seemed to be very helpful to always use freshly prepared plates and pre-cultures from new stock solutions.
Since it is known from former experimental experience, that β-cyclodextrin as an additive facilitates biotransformations of slow and very lipophilic substrates we were interested in the influence of such an additive in our screening methodology. In the case of 3,5-dimethylcyclohexanone **21a** no positive effect was observed. However, the conversion of compound **15a** and substrate **16a** was pushed to completion. Another approach for the optimization of this parallel-mini scale screening was to increase the biocatalytic activity of the cells by the addition of glucose (4g/L) and the use of a different medium. Glucose should influence the co-factor recycling in the cell, whereas terrific broth should facilitate the growth of the cells and increase the biomass during the fermentation. Neither the addition of glucose nor the change of the media gave significantly new results on all tested substrates.

Conclusively, within this set of experiments we were able to establish a "screening"-methodology whereas it is possible to obtain similar biotransformation results compared to shaking flask experiments. All tested substrates **15a, 16a, 18-20a** were converted in preparative scale (mg) previously to give complete conversion in all cases and were isolated in moderate to good yields. Both good and kinetically "slow" substrates can be converted completely to the desired lactone in the multi-well plate (12 and 24) approach.
We were further interested in the use of 96 well plates for screening of larger mutant libraries. Therefore, we performed a screen with 4-methylcyclohexanone **20a** and $CHMO_{Brevi1}$ under optimized conditions in 96-deep well plates (working volume 1mL) and classical 96 well plates (working volume 200µL). The results were summarized in Figure 3.7. Reproducible results and good to excellent conversion rates were obtained by performing the screening in LB_{amp}, with 96-deep-well dishes, sealed with an adhesive foil.

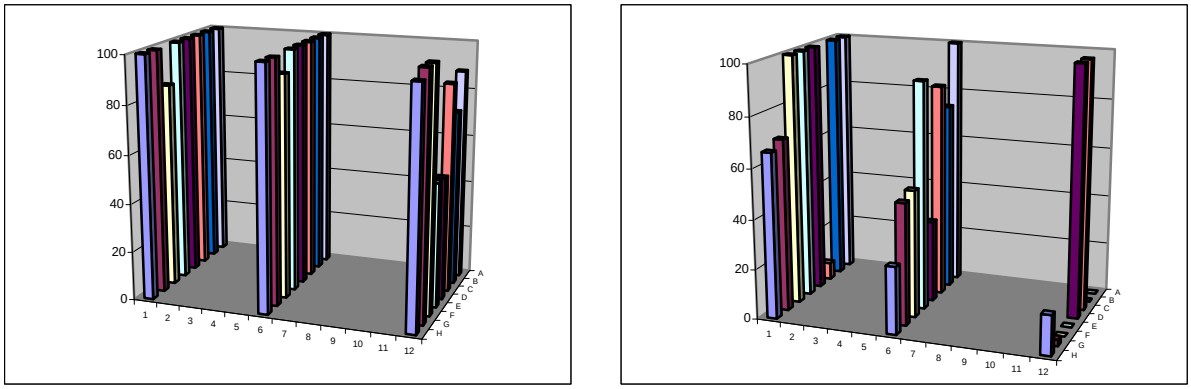


Figure 3.7 96-well plate screening of 4-methylcyclohexanone (**20a**) with $CHMO_{Brevi1}$.

Subsuming, based on the results, screening of whole cell mediated Baeyer-Villiger oxidation was performed in LB_{amp} media, with substrate concentration of 1mg/mL without any additives to minimize any manipulation efforts. Fermentation time was standardized to 24 hours and the fermentation temperature was set to 24°C. Evaluation of the product was performed *via* chiral GC analysis.

Finally, we established a procedure, for a convenient, reproducible and easy to handle screening methodology for 12 to 96-well dishes which reflects fermentation characteristics of shake-flask cultures and represents the basis of an assessment of biocatalyst efficiency and performance on larger scale. In combination with chiral phase GC analysis, a middle throughput screening method, with respect to conversion and stereoselectivity was developed.

3.1.4 Screening Results for Mutant $CHMO_{Acineto}$

While previous studies to alter BVMOs aimed at the modification of a specific property of the enzyme within the transformation of a particular substrate, in this contribution the impact of mutations on a variety of substrates was investigated with the aim to identify general trends. We selected a number of CHMO mutants that displayed enantiocomplementary biooxidation of 4-hydroxycyclohexanone **17a**. These modified enzymes were screened against a library of structurally diverse ketones of different polarity (Tables 3.3-3.6). Baeyer-Villiger oxidation of 6,5'-bicycloketone **15a** with wild type cyclohexanone monooxygenase resulted in the racemic lactone **15b** with a poor 33% conversion. Surprisingly, oxidation of ketone **15a** with all CHMO mutants did not give the desired lactone **15b**.

Biotransformation of 5,5'-bicycloketone **22a** with native CHMO gave (-)-lactone **22b** in very good yield and good enantioselectivity. A single mutation at position 432 (mutant 1-K2-F5) by an exchange of phenylalanine to serine resulted in slight increase in stereoselectivity compared to wild-type $CHMO_{Acineto}$, whereas mutant 1-H7-F4 with amino acid exchanges of L426P and A541V showed a significant decrease of enantiopreference. All other mutations did not have any effect on the enantioselectivity of the biooxidation. Remarkably, the more

lipophilic chloro-bicyclic compound **23a** was transformed to enantiocomplementary lactone **23b** with very good stereoselectivity by this latter mutant strain.

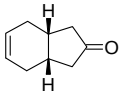
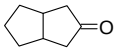
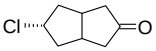
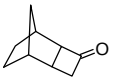
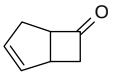
						
Strain		**15a**	**22a**	**23a**	**24a**	**25a**
$CHMO_{Acineto}$	***Conv.; a/n*** ***ee, (+/-)***	rac.	89%, (-)	99%, (-)	70/30, 44%/99%, (-)/(-)	51/49, 95%/99%, (-)/(-)
1-C2-B7 (R)[f]	***Conv.[a]*** ***ee, (+/-)[b]***	n.c.[c] n.a.[d]	++ 90%, (-)	++++ 99%, (-)	++++, 69/31[e] 38%/99%, (-)/(-)	++++, 36/64 99%/57%, (-)/(-)
1-F1-F5 (R)	***Conv.*** ***ee, (+/-)***	n.c. n.a.	++++ 94%, (-)	++++ 99%, (-)	++++, 80/20 29%/99%, (-)/(-)	++++, 41/59 99%/76%, (-)/(-)
1-E12-B5 (R)	***Conv.*** ***ee, (+/-)***	n.c. n.a.	+++ 87%, (-)	++++ 87%, (-)	++++, 49/51 90%/99%, (-)/(-)	++++, 47/53 99%/72%, (-)/(-)
1-H7-F4 (R)	***Conv.*** ***ee, (+/-)***	n.c. n.a.	+++ 17%, (-)	++++ 57%, (+)	++++, 47/53 94%/97%, (-)/(-)	++++, 45/55 99%/89%, (-)/(-)
1-H3-C9 (S)	***Conv.*** ***ee, (+/-)***	n.c. n.a.	+++ 56%, (-)	+++ 96%, (-)	++++, 69/31 38%/99%, (-)/(-)	++++, 29/71 99%/42%, (-)/(-)
1-F4-B9 (S)	***Conv.*** ***ee, (+/-)***	n.c. n.a.	+++ 87%, (-)	++++ 83%, (-)	++++, 72/28 40%/99%, (-)/(-)	++++, 43/57 99%/69%, (-)/(-)
1-K6-G2 (S)	***Conv.*** ***ee, (+/-)***	n.c. n.a.	++++ 94%, (-)	++++ 99%, (-)	++++, 59/41 63%/99%, (-)/(-)	++++, 44/56 99%/78%, (-)/(-)
1-K2-F5 (S)	***Conv.*** ***ee, (+/-)***	n.c. n.a.	+++ 94%, (-)	++++ 99%, (-)	++++, 58/42 65%/99%, (-)/(-)	++++, 44/56 99%/80%, (-)/(-)
2-D19-E6 (R)	***Conv.*** ***ee, (+/-)***	n.c. n.a.	+++ 92%, (-)	+++ 99%, (-)	++++, 84/16 19%/99%, (-)/(-)	++++, 45/55 99%/65%, (-)/(-)

Table 3.3 Biooxidatoin of bicyclic ketones by mutant BVMOs. a) conversion: ++++: >90%, +++: 50-90%, ++: 20-50%, +: <20%; b) sign of optical rotation; c) no conversion, d) not applicable, e) ratio between "normal" and "abnormal" lactone, f) represents the formed enantiomer in the Baeyer-Villiger oxidation of 4-hydroxy cyclohexanone **17a** with $CHMO_{Acineto}$ mutants.

Furthermore, the regiodivergent biooxidation of two racemic fused bicycloketone was investigated (**24a,25a**). In the classical chemical Baeyer-Villiger oxidation the "normal" lactone should be the preferred regioisomer due to the migration of the higher substituted carbon center. In general, compound **24a** (Figure 3.8) was converted to both regioisomers **24b** ("normal" and "abnormal" lactone; ratio 70/30) by wild-type CHMO in very high optical purity for the abnormal lactone and moderate stereoselectivity for the normal lactone. The two mutants 1-H7-F4 and 1-E12-B5 displayed an increase in the enantioselectivity of the "normal" lactone by improving the regioselectivity of the biooxidation towards the optimal 50:50 distribution.

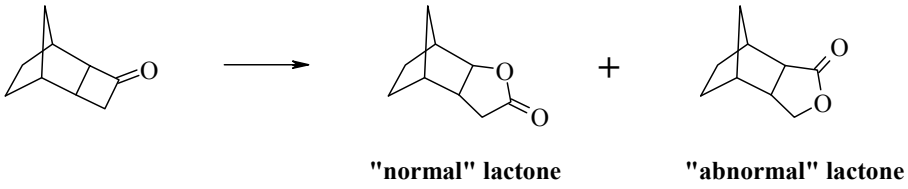


Figure 3.8 Regiodivergent Baeyer-Villiger oxidation of ketone **24a** – formation of "normal" and "abnormal" lactone **24b**.

The racemic fused ketone **25a** was transformed to both regioisomers **25b** by wild-type CHMO in very high enantioselectivity and optimal distribution. In fact, all mutations in the wild type enzyme led either to a decrease of the enantioselectivity or a significant shift in the distribution of both regioisomers. Nevertheless, the microbial Baeyer-Villiger oxidation of fused cyclobutanones (**24a**, **25a**) gave access to both regioismeres in very good yield and stereoselectivity. Based on previous results for the enantiocomplementary biooxidation of 4-hydroxycyclohexanone **17a**, we investigated differently substituted prochiral cyclohexanones (Table 2.4). Surprisingly, no enantiodivergence was observed and neither a change of hybridization (**26a**), modifications in polarity (**28a**) nor stereochemistry of substituents at C-4 showed substantial influence on the stereopreference. Conversion of the prochiral 4-methylcyclohexanone **20a** gave exclusively the (-)-enantiomer **20b** in very high selectivity. This different behavior of the biocatalyst upon change of the hydroxyl substituent into the less polar methyl group indicates an effect of the interaction between the substrate and the active site of the enzyme as no enantiodivergent biotransformation was observed. Hence, transformation of cyclohexanone **21a** with two methyl-substituents in position C-3 and C-5 to the corresponding lactone **21b** did not result in higher or opposite stereopreference for all tested mutants. Stereoselectivity was slightly improved for lactone **26b**, whereas lactone **27b** with a cyclopropyl residue at C-4 was obtained with the same enantioselectivity as the wild type CHMO. Since we observed no significant difference in the stereopreference of all mutants while we decreased the polarity and changed the hybridization of the substituent at the C-4 carbon we decided to test two different ketones **28a** and **29a** with a hydroxyl moiety at the C-4 position. The presence of two methyl groups (C-3 and C-5) and the axial hydroxyl group (C-4) in lactone **28b** resulted in slight increase of enantiopreference. Unfortunately, the change of the stereochemistry at the C-4 carbon into the equatorial OH-group did not give antipodal lactones **29b**, but the substrate acceptance was influenced in some cases significantly. Mutant 1-E12-B5 did not convert ketone **29a** into the desired lactone **29b**. Hence mutations L143F (mutant 1-F1-F5), D41N and F505Y (mutant 1-F4-B9), and mutant 2-D19-E6 resulted in a significant decrease of the conversion down to a maximum of 20%.
Obviously, two methyl residues in the equatorial position at C-3 and C-5 carbon and a hydroxyl group at position C-4 had a significant effect on the stereopreference of all tested mutants compared to the model substrate 4-hydroxycyclohexanone **17a**. Whereas antipodal biooxidation was observed in the case of 4-hydroxycyclohexanone **17a**, no switch in stereopreference was obtained in the 3,5-dimethyl-4-hydroxycyclohxanone series (**28a**, **29a**).

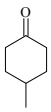
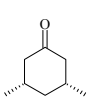
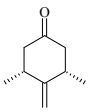
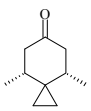
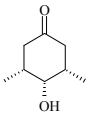
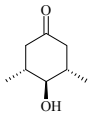
							
Strain		**20a**	**21a**	**26a**	**27a**	**28a**	**29a**
$CHMO_{Acineto}$	***ee, (+/-)***	99%, (-)	99%, (-)	95%, (+)	99%, (+)	96%, (-)	99%, (+)
1-C2-B7 (R)[f]	***Conv.***[a] ***ee, (+/-)***[b]	++++ 99%, (-)	++++ 99%, (-)	++++ 99%, (+)	++++ 99%, (+)	++++ 99%, (-)	++ 96%, (+)
1-F1-F5 (R)	***Conv.*** ***ee, (+/-)***	++++ 99%, (-)	++++ 99%, (-)	++++ 99%, (+)	+% 99%, (+)	++++ 99%, (-)	+% 97%, (+)
1-E12-B5 (R)	***Conv.*** ***ee, (+/-)***	++++ 99%, (-)	++++ 99%, (-)	++++ 99%, (+)	++++ 99%, (+)	++++ 99%, (-)	n.c.[c] n.a.[d]
1-H7-F4 (R)	***Conv.*** ***ee, (+/-)***	-[e] -	++++ 99%, (-)	++++ 99%, (+)	++++ 99%, (+)	++++ 99%, (-)	++++ 99%, (+)
1-H3-C9 (S)	***Conv.*** ***ee, (+/-)***	++++ 99%, (-)	++++ 99%, (-)	++++ 99%, (+)	++++ 99%, (+)	++++ 97%, (-)	++++ 98%, (+)
1-F4-B9 (S)	***Conv.*** ***ee, (+/-)***	++++ 99%, (-)	88% 99%, (-)	+++ 99%, (+)	++++ 99%, (+)	++++ 99%, (-)	+ 98%, (+)
1-K6-G2 (S)	***Conv.*** ***ee, (+/-)***	++++ 99%, (-)	++++ 99%, (-)	++++ 99%, (+)	++++ 99%, (+)	++++ 99%, (-)	++++ 99%, (+)
1-K2-F5 (S)	***Conv.*** ***ee, (+/-)***	++++ 99%, (-)	++++ 99%, (-)	+++ 99%, (+)	++++ 99%, (+)	++++ 99%, (-)	++++ 99%, (+)
2-D19-E6 (R)	***Conv.*** ***ee, (+/-)***	++++ 99%, (-)	++++ 99%, (-)	++++ 99%, (+)	++++ 99%, (+)	++++ 99%, (-)	+ 99%, (+)

Table 3.4 Biooxidation of cyclohexanone derivatives by mutant BVMOs. a) conversion: ++++: >90%, +++: 50-90%, ++: 20-50%, +: <20%; b) sign of optical rotation; c) no conversion, d) not applicable; e) no result, f) represents the formed enantiomere in the Baeyer-Villiger oxidation of 4-hydroxycyclohexanone **17a** with $CHMO_{Acineto}$ mutants.

In the cyclobutanone series (substrates **5-9a** and **12a-13a**) a related trend was observed compared to the 5,5-bicyclic series, although the degree of enantioselectivity turned out to be lower. The mutation F432I (mutant 1-E12-B5) for the precursor **5a** led to the antipodal lactone **5b** (24% ee) compared to wild type CHMO (17% ee). A different mutation at position 432 (Phe→Ser, mutant 1-K2-F5) gave a significant increase in the enantioselectivity (97% ee) compared to the wild type enzyme. Hence, interesting results were obtained in the screening of cyclobutanone **6a**. Again, mutation at position 432 (Phe→Ser, mutant 1-K2-F5) increased the enantioselectivity from racemic (wild type CHMO) up to 91% ee. Biooxidation of benzylcyclobutanone **7a** did not show any significant difference to wild type CHMO transformations. Further surprising results were obtained with phenylcyclobutanone **8a**. The mutation F432S (mutant 1-K2-F5) increased the stereoselectivity significantly compared to wild-type CHMO, while the mutation F432I (mutant 1-E12-B5) had the opposite effect resulting in almost racemic lactone **8b**.

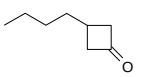
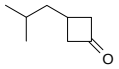
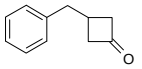
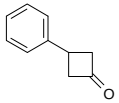
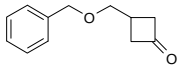
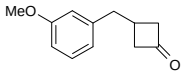
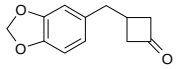
								
Strain		**5a**	**6a**	**7a**	**8a**	**9a**	**12a**	**13a**
$CHMO_{Acineto}$	***ee, (+/)-***	17%, (-)	rac., n.a.	88%, (-)	62%, (-)	53%, (+)	98%, (-)	96%, (-)
1-C2-B7 (R)[e]	***Conv.[a]*** ***ee, (+/-)[b]***	++++ 16%, (-)	++++ 60%, n.a.	++++ 76%, (-)	+ 20%, (-)	++++ 41%, (+)	n.c.[d] n.a.	++ 89%, (-)
1-F1-F5 (R)	***Conv.*** ***ee, (+/-)***	++++ 9%, (-)	++++ 55%, n.a.	++++ 76%, (-)	++++ 93%, (-)	++++ 59%, (+)	++++ 90%, (-)	++++ 92%, (-)
1-E12-B5 (R)	***Conv.*** ***ee, (+/-)***	++++ 24%, (+)	++++ 48%, n.a	++++ 86%, (-)	++++ 8%, (-)	++++ 66%, (+)	++++ 94%, (-)	++++ 96%, (-)
1-H7-F4 (R)	***Conv.*** ***ee, (+/-)***	++++ 97%, (-)	++++ 96%, n.a	++++ 90%, (-)	++++ 95%, (-)	++++ 70%, (-)	++++ 95%, (-)	++++ 97%, (-)
1-H3-C9 (S)	***Conv.*** ***ee, (+/-)***	++++ 18%, (-)	++++ 60%, n.a	++++ 76%, (-)	++++ 72%, (-)	++++ 40%, (+)	n.c. n.a.	n.c. n.a.
1-F4-B9 (S)	***Conv.*** ***ee, (+/-)***	++++ 19%, (-)	++++ 63%, n.a	++++ 54%, (-)	++++ 10%, (-)	++++ 24%, (+)	++++ 95%, (-)	+++ 95%, (-)
1-K6-G2 (S)	***Conv.*** ***ee, (+/-)***	++++ 92%, (-)	++++ 87%, n.a	++++ 77%, (-)	++++ 94%, (-)	++++ 75%, (-)	++++ 91%, (-)	++++ 91%, (-)
1-K2-F5 (S)	***Conv.*** ***ee, (+/-)***	++++ 97%, (-)	++++ 91%, n.a	++++ 78%, (-)	++++ 96%, (-)	++++ 83%, (-)	++++ 91%, (-)	++++ 91%, (-)
2-D19-E6 (R)	***Conv.*** ***ee, (+/-)***	++++ rac, n.a.[c]	++++ 57%, n.a	++++ 76%, (-)	++++ 82%, (-)	++++ 7%, (+)	+++ 92%, (-)	+++ 95%, (-)

Table 3.5 Biooxidation of cyclobutanone derivatives by mutant BVMOs. a) conversion: ++++: >90%, +++: 50-90%, ++: 20-50%, +: <20%; b) sign of optical rotation; c) not applicable, d) no conversion, e) represents the formed enantiomer in the Baeyer-Villiger oxidation of 4-hydroxy cyclohexanone **17a** with $CHMO_{Acineto}$ mutants.

Again, in the case of more polar precursor **9a** the mutation F432S (mutant 1-K2-F5) led to the antipodal lactone **9b** compared to wild type CHMO. Another mutation at position 432 (Phe→Ile, mutant 1-E12-B5) gave a slight increase in enantioselectivity compared to the wild type enzyme.

Within the investigations of cyclobutanone **12a** no influence on the stereopreference but on the substrate acceptance was observed. Mutants 1-C2-B7 and 1-H3-C9 did not give the desired lactone **12b** compared to wild type CHMO. A similar trend was observed for the cyclobutanone precursor **13a**. Whereas, the obtained enantioselectivities indicated a similar trend compared to wild type CHMO transformations, again the same mutants 1-C2-B7 and 1-H3-C9 showed low (28% conversion) or no substrate acceptance.

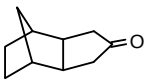
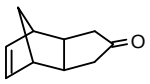
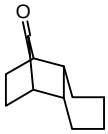
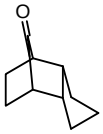
					
Strain		**30a**	**31a**	**32a**	**33a**
$CHMO_{Acineto}$	***ee, (+/-)***	n.a.	n.a.	97%, (-)	97%, (-)
1-C2-B7 (R)[e]	***Conv.[a]*** ***ee, (+/-)[b]***	++ 92%, (+)	n.c. n.a.	++++ 91%, (-)	++++ 96%, (-)
1-F1-F5 (R)	***Conv.*** ***ee, (+/-)***	n.c. n.a.	+ ~35%, (-)	++++ 78%, (-)	++++ 92%, (-)
1-E12-B5 (R)	***Conv.*** ***ee, (+/-)***	n.c. n.a.	n.c. n.a.	++++ 48%, (-)	++++ 48%, (-)
1-H7-F4 (R)	***Conv.*** ***ee, (+/-)***	+++ 12%, (+)	++ ~60%, (+)	n.c. n.a.	++++ 79%, (-)
1-H3-C9 (S)	***Conv.*** ***ee, (+/-)***	+++ 69%, (+)	+++ ~50%, (-)	++++ 94%, (-)	++++ 96%, (-)
1-F4-B9 (S)	***Conv.*** ***ee, (+/-)***	n.c. n.a.	n.c. n.a.	++ 9%, (-)	++++ 82%, (-)
1-K6-G2 (S)	***Conv.*** ***ee, (+/-)***	++ 72%, (+)	+++ ~90%, (-)	++++ 94%, (-)	++++ 93%, (-)
1-K2-F5 (S)	***Conv.*** ***ee, (+/-)***	n.c. n.a.	+++ ~90%, (-)	n.c. n.a.	++++ 93%, (-)
2-D19-E6 (R)	***Conv.*** ***ee, (+/-)***	+ 98%, (+)	n.c. n.a.	n.c. n.a.	++ 81%, (-)

Table 3.6 Biooxidation of bridged bicycloketones by mutant BVMOs. a) conversion: ++++: >90%, +++: 50-90%, ++: 20-50%, +: <20%; b) sign of optical rotation; c) no conversion, d) not applicable, e) represents the formed enantiomer in the Baeyer-Villiger oxidation of 4-hydroxy cyclohexanone **17a** with $CHMO_{Acineto}$ mutants.

In a number of previous studies it became apparent that wild-type CHMO does not accept sterically demanding ketones. Therefore, we focused our study on this class of substrates (Table 3.6) and obtained very interesting results with *exo* tricyclic ketones (**30a**, **31a**). By multiple mutations an expansion in substrate acceptance was observed. The most interesting mutations are L426P and A541V in mutant 1-H7-F4 and various amino acid exchanges at position 432. The saturated *exo*-compound **30a** gave 92% enantioselectivity with mutant 1-C2-B7 and essentially racemic lactone **30b** with mutant 1-H7-F4 (12% ee). Three mutations in 1-H3-C9 (L220Q, P428S, T433A) resulted in increased enantioselectivity of the desired lactone **30b** (69% ee). Again, a mutation at position 432 (Phe→Tyr, mutant 1-C2-B7), resulted in lactone **30b** with significant increase in stereoselectivity and decreased conversion (20%). Interesting results were obtained with the unsaturated *exo*-tricyclic ketone **31a**, whereas mutant 1-K2-F5 with a mutation at position 432 produced (-)-lactone **31b** with high enantioselectivity (~90% ee; no baseline separation on chiral phase GC). Biooxidation with mutant 1-H7-F4 resulted in formation of the antipodal (+)-product 24b (~60% ee). The same behavior as in mutant 1-K2-F5 was also observed with strain 1-K6-G2 expressing a CHMO also modified at position 432 (Phe→Ser) plus an additional mutation at position 78. These finding clearly indicate the pivotal role of position 432 in $CHMO_{Acineto}$.

In the *endo*-tricyclic series of type (**32a**, **33a**), again mutations at position 432 showed the strongest effects. The exchange of phenylalanine by isoleucine (mutant 1-E12-B5) resulted in a significant decrease in enantioselectivity, whereas mutation F432S (mutant 1-K6-G2) had no effect on the stereopreference. Some mutations, like in 1-K2-F5, 2-D19-E6, and 1-H7-F4 showed a significant decrease of substrate acceptance of ketone **32a** and gave no satisfactory results. Ketone **33a** is well accepted by all mutants and again, a single mutation at position 432 (Phe→Ile, mutant 1-E12-B5) showed the strongest effect on the stereopreference. Biooxidation of ketone **33a** with mutant 1-E12-B5 gave a slight decrease of enantioselectivity (48% ee).

In Table 3.7 the most interesting results were highlighted, again, to show the importance of specific mutations on selected subtrates. As mentioned above some of the most important positions in the protein cyclohexanone monooxygenase are 432, 426, 541 as identified within this study. Amino acid exchanges at this position can influence substrate specificity as well as stereopreference to some extent. In the case of bicyclic ketones (**22a**, **23a**) within those mutations a significant effect on the stereoselectivity was observed. Similar results were obtained with the fused cyclobutanone derivative. Distribution of both stereoisomers was pushed to a 1:1 ratio and the enantioselectivity was increased to over 90% for both isomers.

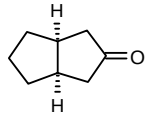
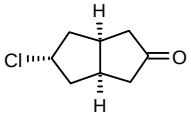
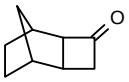
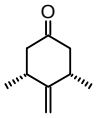
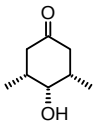
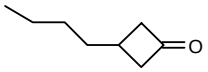
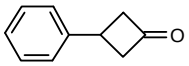
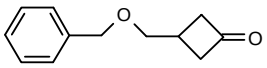
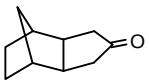
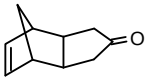
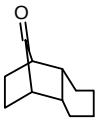
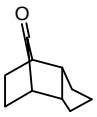
substrate		mutant	mutation	(+/-)[a]	ee [%]	conv.[b]
22a		wild type	-	-[53c]	89	++++
		1-K2-F5	F432S	-	94	++++
		1-H7-F4	L426P, A541V	-	17	++++
23a		wild type	-	-[53c]	>99	++++
		1-K2-F5	F432S	-	99	++++
		1-H7-F4	L426P, A541V	+	57	++++
24a		wild type	-	-/-	44/>99	++++/ (70/30)[e]
		1-K2-F5	F432S	-/-	65/>99	++++/ (58/42)
		1-H7-F4	L426P, A541V	-/-	94/>99	++++/ (47/53)
21a		wild type	-	-[55]	>99	++++
		1-K2-F5	F432S		>99	++++
		1-H7-F4	L426P, A541V	-	>99	++++
26a		wild type	-	+[52d]	92	++++
		1-K2-F5	F432S	+	>99	++++
		1-H7-F4	L426P, A541V	+	>99	++
28a		wild type	-	-[55]	96	++++
		1-K2-F5	F432S	-	>99	++++
		1-H7-F4	L426P, A541V	-	>99	++++
5a		wild type	-	-	17	++++
		1-K2-F5	F432S	-	97	++++
		1-E12-B5	F432I	+	24	++++
8a		wild type	-	-[70a]-	62	++++
		1-K6-G2	K78E, F432S		96	++++
		1-E12-B5	F432I	-	8	++++
9a		wild type	-	+[70b]	53	++++
		1-K2-F5	F432S	-	83	++++
		1-E12-B5	F432I	+	66	++++
30a		wild type	-	n.a.[c]	n.a.	n.c.[d]
		1-C2-B7	F432Y, K500R	+	92	+
		1-H7-F4	L426P, A541V	+	12	++
31a		wild type	-	n.a.	n.a.	n.c.
		1-K2-F5	F432S	-	ca 90	++
		1-H7-F4	L426P, A541V	+	ca 60	++
32a		wild type	-	-[52c]	96	++++
		1-K6-G2	K78E, F432S	-	94	++++
		1-E12-B5	F432I	-	48	++++
33a		wild type	-	-[52c]	96	++++
		1-K2-F5	F432S	-	93	++++
		1-E12-B5	F432I	-	48	++++

Table 3.7 Summary of screening results of CHMO produced by directed evolution; a) sign of optical rotation; b) conversion: ++++: >90%, +++: 50-90%, ++: 20-50%; +: <20% c) not applicable, d) no conversion, e) ratio between "normal" and "abnormal" lactone

Surprisingly, the class of ketones which is mostly related to the model substrate 4-hydroxy cylohexanone **17a** did not display any significant effets. For all tested substrates (e.g. **21a**, **26a**, **28a**), a slight increase but no inversion of enantioselectivity was observed for mutants 1-H7-F4 and 1-K2-F6. Cyclobutanones **5a**, **8a** and **9a** were mostly affected by mutations at

position 432. Structurally more demanding ketones (**30a, 31a**), not accepted by wild type CHMO, were not only biooxidized due to mutations at positions 432, 426, 541 but also gave lactones with different enantioselectivities (**30b**, **31b**). Finally, biooxidation results of tricyclic ketones **32a** and **33a** were strongly dependent on the mutations in the protein. Again, mutation at position 432 gave a significant decrease of enantioseletvitiy.

AA Position	Mutations	Effect	Substrate
432	Phe→Ser	extended substratprofile	**31a**
	Phe→Ser	significant influence on enantioselectivity	**22a, 5a, 8a, 9a,**
	Phe→Ile	significant influence on enantioselectivity	**8a, 9a, 32a, 33a**
	Phe→Tyr	extended substratprofile	**30a**
426	Leu→Pro	significant influence on enantioselectivity	**22a, 23a, 24a,**
	Leu→Pro	extended substratprofile	**30a, 31a**

Table 3.7 Summary of most important CHMO mutations and their influence on the biocatalytic performance.

Within this study, we could demonstrate that a limited number of modifications within a protein can significantly alter both substrate acceptance and stereopreference compared to the wild-type enzyme. In a number of cases, new substrates were identified and enantiocomplementary biooxidations were realized. The results obtained also confirm the existence of certain "hot spots" within CHMO of paramount influence to both substrate specificity and stereoselectivity. In particular, mutations at position 432 have a significant impact on the characteristics of mutant biocatalysts (Table 3.7). The identification of such hot spots offers the option of modifications of the enzyme in subsequent rounds of biocatalyst optimization either by rational design or directed evolution.[103]

A more profound understanding of these modifications and the resulting changes in interactions between active site and substrate will require future docking studies. A homology model of CHMO would form the basis of a future study aimed at understanding the source of enhanced or inverted stereoselectivity.

[103] CASTing has recently emerged as an alternative to traditional forms of random mutagenesis: a) Reetz, M.T.; Bocola, M.; Carballeira, J.D.; Zha, D.; Vogel, A. *Angew. Chem. Int. Ed.* **2005**, *44*, 4192-4196; b) Reetz, M.T.; Wang, L.-W.; in part Bocola, M. *Angew. Chem. Int. Ed.* **2006**, *45*, 1412-1415.

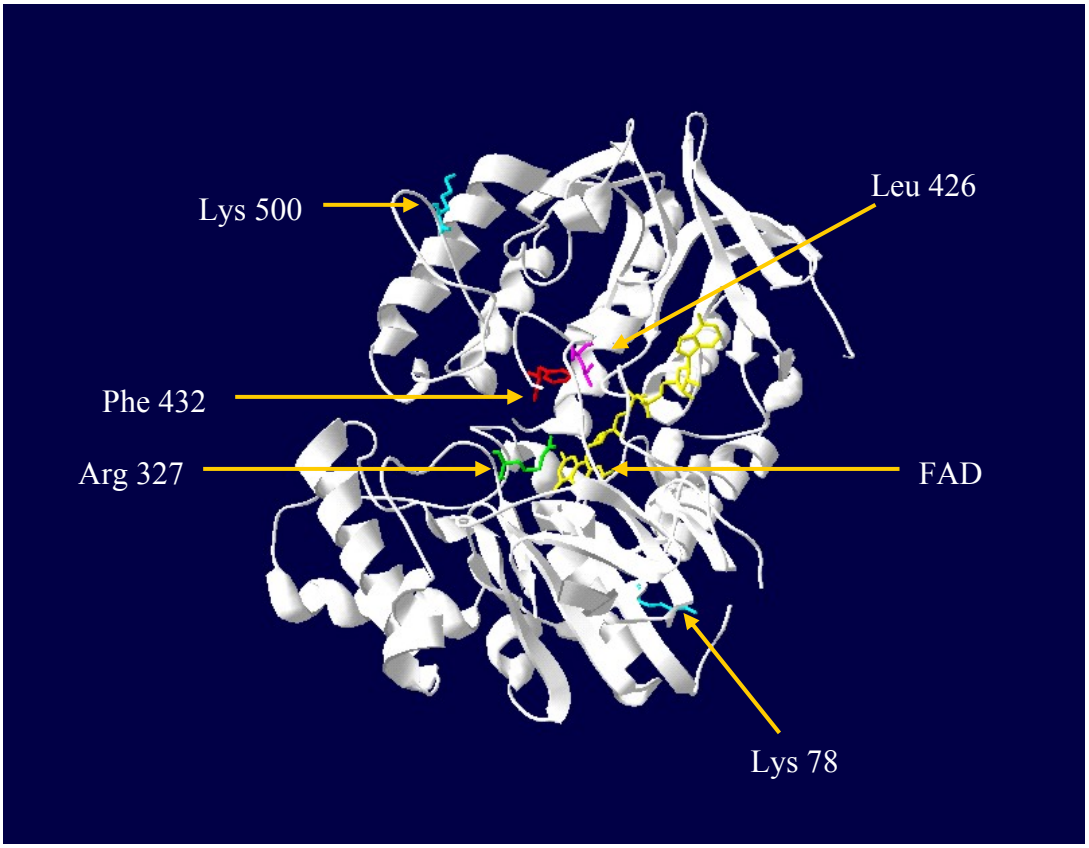


Figure 3.9 Presentation of the homology approximation of CHMO (40.3% sequence identity to 1W4X of PAMO, estimated by Deep View/Swiss-PdbViewer 3.7 (SP5)). This model shows the overall fold with all mutation sites which gave the most interesting results.

According to the recently published x-ray structure of $PAMO_{Thermo}$ we were able to design a homology approximation of $CHMO_{Acineto}$ (Figure 3.9). In fact the sequence homology between $CHMO_{Acineto}$ and $PAMO_{Thermo}$ is about 40% and therefore a sufficient estimation of the structure of $CHMO_{Acineto}$ could be assigned. Hence, incorporation of FAD (highlighted in yellow) into the active site and the FAD interacting Arginine at position 327 (highlighted in green) was performed by comparison to PAMO. Additionally, we highlighted all important amino acids, which were mutated. Alanin 541 could not be seen in the homology approximation because of its position on the outer surface of the protein. In general, all mutation sites are distributed randomly around the whole enzyme with two exceptions. Phe at position 432 and Leu at postion 426 are located rather close to the active site and mutations therin are able to influence the interaction between substrate and enzyme during the catalytic process. Figure 3.10 provides a closer look at the active site of $CHMO_{Acineto}$. However, mutations that are located at the outer sphere of the protein like Lys 500 or Ala 541 (which cannot be seen in our homology model) seem to display also a remarkable effect in the biocatalytic performance of the protein.

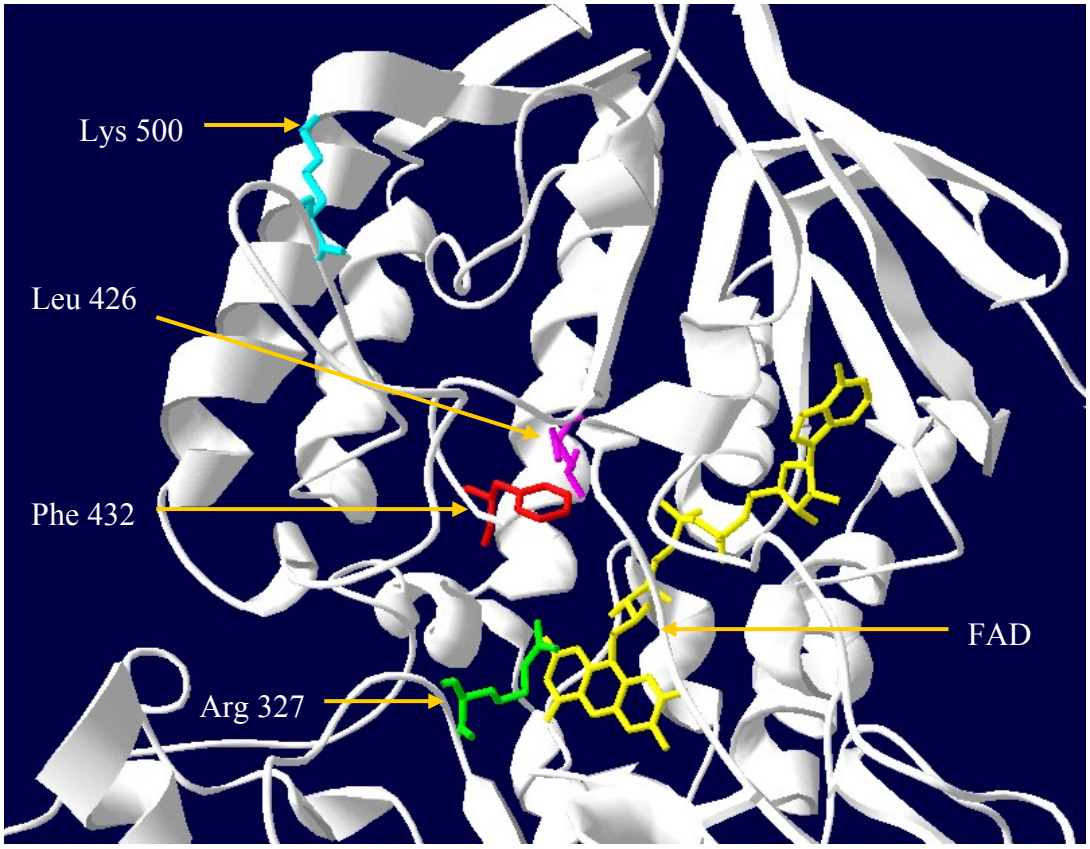


Figure 3.10 This part is a zoom into the active site showing the FAD cofactor as solid yellow sticks and the catalytic arginine in green. The red and margenta color highlights the presence of two additional amino acids close to the active center which turned to be the most interesting mutation sites of CHMO.

However, as opposed to naturally evolved BVMOs,[66] where two classes of enzymes with enantiocomplementary properties and different substrate profiles were established, no explicit trend was identified for the effect of specific amino-acid exchanges on both substrate acceptance and stereopreference. This study accumulated a multitude of novel data on structurally diverse substrates, which may be compiled into a more comprehensive model on molecular level. So far, this remains a major challenge, as previous studies suggested substantial conformational changes of such enzymes during the complete catalytic cycle.[35]
In summary, we have demonstrated the potential of random mutagenesis for the wide-spread modification of a Baeyer-Villiger monooxygenase.

4 Optimization for Up-Scale Fermentation Process

As biocatalysis moves from laboratory to production scales, economic considerations become increasingly important.[104] Biotechnology needs enzymes which are stable and active over long periods of time, enzymes which are active in non-aqueous solvents, and enzymes which can accept different substrates (different from their individual substrates in nature). High stereoselectivity and good substrate acceptance can be ensured by choosing the most appropriate enzyme. Suitable biocatalysts can be obtained by screening recombinant whole-cells or isolated enzymes from various microorganisms.[105] When a suitable biocatalyst with a good performance was identified, optimization *via* various molecular biological methodologies, as already outlined (e.g. directed evolution or gene shuffling) can be applied.[106]

Yield can be easily improved for example by choosing the best substrates, and process conditions like temperature, pH value, media, and usage of highly productive recombinant *E.coli* strains. Many different aspects have to be involved step by step in the optimizing process to improve previous results. Especially the importance of the linkage of co-factor recycling, the essential amount of molecular oxygen and product formation in the case of redox biocatalysis has to be determined in details.

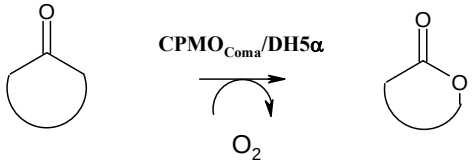


Figure 4.1 Microbial Baeyer-Viliger oxidation of various cyclic ketones for industrial application.

One of the main topics of this chapter was the optimization of the Baeyer-Villiger fermentation process (Figure 4.1) for recombinant *E.coli* expressing CPMO (cyclopentanone monooxygenase) from *Comamonas*. Based on previous results obtained by small scale biotransformations, the development of an applicable and efficient up scaling process became an issue at stake.[43,52,53]

Furstoss *et al.*[61] published a very convenient and efficient methodology named "SFPR-concept", which overcomes several difficulties in microbial fermentation processes. While in preceding work this concept was exclusively applied to a single Baeyer-Villiger biooxidation, we wanted to evaluate the possibility of this "two-in-one" resin based SFPR[61,107,108] concept

[104] a) Wandrey, C.; Liese, A.; Kihumbu, D. *Org. Proc. Res. Dev.* **2000**, *4*, 286-290; b) Schmid, A.; Dordick, J.S.; Hauer, B.; Kiener, A.; Wubbolts, M.; Withold, B. *Nature* **2001**, *409*, 258-268; c) Straathof, A.J.J.; Panke, S.; Schmid, A. *Curr. Opin. Biotechnol.* **2002**, *13*, 548-556.

[105] Demirjian, D.C.; Shah, P.C.; Morís-Varas, F. *Top. Curr. Chem.* **1999**, 200, 1-29.

[106] a) Huisman, G.W.; Gray, D. *Curr. Opin. Biotechnol*. **2002**, *13*, 352-358; b) Zhao, H.; Chockalingam, K.; Chen, Z. *Curr. Opin. Biotechnol*. **2002**, *13*, 104-110.

[107] a) Vicenzi, J.T.; Zmijewski, M.J.; Reinhard, M.R.; Landen, B.E.; Muth, W.L.; Marler, P.G. *Enzyme Microb. Technol.* **1997**, *20*, 494-499; b) D'Arrigo, P.; Lattanzio, M.; Fantoni, G.P.; Servi, S. *Tetrahedron: Asymm*.

as a general tool in whole-cell mediated Baeyer-Villiger fermentation processes in the present study. This included the extension to highly different substrates (Figure 4.2) and the utilization of a different recombinant *E.coli* based expression system. We chose recombinant cells producing cyclopentanone monooxygenase (CPMO) from *Comamonas* NCIMB 9872,[43] as this enzyme displayed a remarkable substrate profile and stereoselectivity in previous studies.[96]

The ultimate goal of our ongoing research program is to reach substrate concentrations of grams per liter fermentation volume together with an easy work up and purification procedure of the desired product for subsequent industrial scale applications.

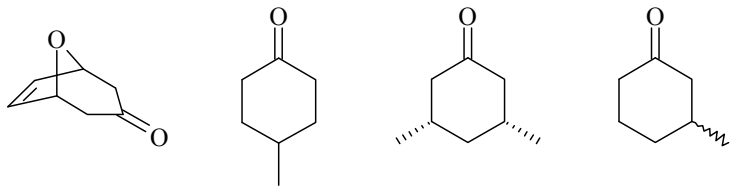


Figure 4.2 Fermentation optimization of microbial BV-oxidation with different prochiral cyclic ketones.

Among other issues one aim of this thesis and of this part especially was the optimization of the enantioselective microbial Baeyer-Villiger oxidation of bridged bicyclic ketone **16a**, which potentially offers a new entry to various C-nucleosides and poly substituted tetrahydrofurans (Figure 4.3).

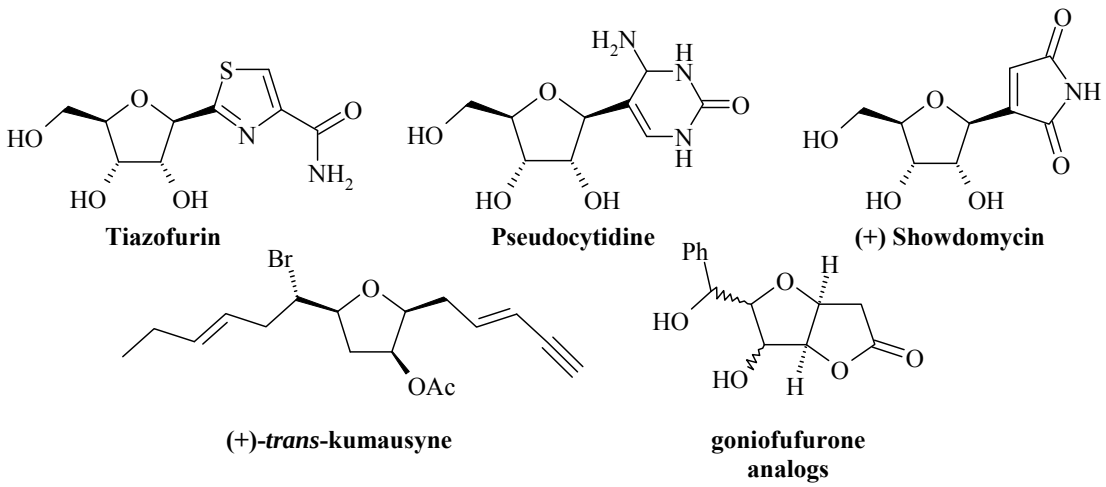


Figure 4.3 Various C-nucleosides and polysubstituted tetrahydrofurans.

1998, *9*, 4021-4026; c) Conceicao, G.J.A.; Moran, P.J.S.; Rodrigues, J.A.R. *Tetrahedron Asymm.* **2003**, *14*, 43-45.

[108] a) Hilker, I.; Gutierrez, M.C.; Alphand, V.; Wohlgemuth, R.; Furstoss R. *Org. Lett.* **2004**, *6*, 1955-1958; b) Gutièrrez, M.-C.; Furstoss, R.; Alphand, V. *Adv. Synth. Catal.* **2005**, *347*, 1051-1059; c) Hilker, I.; Wohlgemuth, R.; Alphand, V.; Furstoss R. *Biotechnol. Bioeng.* **2005**, *92*, 702-710.

C-Nucleosides possess a carbon-carbon linkage between the carbohydrate and the heterocyclic moiety, and have become an increasingly interesting area in organic and medicinal chemistry because of their potential utility as therapeutic agents such as antiviral, anticancer and anti-HIV drugs.
D-(+)-Showdomycin was chosen as a model substrate for the applicability of the presented enantioselective synthetic approach. This compound is a natural C-nucleoside displaying biolocical activity as an antibiotic and cytostytic and was first isolated from *Streptomyces showdoensis* by Nishimura and coworkers.[109]

4.1 Batch Fermentation

Several problems and disadvantages of the microbial Baeyer-Villiger oxidation have to be solved for the biotransformation to ultimately become appealing for industrial scale applications. Critical parameters in the optimization of fermentation conditions include temperature, pH, media composition, oxygen availability, efficiency of co-factor recycling, and utilization of highly productive recombinant *E.coli* strains, which have to be fine-tuned in an iterative process.

4.1.1 Preparation of the Biocatalyst - "Growing-" vs. "Non Growing" Conditions

Due to problems of reproducibility the starting point of all our batch fermentations was the cultivation of the recombinant host organism to obtain highly active biocatalyst. In general, cultures from frozen glycerin stocks were plated on an agar-agar petri-dish and were incubated overnight at 37°C. One single colony was picked to inoculate a preculture, which was then incubated overnight. It is necessary to reach the stationary phase of the pre culture to obtain similar and comparable growing conditions in the following batch-fermentation. Fresh media (1-2L) was inoculated with 2% of such an overnight culture. Optical density (OD_{590}) was measured every hour to control the growth of the cell culture.
Figure 4.4 describes a typical growing curve of bacteria. At first the bacteria enters the lag phase. This stage of growth is characterized by slow to no cell division. Dependent on the fermentation conditions and nutrient media, the cell machinery starts with the preparation and production of all required components for a fast and effective cell division.
The next stage, so called log phase, represents the exponential growing of the microorganism. As long as fermentations conditions have not been changed and the amount of required nutrients is constant, a continuous growth can be observed. Finally the stationary phase is reached. In general, the carbon and/or the nitrogen source are running low and therefore only the essential components, which prolong the lifetime of the cells, are produced and no further growth can be observed. The number of growing and dying cells is balanced. In the death or

[109] Nishimura, H.; Mayama, M.; Komatsu, Y.; Kato, H.; Shimaoka, N.; Tanaka, Y. *J. Antibiot. Ser. A.* **1964**, *17*, 148-155

decline phase the number of cells that are dying is greater than the number that is growing. The death phase may, but not necessarily, involve cell lysis.

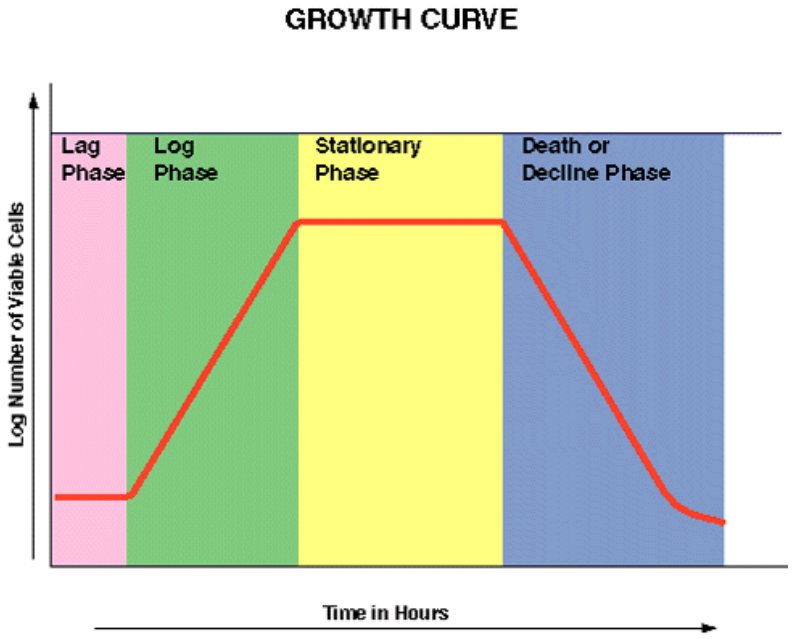


Figure 4.4 Typical cell growth of microorganisms

Due to the above described classical cell growth of microorganism two different approaches for an efficient batch fermentation can be followed. The biotransformation can be started during the early log or very late log phase. Under the so called "growing conditions" the protein production and the biotransformation take place in a parallel manner. After the recombinant microorganism reaches the exponential growing phase (early log phase) the cells are very robust and show very strong metabolic activity. Expression of the active $CPMO_{Coma}$ enzyme was initated with IPTG at 25°C and at OD_{590} of about 0.7. After 2 hours of induction the substrate was added and the biotransformation was started. This method represents one of the most common protocols used for protein expression and isolation.
On the other hand, biotransformations under "non growing" conditions have been performed in a completely different way. The concept behind this strategy is to maintain good and unhampered cell growth without any negative influence of an unnatural substrate until a specific high optical density was achieved. Shortly before the cells enter the stationary phase (late log phase) the induction of the production of biocatalyst was performed. After time dependent production of the protein the desired substrate can be added and the biotransformation can be started.

However, the presence of an unnatural substrate up to very high concentrations may influence the performance of the overall biotransformation. As long as the substrate concentration is maintained below any inhibition (substrate and/or product) or toxicity levels satisfying biotransformation results can be obtained. Both strategies have advantages and disadvantages

and within this thesis we investigated the best fermentation conditions with respect to our model substrates (Figure 4.2).

4.1.2 The Carbon Source - Glucose vs. Glycerol

BVMOs are flavin-enzymes which require nicotinamide phosphate as reducing agent. Previous studies with different recombinant *E.coli* overexpressing $CHMO_{Acineto}$ demonstrated the importance of a sufficient amount of NADPH during fermentation.[110] Both glycerol and glucose represent very efficient carbon sources for *E.coli*. Glucose metabolism in *E.coli* provides three key sources of NADPH which represents the crucial driving force for the redox reaction described in this thesis.

Figure 4.5 gives an overview of the main carbohydrate metabolism of *E.coli* focused on the production of NADPH. D-Glucose was converted to D-glucose-6-phosphate with consumption of ATP. This very important intermediate can enter both the Entner-Doudoroff pathway as well as the primary carbohydrate metabolism. The oxidation of D-glucose-6-phosphate to D-glucono-1,5-lactone-6-phosphate leads to 6-phospho-D-gluconate and thereby **1 equivalent** of $NADP^+$ was reduced to the desired **NADPH**. 6-Phospho-D-gluconate is capable to enter the pentose-phosphate pathway and another **equivalent** of **NADPH** is produced, whereas 6-phospho-D-gluconate is transformed to D-ribulose-5-phosphate. Two molecules of D-ribulose-5-phosphate can be converted to dihydroxyacetone phosphate and subsequent transformation to pyruvate opens an entry point to the citric acid cycle. On the other hand D-glucose is converted to D-glucose-6-phosphate with subsequent transformation to fructose-6-phosphate, in general. Continuous metabolic degradation leads to glyceraldehy-3-phosphate and dihydroxyacetone phosphate. Again, conversion of dihydroxyacetone phosphate to pyruvate opens the metabolic pathway of citric acid cycle.

[110] Walton, A.Z.; Stewart, J.D. *Biotechnol. Prog*. **2004**, *20*, 403-411.

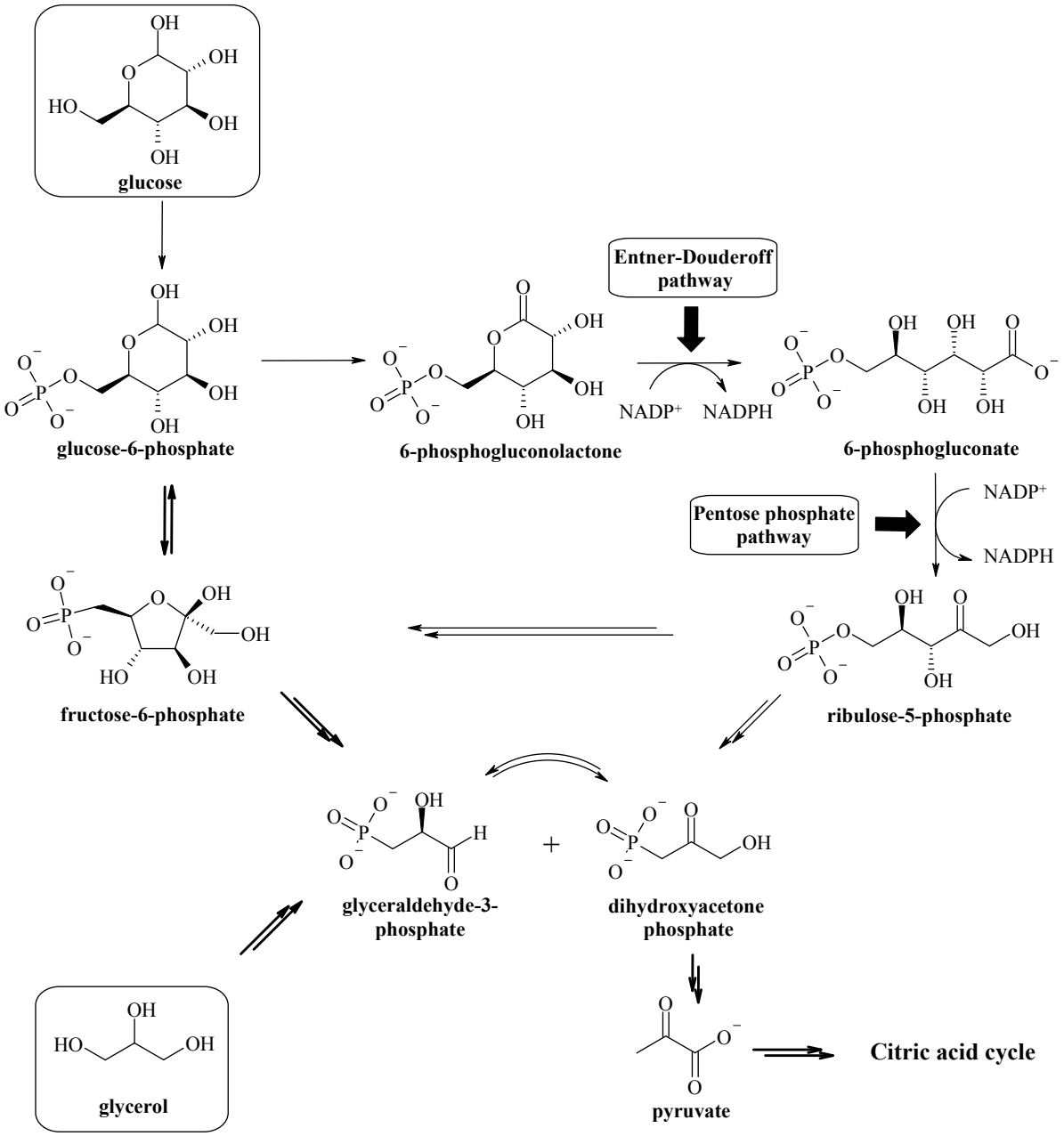


Figure 4.5 Carbohydrate metabolism in *E.coli*.

The third way for production of NADPH is represented by the citrate acid cycle (Figure 4.6), whereas pyruvate is transformed to acetyl–coenzyme A and ultimately gives isocitrate within several steps. Decarboxylation and subsequent oxidation of isocitrate to α-ketoglutarate yields **another equivalent** of **NADPH**.

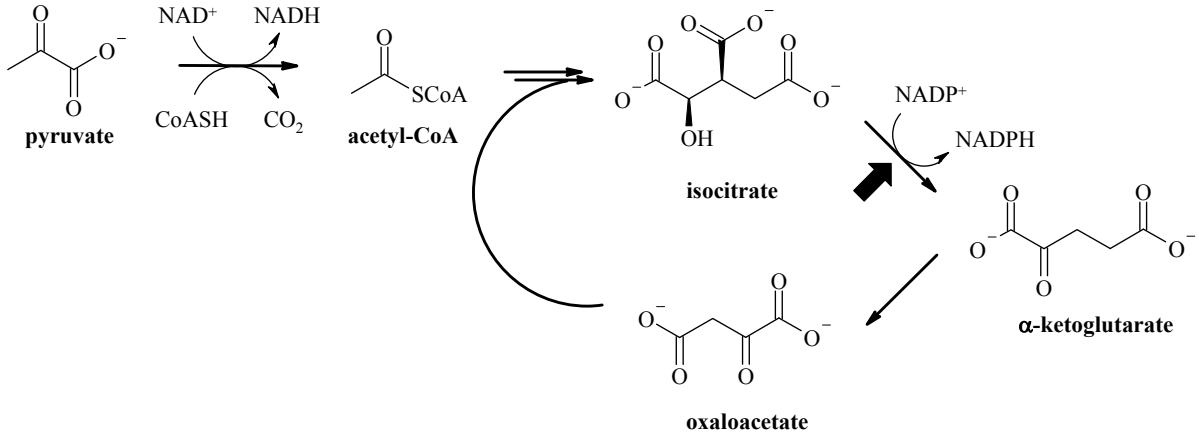


Figure 4.6 Abridgement of the citrate cycle (TCA) focused on the production of NADPH.

In comparison, glycerol enters the carbohydrate metabolism of *E.coli* exclusively at the stage of the citric acid cycle. Whereas glycerol is transformed to glyceraldehy-3-phosphate and dihydroxyacetone phosphate, subsequent reaction of dihydroxyacetone phosphate to pyruvate opens access to the classical citric acid cycle. Based on these theoretical considerations, the use of glucose as carbon source turned out to be the better choice compared to glycerol, in particular when NADPH as a cofactor is required. The utilization of glucose yields in a **three times higher** production of NADPH than in the case of glycerol. Due to this we performed our biotransformations in the presence of a specific amount of glucose (4g/L).

4.2 Parameter Optimization for the Fermentation Process

4.2.1 LB Media vs Terrific Broth (TB)

At the beginning of the optimization of the batch fermentation media composition was identified as critical parameter. Due to the fact, that all our shaking flask experiments were performed under growing conditions in LB media we decided to start the up-scaling experiments of the fermentation with the same media for a better comparison. It is known in the literature that cell densities achieved in LB media were limited to an OD_{590} of 5-6 because of the composition of the media, whereas much higher cell densities can be obtained with TB media. Our investigations were in agreement with the literature. In a typical growing experiment the fermentation media (1L TB supplemented by 200mg/L ampicillin) was inoculated with an overnight culture (2 vol%) of the recombinant microorganism (DH5α/CPMO) at 37°C and a maximum aeration of 5L/min. Production of biomass was measured by OD_{590}. Terrific broth led to very high concentration of biomass and rapid growth of cells. After 7 to 9 hours an OD of 10-12 was obtained. In contrast LB media gave much lower cell density and slower growth of cells. LB media supplemented by glucose (4g/L) as an additional carbon source led to comparable results as TB (Figure 4.7).

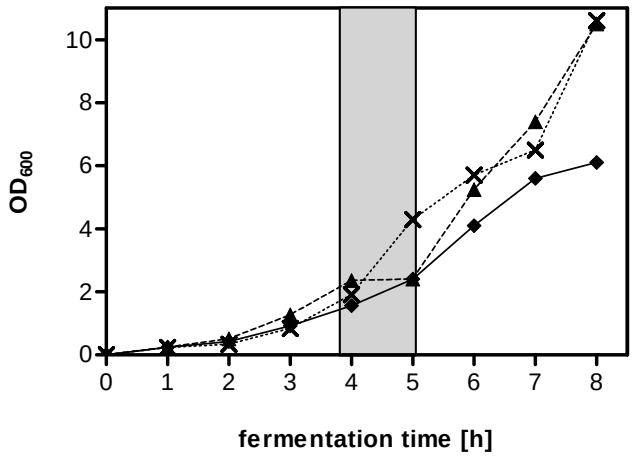


Figure 4.7 LB vs. TB – cell growth; the highlighted part displays the 2nd lag phase of cells growing in LB with glucose -**x**- OD_{590}-TB, -▲- OD_{590}-LB+glu; -♦- OD_{590}-LB.

In both cases the growth of the bacteria starts with the typical lag phase and enters into the log phase within the first 3 hours. Whereas a continuous cell division can be observed in the case of TB media, the cells seemed to stop the cell growth in LB media again to enter another kind of lag phase. After another hour of fermentation an exponential growth was observed again. Nevertheless the overall cell densities reached were comparable for TB and LB media.
In Figure 4.8 results of a closer investigation of the relationship between the cell growth and the glucose consumption during the fermentation in LB media are compiled. During the first 3 hours a usual slow cell growth (log phase) was observed and no glucose was consumed. Afterwards, a kind of a second "lag" phase as mentioned above was entered; the cells stopped growing and no glucose was consumed. Upon ongoing fermentation time the glucose consumption increased significantly and the cells entered a second "log" phase. Continuous growing of the cells was observed up to similar optical densities as described for terrific broth within the same reaction time.

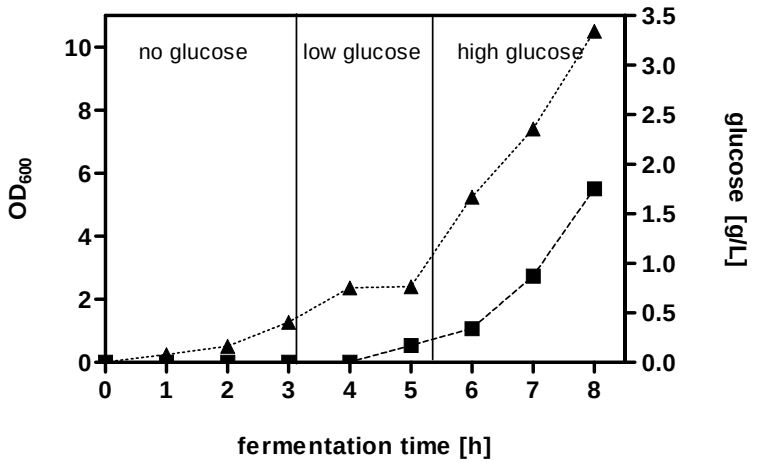


Figure 4.8 Glucose consumption vs. cell growth LB; -■- glucose consumption [g/L]; -▲- OD_{590}.

Finally we found, that TB media and LB media with glucose as an additive give similar results with respect to the overall growing performance and the produced biomass. However, due to the higher expenses in the case of LB media with glucose (high amount of consumed glucose) we decided to perform the following experiments in TB exclusively.

4.2.2 The in *situ* Substrate Feed and Product Removal Concept ("SFPR")

The concentrations of substrate and product in the liquid phase have to be controlled during the whole fermentation process to ensure maximum cell activity. This was realized by using an adsorbent material, which was "pre"-loaded with ketone (Figure 4.9). In the present study we exclusively used cells under growing conditions; therefore a rapid biotransformation was mandatory as the expression system loses efficiency upon reaching the stationary phase.

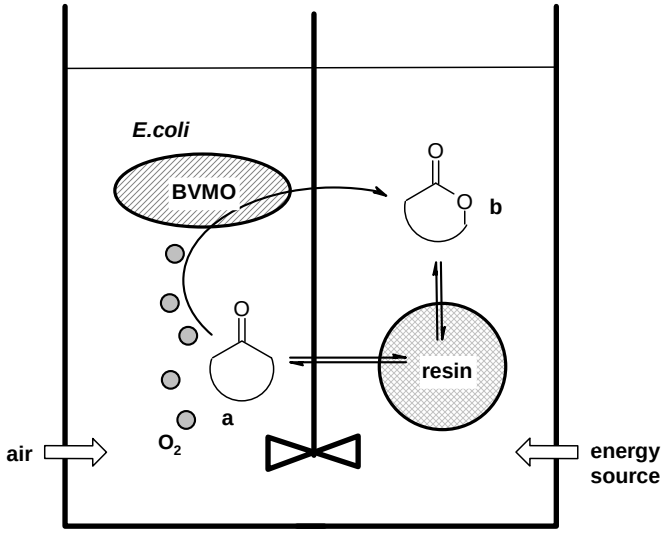


Figure 4.9 Principle of *in situ* Substrate Feeding / Product Removal concept (SFPR).

An illustration of the SFPR process during the biotransformation is shown in Figure 4.9. Initially, the loaded adsorbent is added to the fermentation broth and after a short period of time equilibrium between the adsorbent and the aqueous phase is reached. Thus, a defined amount of substrate ketone (Figure 4.2) can be transferred into the cell and will be oxidized by the BVMO. The product will leave the cell and can be re-adsorbed by the solid support. The equilibrium between the adsorbent and the aqueous phase has to be adjusted to concentrations below the inhibition level of the enzyme and toxic effects to the cells. One of the most important properties of the adsorbent will be a high capacity or high load at the equilibrium concentration chosen for the biotransformation. The load is defined and measured as described below:

$$X^{eq} = \frac{m_i}{m_{dry\ ads.}}$$

X^{eq}	equilibrium load
m_i	weight of compound i
$m_{dry\ ads.}$	dry weight of adsorbent

Equation 4.1 Calculation of load of resin.

A high load reduces the amount of adsorbent required per gram of substrate during the biotransformation, decreasing costs and influences on the whole-cell system, in general. According to the literature[61] tests of all the desired substrates were performed with different resins.

4.2.3 Screening of Adsorbent Material

Based on the results published by the group of Furstoss and his co-workers for commercially available resins, similar adsorbents were tested and the same "trend" for different substrates was observed. Two "resin families" could be identified, those with "Γ" shape adsorption curves and those with "S" shape curves. We determined a "working" load X^{eq} which corresponds to the optimal initial ketone concentration for the biotransformation depending on the kinetic studies for each substrate. The adsorption equilibria of *rac*-3-methylcyclohexanone (**34a**) were measured on different materials whereas the aqueous phase concentration c^{eq}_{ketone} in dependence of the equilibrium load X^{eq} was related.

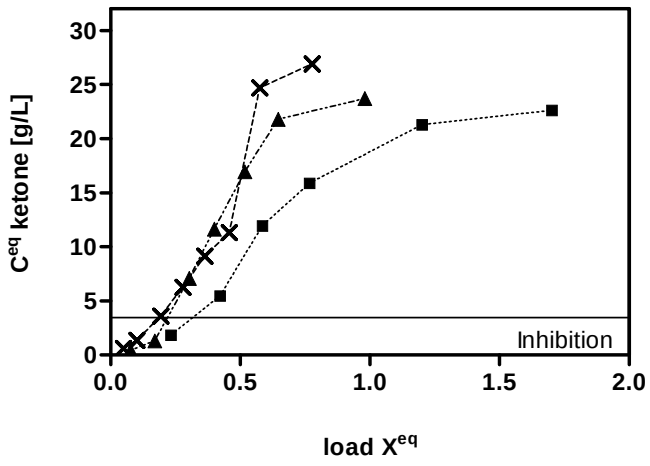


Figure 4.10 Tests with *rac*-3-methylcyclohexanone **34a** and different "Γ"-shape resins; -■- XAD-16, -▲- Sepabeads SP 207, -**x**- XAD-2000.

As shown in Figure 4.10 "Γ" resins were less efficient adsorbents because of their low working load X^{eq}. A high amount of resin would be necessary to maintain the concentration

c^{eq}_{ketone} below the inhibition level. The best performance was observed for XAD-16 which showed the higher working load X^{eq} but both, Sepabeads SP 207 and XAD-2000 displays a typical "Γ"-behavior with a low load X^{eq} in general. In contrast, the results of the tested resins in Figure 4.11 showed the appropriate effects.

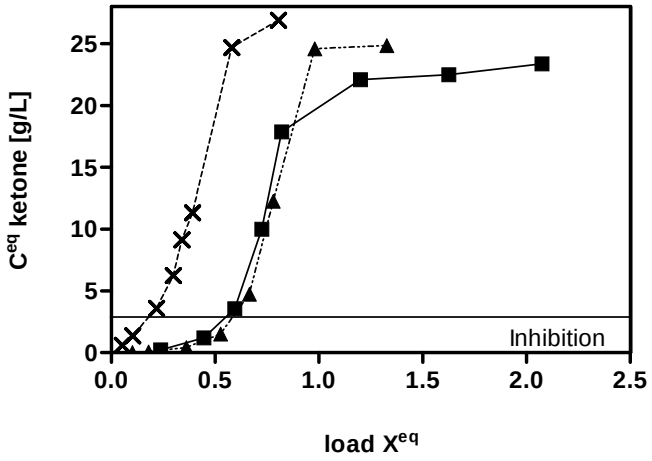


Figure 4.11 Tests with *rac*-3-methylcyclohexanone **34a** and different "S"-shape resins; -■- Optipore L-493, -▲- Lewatit VPOC 1163, -**x**- activated charcoal.

Figure 4.11 shows the adsorption equilibria of *rac*-3-methylcyclohexanone (**34a**) which were determined on different "S" shape resins The "S" type resins were much more efficient adsorbents than the "Γ" type because of their high working load X^{eq}. Hence, only low amounts of resin would be necessary to keep the concentration c^{eq}_{ketone} on an appropriate level.

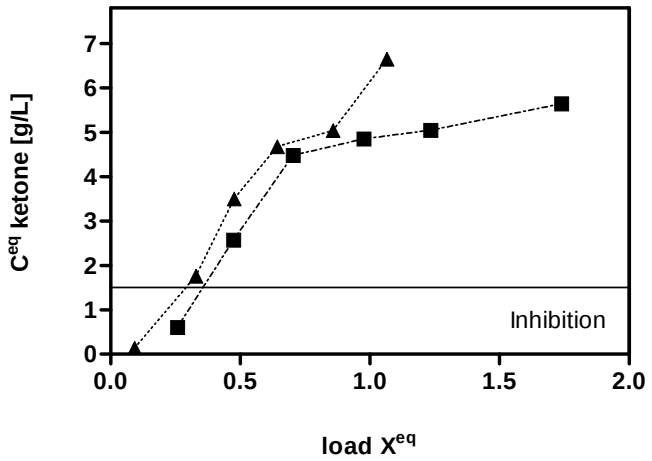


Figure 4.12 Tests with 3,5-dimethylcyclohexanone **21a** and different "Γ"-shape resins; -■- XAD-16, -▲- Sepabeads SP 207.

Resin studies with the more volatile and polar 3,5-dimethylcyclohexanone **21a** gave similar results. Since the maximum concentration of enzyme inhibition is much lower than in the corresponding 3-methylcylcohexanone **34a** series a very efficient resin was mandatory. Tests with the "Γ"-shape resins (Figure 4.12) resulted in a low working load X^{eq}, whereas the "S"

shape resins gave satisfactory results with respect to the adsorption equilibrium (Figure 4.13). The major problem for biotransformations with this compound was the high volatility and therefore subsequent attempts for a successful biotransformation failed, so far.

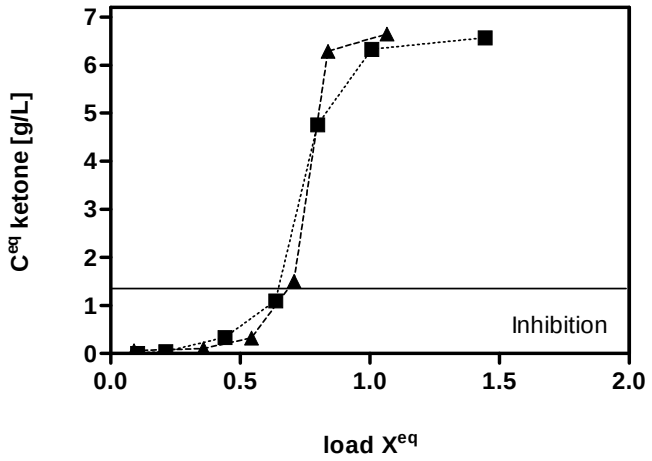


Figure 4.13 Tests with 3,5-dimethylcyclohexanone **21a** and different "S"-shape resins; -■- Optipore L-493, -▲- Lewatit VPOC 1163.

The most interesting compound for subsequent applications in the biooxidation of gram-quantities was 8-oxabicyclo[3.2.1]oct-6-en-3-one **16a**. Interestingly this compound is very water soluble and the adsorption on the resin was much more difficult than for less polar substrates. Eventually, a more polar resin would be a better choice for future experiments; however for our purposes we identified the best resin within the collection of investigated polymers, so far, and finally used Lewatit VPOC 1163 for the fermentation optimization (Figure 4.14).

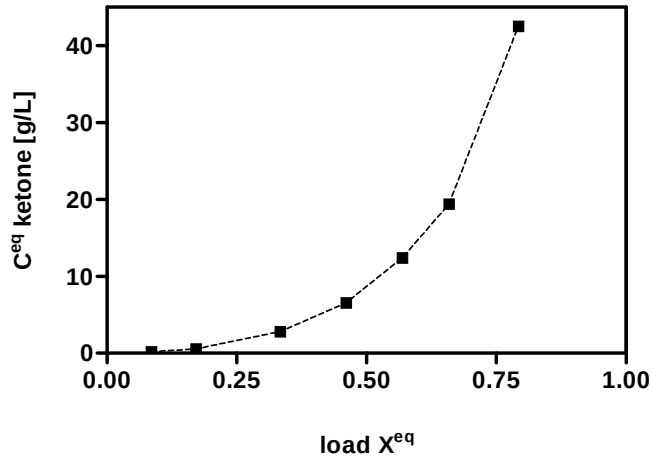


Figure 4.14 Tests with 8-oxabicyclo[3.2.1]oct-6-en-3-one **16a** and "S"-shape resin; -■- Optipore L-493.

We observed,[61] that resins can be classified into two types according to the shape of the adsorption isotherm: the "Γ"-shape group and the "S"-shape group. The latter type is characterized by a better load at low concentration. This classification is independent of the

substrate used. Based on our results two "S"-shape resins (Optipore L-493 and Lewatit VPOC 1163) could be identified which display nearly the same performances with respect to the working load X^{eq}. As only Lewatit VPOC 1163 was commercially available this resin was utilized within the following investigations. All results of the resin studies were summarized in Figure 4.15.

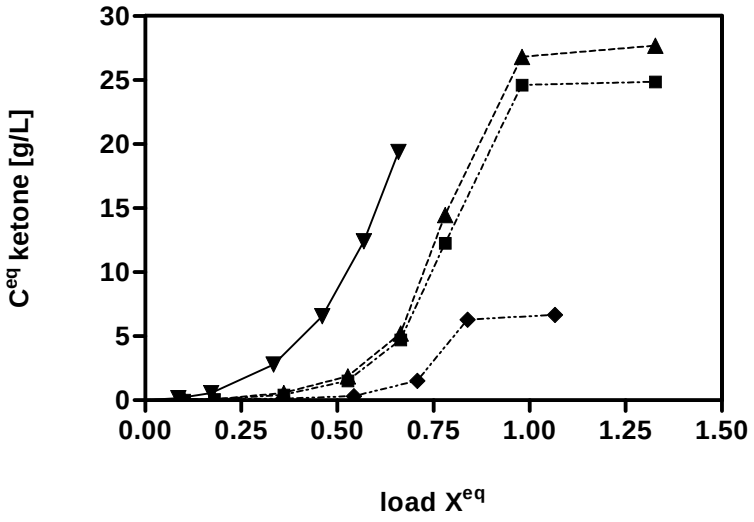


Figure 4.15 Tests with "s"-shape resin Lewatit VPOC 1163 and 4 different substrates. -▲- 4-methylcyclohexanone **20a**; -♦-3,5-dimethylcyclohexanone **21a**; -■- *rac* 3-methylcyclohexanone **34a**; -▼- 8-oxabicyclo[3.2.1]oct-6-en-3-one **16a**.

4.2.4 Activity Tests

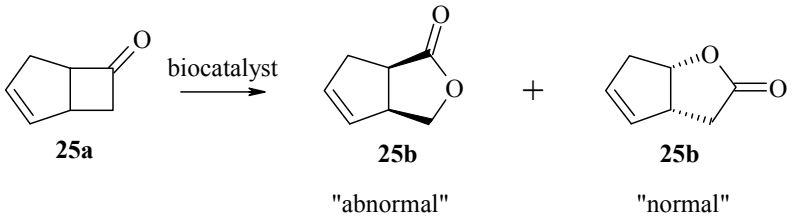


Figure 4.16 Model reaction for activity tests of the biocatalyst.

After optimization of cell growth and biocatalyst production, a control for activity of the cells became important, and therefore we used a fast and very reliable "activity test".

These tests were performed with a model reaction, which is well known from previous CHMO studies (Figure 4.16). The most readily accepted bicyclo[3.2.0]hept-2-en-6-one **25a** was converted in a shaking flask experiment to the desired normal and abnormal lactones (**25b**). A standard solution of bicyclo[3.2.0]hept-2-en-6-one in ethanol was added up to a concentration of 0.5g/L to a 20 mL sample of the growing culture from the fermentation run. Conversion of 80% or higher after 1 hour has to be achieved for cells with a high enzymatic activity (Figure 4.17). Upon passing this reactivity test, an efficient batch of recombinant cells can be assumed and biotransformations are expected to deliver top performance.

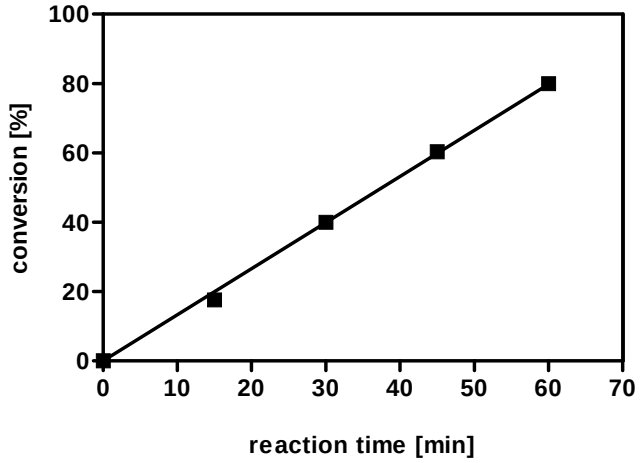


Figure 4.17 Conversion vs reaction time of the activity test of the biocatalyst.

4.2.5 Biotransformation of 4-Methylcyclohexanone 20a

4.2.5.1 "Growing"-Conditions

Based on previous shaking flask experiments we decided to start the "up scale" of the microbial Baeyer-Villiger oxidation in a batch fermentation process under "growing" conditions. Several parameters like oxygen saturation, time-point of induction, concentration of an auxiliary and influence of glucose were tested. The model reaction for this process was the conversion of 4-methylcyclohexanone **20a** to the corresponding lactone.

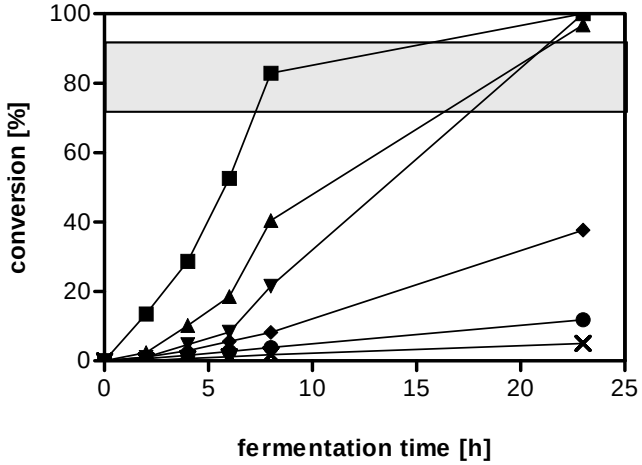


Figure 4.18 Kinetic studies for 4-methylcyclohexanone **20a** -■- 3mM; -▲-10mM; -▼-20mM; -♦-30mM; -●-40mM; -**x**-50mM.

Initially, inhibition level for our model reaction and CPMO had to be determined. All kinetic experiments were performed in 10mL scale shaking flask experiments under "growing" conditions. The obtained data showed a significant decrease in conversion of substrate **20a** within a concentration range of 25-30mM (Figure 4.18). Enzyme inhibition and possible

toxicity of the substrate was observed. Based on these kinetic studies for 4-methyl cyclohexanone **20a** all fermentations were performed with a maximum concentration of 30mM (3.36g/L).

A first set of experiments aimed at the optimization of the fermentation process without using a solid support reservoir. The fermentation media (1L TB supplemented by 200mg/L ampicillin) was inoculated with an overnight culture (2 vol%) of the recombinant microorganism (DH5α/CPMO) at 37°C. After 2 hours of growing (OD_{590}~1, 0.43g/L dcw), the fermentation temperature was decreased to 25°C and IPTG (0.25mM) was added to induce protein production. Subsequent addition of substrate **20a** (3.36g, 30mM) initiated the biotransformation under "growing" conditions. The results during the optimization process were compiled in Table 4.1.

Experiments	**1**	**2**	**3**	**4**	**5**
Substrate c [mM]	30	30	30	30	30
T [°C]	25	25	25	25	25
pH	7	7	7	7	7
O_2 sat. [%]	100	30	100	50	100
Glucose [g/L]	-	-	4	4	4
Fermentation time [h]	21	22	23	21	23
β-cyclodextrin [mol%]	-	-	-	-	10
Calculated Yield [%]	**56**	**63**	**72**	**84**	**81**

Table 4.1 Data for biooxidation of 4-methylcyclohexanone **20a** under "growing" conditions (without solid support reservoir).

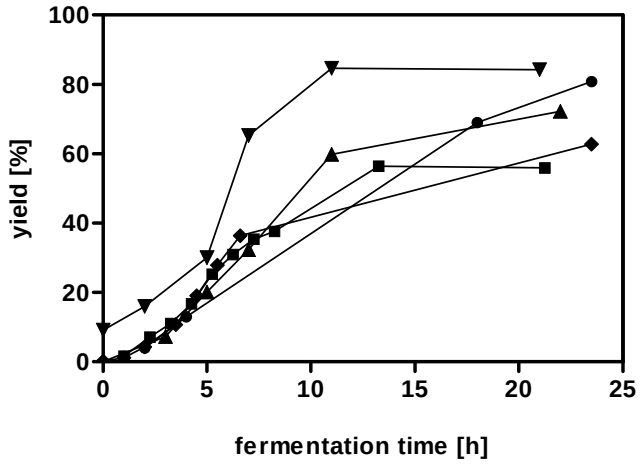


Figure 4.19 Conversion data of 4-methylcyclohexanone **20a** biooxidized under "growing conditions"; -■- exp #1; -♦- exp #2; -▲- exp #3; -▼- exp#4; -●- exp #5.

The oxygen saturation was set at 100% and the progress of the fermentation was determined by GC analysis with an internal standard to calculate the absolute yield of the fermentation process at any time without product isolation. The fermentation reached complete conversion after 21hours, however, with a moderate 55% calculated overall yield. Due to the loss of 45%

of starting material and the volatility of the substrate we decided to decrease the oxygen saturation to avoid evaporation of the starting material. The second experiment was performed under the same reaction conditions with an oxygen saturation of 30%. A slight increase in yield to 63% was observed. A substantial increase in efficiency of the biooxidation was achieved by addition of glucose (4g/L) after induction of protein production, with an increase in yield to 72% (experiment #3).

An acceptable trade-off between sufficient aeration (to ensure both biooxidation as well as microbial growth) and minimized ketone loss (due to the air flow) was found when running the fermentation with oxygen saturation at 50% and addition of glucose (84% yield). Finally, the influence of a β-cyclodextrin additive was tested, which may act either as transportation auxiliary for the substrate into the cell or as an additional reservoir for the substrate within the fermentation broth by a host-guest interaction. With an oxygen saturation of 100%, 4g/L of glucose and 10mol% of β-cyclodextrin a total yield of 81% was obtained. Nevertheless, the process is limited to a final substrate concentration of 3.36g/L (30mM).

4.2.5.2 SFPR-based Biotransformation of 4-Methylcyclohexanone 20a under "Growing" conditions

A second set of experiments aimed at the optimization of the fermentation process with using a solid support reservoir under the same "growing"-conditions as described above. Again the fermentation media (1L TB supplemented by 200mg/L ampicillin) was inoculated with an overnight culture (2 vol%) of the recombinant microorganism (DH5α/CPMO) at 37°C. After about 2 hours of growing (OD_{590}~1, 0.43g/L dcw), the fermentation temperature was decreased to 25°C and IPTG (0.25mM) was added to induce protein production. Subsequently, the pre-loaded resin was added and the biotransformation was monitored by GC-MS. The resin and the aqueous fermentation solution were extracted separately with dichloromethane or ethylacetate and an internal standard (methylbenzoate) to monitor the progress of the reaction.

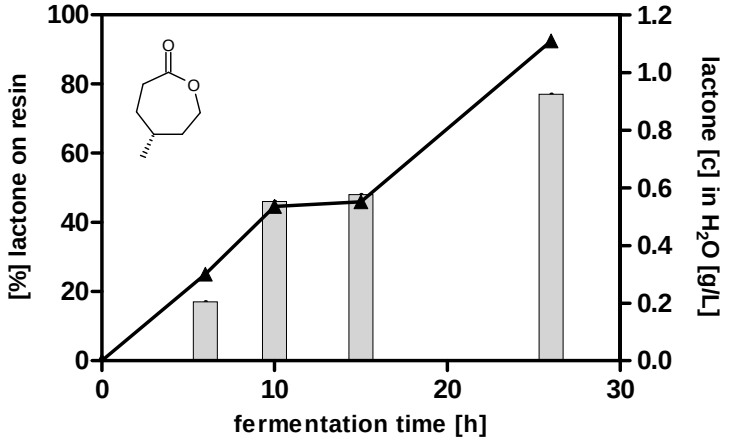


Figure 4.20 Fermentation of 10g/L 4-methylcyclohexanone **20a** with CPMO "growing cells"; -▲- g/L lactone in aqueous phase; ▭ percent lactone of organic compounds adsorbed to the resin.

Based on our results from resin and kinetic studies, 10g/L of 4-methylcyclohexanone **20a** were successfully converted to the corresponding lactone **20b** in a first attempt by CPMO producing "growing" cells**.** The biotransformation was performed under the same reaction conditions as outlined in experiment 5 (Table 4.1) and gave lactone **20b** after 26 hours with 77% yield (Figure 4.20).

4.2.6 SFPR-based Biotransformation under "Non-Growing" Conditions

Most of the critical aspects of the above set of experiments could be overcome upon applying the SFPR concept. The effect of the non-natural substrate on the biocatalyst (recombinant whole-cells) was reduced to a minimum and therefore the problems of toxicity and enzyme stability were solved.
Three different substrates were transformed applying the "SFPR" procedure described above. Commercially available prochiral (4-methylcyclohexanone, **20a**) and racemic (*rac*-3-methylcyclohexanone, **34a**) ketones were used to prove the concept and compare SFPR fermentations to previously reported results for CPMO biooxidations. Baeyer-Villiger oxidation of the third substrate 3,5-dimethylcyclohexanone **21a** turned out to be the first example where we observed complete enantiodivergent transformation with our set of recombinant *E.coli*. Therefore we tested ketone **21a** with several resins, but it turned out to be too volatile for big scale fermentations and all our efforts for a successful biotransformation failed and no results were obtained. Fermentation under classical "non growing" conditions were performed, but at one hand we were not able to determine any conversion of the starting material and at the other hand we observed a rapid decrease in the concentration of the starting material during the experiment. Due to its volatility and the fact that we bubble air through the fermentation broth we lost most of the compound during the experiment. Compound **21a** was analyzed in the lab-atmosphere by its characteristic odor.
CPMO is able to give antipodal lactone **20b** compared to the majority of other BVMOs.[111] Ketone **34a** is transformed in a regioselective process to the proximal lactone **34b-prox**. The attractive prochiral precursor 8-oxabicyclo[3.2.1]oct-6-en-3-one **16a** represented our benchmark experiment using a highly polar and structurally demanding substrate.

Based on the above results the critical limitation of the biotransformation seems to be toxicity to the whole-cells eventually accompanied by enzyme inhibition. When approaching a substrate concentration of 30mM (3.36g/L) for **20a** a significant decrease of cell growth was observed. Such stagnation in biomass consequently results in a slow biooxidation during the fermentation process due to low amount of active enzyme. As we wanted to maximize the amount of biomass, in the subsequent set of experiments implementing the SFPR concept a different biotransformation strategy was chosen. Under "growing" conditions the biotransformation took place at the early log phase of the growth of the cells.

[111] Wang, S.; Kayser, M.M.; Iwaki, H.; Lau, P.C.K. *J. Mol. Catal. B: Enzym*. **2003**, *22*, 211-218.

Biotransformations with "non-growing" cells were investigated in the very late log or the beginning of the stationary phase.

Therefore the growth of the cells was continued at 37°C and under 5vvm of air until an optical density of 7-8 was reached (or when the change in OD_{590} increased by less than 0.5 AU over a 30min period). Using this strategy, cell growth was not decreased by the presence of a toxic substrate. As soon as the maximum amount of biomass was produced, the fermentation temperature was decreased to 25°C and protein production was induced by addition of IPTG (0.25mM). At this temperature the cell division rate was slowed down significantly. Glucose (4g/L) was added 1hour after induction. Another hour later cells were sampled and tested for their biooxidation activity. These tests were performed with the model reaction using the highly reactive ketone bicyclo[3.2.0]hept-2-en-6-one **25a** known from other BVMO biotransformations in a shake flask experiment as mentioned before. In a third optimization experiment we performed the biotransformation with 15g/L of 4-methylclohexnone **20a** under "non-growing" conditions and applying the "SFPR" concept and obtained 86% of the desired lactone **20b** in only 20 hours of fermentation time (Figure 4.21).

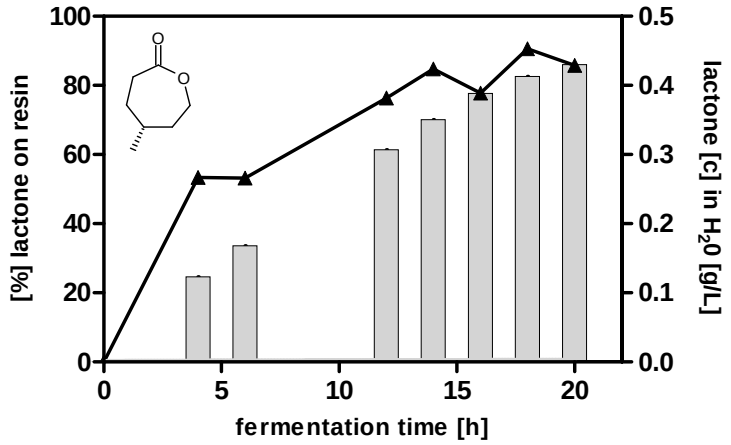


Figure 4.21 Fermentation of 15g/L 4-methylcyclohexanone **20a** with CPMO "non growing cells"; -▲- g/L lactone in aqueous phase; ☐ lactone of organic compounds adsorbed to the resin.

This represents a five-fold increase in substrate concentration and an improved yield compared to "growing" cell fermentation conditions without using the "SFPR" concept. The combination of "non-growing" cells and the "SFPR" concept turned out to be the best conditions for a biooxidative whole-cell Baeyer-Villiger process. No problems with respect to substrate volatility were observed for this precursor. The performance of the CPMO producing cells was in the range previously reported in the literature on an overexpression system for cyclohexanone monooxygenase (CHMO).[110] The fastest biotransformation and therefore the highest BVMO activity was determined within the first 12 hours (60% conversion) and afterwards a moderate loss of activity was observed (Figure 4.21).

The second example for the "SFPR" concept was demonstrated with racemic 3-methylcyclohexanone **34a** which is oxidized regioselectively to the proximal lactone **34b-prox** (Figure 4.22).[112]

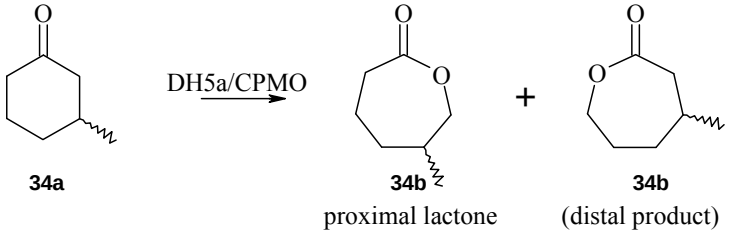


Figure 4.22 Regioselective Baeyer-Villiger oxidation of *rac*-3-methylcyclohexanone by CPMO yielding only proximal lactone **34b-prox**.

Biotransformation with 15g/L *rac*-3-methylcyclohexanone **34a** was successfully performed using the "SFPR" concept. Again, the majority of starting material (80%) was consumed within the first 10 hours of the biotransformation. The corresponding lactone **34b-prox** was isolated in 90% yield (96% conversion) after 16 hours of fermentation time (Figure 4.23).

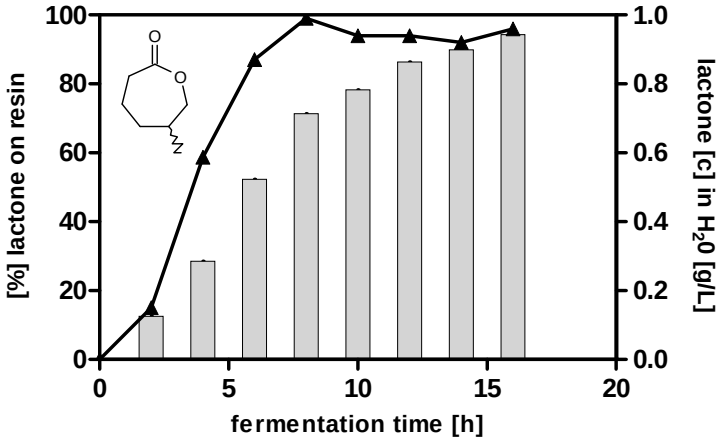


Figure 4.23 Fermentation of 15g/L *rac*-3-methylcyclohexanone **34b** with CPMO "growing cells"; -▲- g/L lactone in aqueous phase; ▢ lactone of organic compounds adsorbed to the resin.

After the growth of the cells and the induction of the biocatalyst took place the active cells can be stored at 4°C for a few days without losing their biocatalytic activity. Based on that data we performed another experiment whereas we tried to use a "repeated cell cycle" approach to increase the overall concentration of the substrate. Therefore, ketone **34a** (10g/L) was oxidized according to above described fermentation procedure using the SFPR concept. Conversion rates up to 30 % of the starting material were obtained within the 1st cell cycle. Afterwards the resin was filtered off and was re-suspended in a new batch of recombinant whole-cells, which was stored previously at 4°C. Continuous biotransformation with the

[112] Wang, S.; Kayser, M.M. *J. Org. Chem*. **2003**, *68*, 6222-6228.

second cell cycle gave the desired lactone **34b** in over 90 % yield (Figure 4.24). We suppose that it is possible to increase the substrate concentration by two fold by applying this strategy.

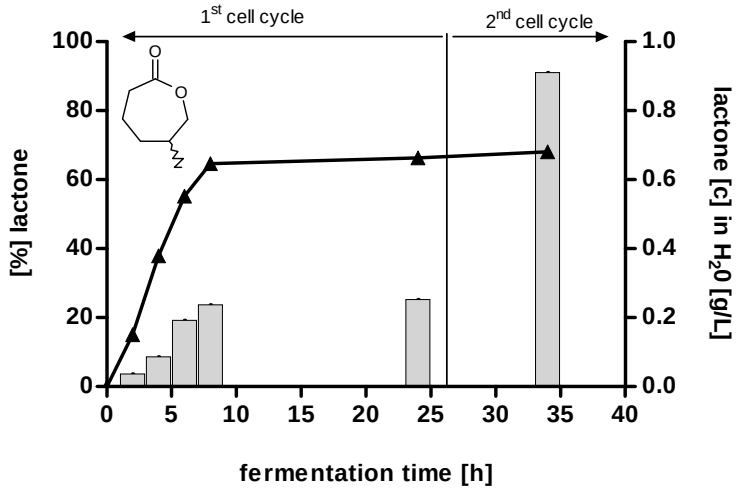


Figure 4.24 Fermentation of 10g/L *rac*-3-methylcyclohexanone **34a** with CPMO "growing cells"and the use of two cell cycles; -▲- g/L lactone in aqueous phase; ▭ lactone of organic compounds adsorbed to the resin.

Ketone **16a** (8-oxabicyclo[3.2.1]oct-6-en-3-one) represents an interesting starting material for biooxidative desymmetrization offering access to lactone **16b** as versatile platform for subsequent synthetic transformations. The prochiral compound possesses a relatively high water solubility and is only moderately stable at slightly elevated temperatures (>35°C) and under acidic conditions.

	growing cells	**growing cells**	**non-growing cells**	**non-growing cells**
Exp. Set up	shaking flask	bench top fermenter	bench top fermenter	bench top fermenter
Ferment. Conc.	0.4 g/L	0.7 g/L	2 g/L	5 g/L
Ferment. Temp	20°C	24°C	24°C	24°C
Ferment. Media	LB	LB	TB	TB
Resin	-	-	-	Lewatit VPOC 1163
pH:	7	7	7	7
O2-sat.:	100%	100%	100%	100%
Add. C-source	-	4 g/L glucose	4 g/L glucose	4 g/L glucose
β-Cyclodextrin	1 eq.	10 mol%	10 mol%	10 mol%
Ferment. Tim	4d	36h	24h	36h
Conversion	not complete	100%	100%	85%
Work up	extr. with CH_2Cl_2	extr. with CH_2Cl_2	cont. extr. with CH_2Cl_2	cont. extr. with CH_2Cl_2
Yield	**60%**	**81%**	**72%**	**79%**

Table 4.2 Fermentation results for 8-oxabicyclo[3.2.1]oct-6-en-3-one **16a**.

The substrate showed a very slow kinetics and therefore highly active cells and long reaction times were required. The first experiments were performed in a typical shaking flask experiment with a substrate concentration of 0.4 g/L at 20°C under growing conditions with 1 equivalent of β-cyclodextin as an additive (Table 4.2). The reaction time took about 4 days and no complete conversion was achieved. The desired product was isolated after extractive work up with CH_2Cl_2 followed by chromatographic purification to give 60% yield. The second experiment demonstrates the influence of glucose addition during the fermentation. Hence, we changed the experimental set up and performed the transformation in a bench top fermenter. All parameters were similar to the shaking flask experiment with the exception of the addition of 4g/L glucose during the biooxidation. Within this experimental set up we were able to reduce the amount of β-cyclodextrin to some extent from 1 equivalent to only 10mol% of the ketone. We wanted to investigate a cost efficient process also and therefore it was mandatory to reduce the amount of any expensive additives like β-cyclodextrin. Decreasing the amount of β-cyclodextrin did not show any negative effect on the overall fermentation performance down to an amount of 10mol%. No further investigations were performed to identify the specific role of β-cyclodextrin as an additive. However, there are hypotheses in the literature, that it could facilitate the transportation of substrates through the cell membrane as well as to act like a reservoir and therefore as a protection for the ketone to maintain the substrate concentration below a defined concentration in the fermentation process.
Nevertheless the reduction of β-cyclodextrin is a big advantage of the overall process to cut fermentation expenses. By changing the process conditions from "growing" to "non-growing" cells another progress for the fermentation was achieved. We increased the substrate concentration in former experiments from 0.7 to 2 g/L and shortened the reaction time to 24 hours. The continuous extraction of the crude fermentation solution with CH_2Cl_2 gave the desired lactone after purification in 72% yield. Further attempts failed to increase the fermentation concentration of ketone **16a** to more than 2.0g/L (70% isolated yield) because of substrate inhibition. All these experiments were carried out in the absence of a resin to act as substrate reservoir and are summarized in Figure 4.25.

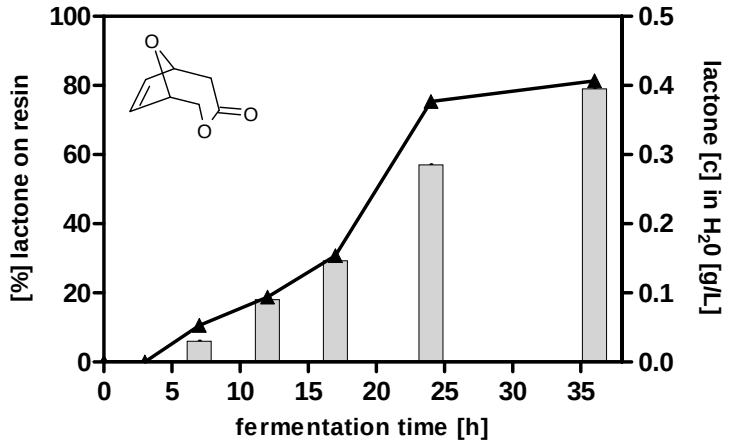


Figure 4.25 Fermentation of 5g/L 8-oxabicyclo[3.2.1]oct-6-en-3-one **16a** with CPMO "non growing cells"; -▲- g/L lactone in aqueous phase; ▭ lactone of organic compounds adsorbed to the resin.

Upon switching to the SFPR methodology it was interesting to note that only 29% conversion of the starting material was observed after the first 14 hours. The following 22 hours of the biotransformation yielded in total 79% of lactone **16b**. In contrast to previous experience in shake-flask experiments, no hydrolytic degradation of the lactone product was observed upon prolonged fermentation times. The addition of β-cyclodextrin (10mol%) was mandatory for a successful biotransformation, which supports the hypothesis to facilitate membrane transport. Ultimately, substrate concentrations could be increased to 5g/L upon applying the "SFPR" concept.

4.3 Conclusion

We demonstrated that the microbial Baeyer-Villiger oxidation can be performed in multiple gram scale using the SFPR concept. Together with previous studies on CHMO, this technique can be regarded as a general tool to improve the performance of recombinant whole-cell expression systems for BVMOs. In particular, the method circumvents toxicity and inhibition problems and reduces loss on volatile substrates to some extent. Figure 4.26 summarizes the optimization results for the microbial Baeyer-Villiger oxidation in bench-top fermenter scale based on the transformation of *rac*-3-methylcylcohexanone **34a**. We were able to increase the substrate concentration from 3g/L to 15g/L. Due to the optimization of the biocatalyst itself by adopting the best growing conditions the fermentation time were reduced from 36 hours to only 16 hours.

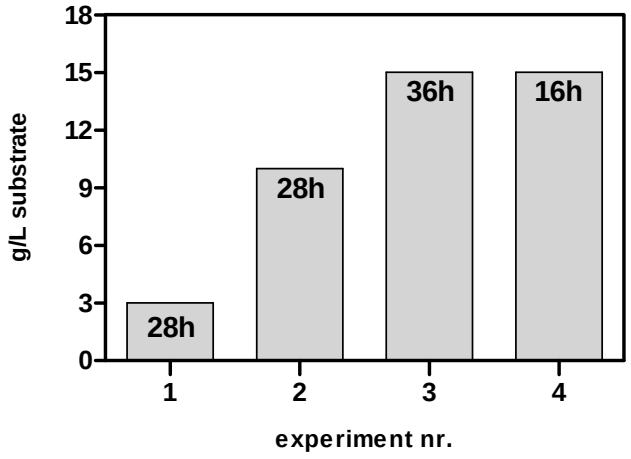


Figure 4.26 Summary of fermentation optimization based on *rac*-3-methylcyclohexanone **34a**; #1 fermentation under "growing conditions" w/o resin support; #2 fermentation under "growing conditions" with resin support; #3 fermentation under "non-growing conditions" with resin support; #4 fermentation under " non-growing conditions" with resin support and optimized growing conditions;

A further increase of substrate concentration may be realized by combination of the SFPR with "stationary phase" cells in minimal media together with the application of repeated fermentation cycles. Such future studies aiming at industrial scale fermentations may also

include optimization of the fermentation equipment as recently outlined by the group of Wohlgemuth.[113] Together with progress in the development of a tool-box of BVMOs, this work should contribute to establish the microbial Baeyer-Villiger oxidation as a standard technique in synthetic chemistry, which enables access to gram quantities of chiral lactones as versatile intermediates in bioactive compound preparation.

[113] Hilker, I.; Baldwin, C.; Alphand, V.; Furstoss, R.; Woodley, J.; Wohlgemuth, R. *Biotechnol. and Bioeng.* **2006**, *93*, 1138-1144.

5 Formal Total Synthesis of D-(+)-Showdomycin

5.1 Introduction

In recent years, nucleoside analogs received considerable attention in synthetic chemistry owing to their biological and pharmaceutical properties. Nucleosides are generally considered to be compounds which posses a carbohydrate moiety and an N-glycosyl-linked heterocyclic aglycon. C-Nucleosides differ from the more common nucleosides in that the carbohydrate and the heterocyclic moiety are connected by a C-C rather than a C-N bond. Since pseudouridine was isolated from transfer RNA in 1957 as the first reported C-nucleoside,[114] a number of naturally occurring C-nucleosides (Figure 5.1) have been isolated mainly from the culture filtrates of various *Streptomyces* species.[115]

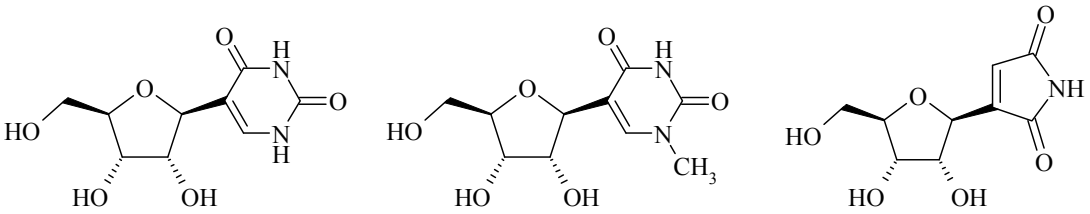


Figure 5.1 Naturally occurred C-nucleosides.

The carbon-carbon bond is responsible for the resistance of such C-nucleosides to enzymatic and hydrolytic cleavage. C-Nucleosides are furthermore suitable compounds for the construction of triplex DNA in gene therapy. Based on their unique structural features most naturally occurring C-nucleosides exhibit antibiotic, anticancer, and/or antiviral activities.[114,116] Back in the 1960s, five-membered ring nucleoside antibiotics such as D-(+)-showdomycin were isolated from bacterial culture and showed significant activity against a broad series of viruses in cell culture. D-(+)-Showdomycin shows an antibiotic activity against gram + and gram – bacteria and possesses a significant antitumor activity against Ehrlich mouse ascites tumor *in vivo* and against cultured HeLa cells.[117]

Methods for the synthesis of C-nucleosides have been studied extensively.[118] However, synthetic drawbacks in terms of low yield and/or poor enantioselectivity have been frequently encountered. Two major synthetic approaches for the preparation of such compounds have been established over the last decades. At one hand a pre-synthesized heterocyclic base unit

[114] Towsend, L.B. *Chemistry of Nucleosides and Nucleotides*; Plenum Press: New York, **1994**, 421-535.

[115] Chambers, R.W. *Prog. Nucleic Acid Res. Mol. Biol*. **1966**, *5*, 349-398.

[116] a) Guntaka, R.V.; Varma, B.R.; Weber, K.T. *Int. J. Biochem. Cell Biol*. **2003**, *35*, 22-31; b) Navarre, J.; Guianvarch, D.; Giorgio, A.F.; Condom, R.; Benhida, R. *Tetrahedron Lett*. **2003**, *44*, 2199-2202; c) Togo, H.; He, W.; Waki, Y.; Yokoyama, M. *Synlett* **1998**, 700-717.

[117] Darnall, K.R.; Townsend, L.B.; Robins, R.K. *Proc.Natl.Acad.Sci*. USA **1967**, 57, 548-553.

[118] Wu, Q.; Simons, C. *Synthesis* **2004**, *10*, 1533-1553.

was directly attached to an appropriate carbohydrate moiety. On the other hand introduction of a functional group at the anomeric center of a sugar derivative with subsequent construction of aglycon moiety leads to the desired C-nucleosides. We focused our work on the second approach, because we were interested in the total synthesis of natural and/or unnatural C-nucleosides starting from non-sugar precursors by applying a biocatalytic methodology for the introduction of chirality. Hence, this strategy allows a maximum flexibility in the synthesis of different C-nucleosides by variation of the heterocyclic base moiety. Development of methodologies for the creation of four asymmetric centers in the ribose skeleton as well as a stereochemical control at the anomeric center turned out to be the most challenging aspects of this approach.

D-(+)-Showdomycin (Figure 5.1), first isolated from *Streptomcyces showdoensis* by Nishimura *et al*.,[109] was chosen as a model target for our investigations based on its interesting pharmacological properties as antibiotic and antiviral compound.

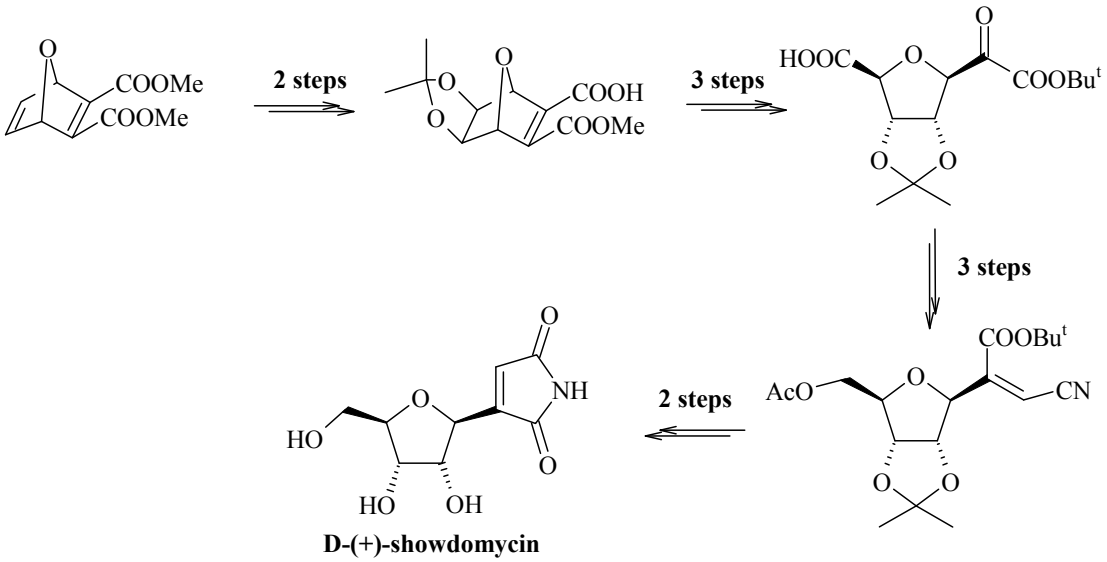


Figure 5.2 Different approaches for the synthsis of D-(+)-showdomycin as published by Ohno *et al*.

Several synthetic approaches for the synthesis of D-(+)-showdomycin were established during the last two decades. In 1984, Ohno *et al*. presented a chemoenzymatic and enantioselective synthetic route towards D-(+)-showdomycin. The crucial step for the introduction of chirality in this approach was based on asymmetric hydrolysis by esterases. The desired C-nucleoside was prepared *via* 10 synthetic steps and an overall yield of only 8% (Figure 5.2).[119]

[119] Ohno, M.; Ito, Y.; Arita, M.; Shibata, K.; Adachi, K.; Sawai, H. *Tetrahedron* **1984**, *40*, 145-152.

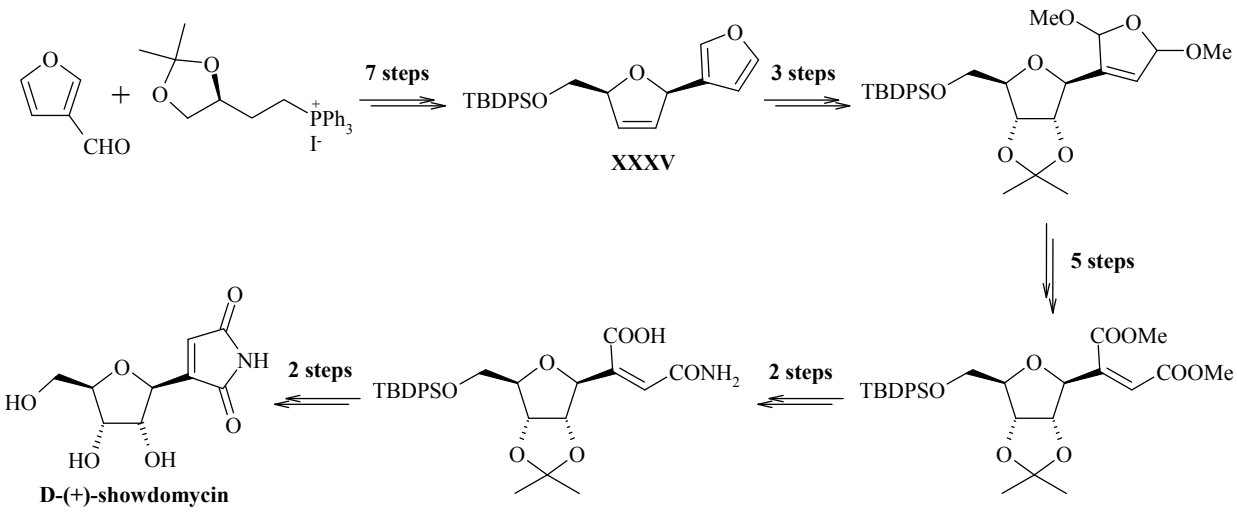


Figure 5.3 Synthesis of D-(+)-showdomycin by Kang *et al*.

Kang *et al.* presented a completely different approach and started the synthesis from *syn*-2,5-disubstitutet tetrahydrofurans (Figure 5.3).[120] This synthetic approach starting from a chiral precursor was accomplished practically in 26% starting from *syn*-2,5-disubstituted 2,5-dihydrofuran **XXXV** within 19 synthetic steps.

Another, very convenient and stereocontolled entry towards C-nucleosides (especially D-(+)-showdoycin), starting from inexpensive non-carbohydrate substances was published by Noyori *et al*. in 1984 (Figure 5.4).[121] The ribose skeleton **36** was prepared by the efficient [3+4] cycloaddition reaction between tetrabromo acetone and furan aided by $Fe_2(CO)_9$ or activated zinc and subsequent oxidative modifications of the resulting oxabicyclic structure. The unsaturated prochiral oxabicyclic ketone **16a** can be transformed to diol **37** *via* osmylation. Hence protection of diol **37** gives acetonide **38**, and subsequent Baeyer-Villiger oxidation yields racemic lactone **36**. Expensive separation of the two enantiomers (+/-)-**36** *via* ring opening and formation of diastereomers **XXXIX** and **XXXX** by Pikle's resolution method results in the optical pure lactone **(+)-36**. The synthetic sequence continues by the introduction of a formyl group or its synthetic equivalent at α-position to lactone **46** *via* a knoevenagel type condensation with the Bredereck reagent[122] *tert*.-butoxybis(dimethylamino)methane. Ozonolysis of the obtained aminomethylene lactone **46** and following Wittig condensation gives the desired protected D-(+)-showodmycin **51**. The major drawback of this approach is the low overall yield (10 steps, 2.4% yield) for D-(+)-showdomycin. A closer look at this synthetic approach points out two critical steps that are responsible for the dramatic decrease of the over-all yield: (i) chemical Baeyer-Villiger oxidation (c) towards racemic **36**; (ii) ozonolysis of aminomethylene lactone **46** which gives the desired product **51** in only 30% yield. Based on this synthetic approach, the emphasis of

[120] Kang, S.H.; Lee, S.B. *Tetrahedron Lett*. **1995**, *23*, 4089-4092.

[121] Sato, T.; Hayakawa, Y.; Noyori, R. *Bull. Chem. Soc. Jpn*. **1984**, *57*, 2515-2525 and references therein.

[122] Bredereck, H.; Simchen, G.; Rebsdat, S.; Kantlehner, W.; Horn, P.; Wahl, R.; Hoffmann, H.; Grieshaber, P. *Chem. Ber*. **1968**, *101*, 41-50.

this work was the optimization of this very stereoselective and chemically convenient synthetic approach towards D-(+)-showdomycin.

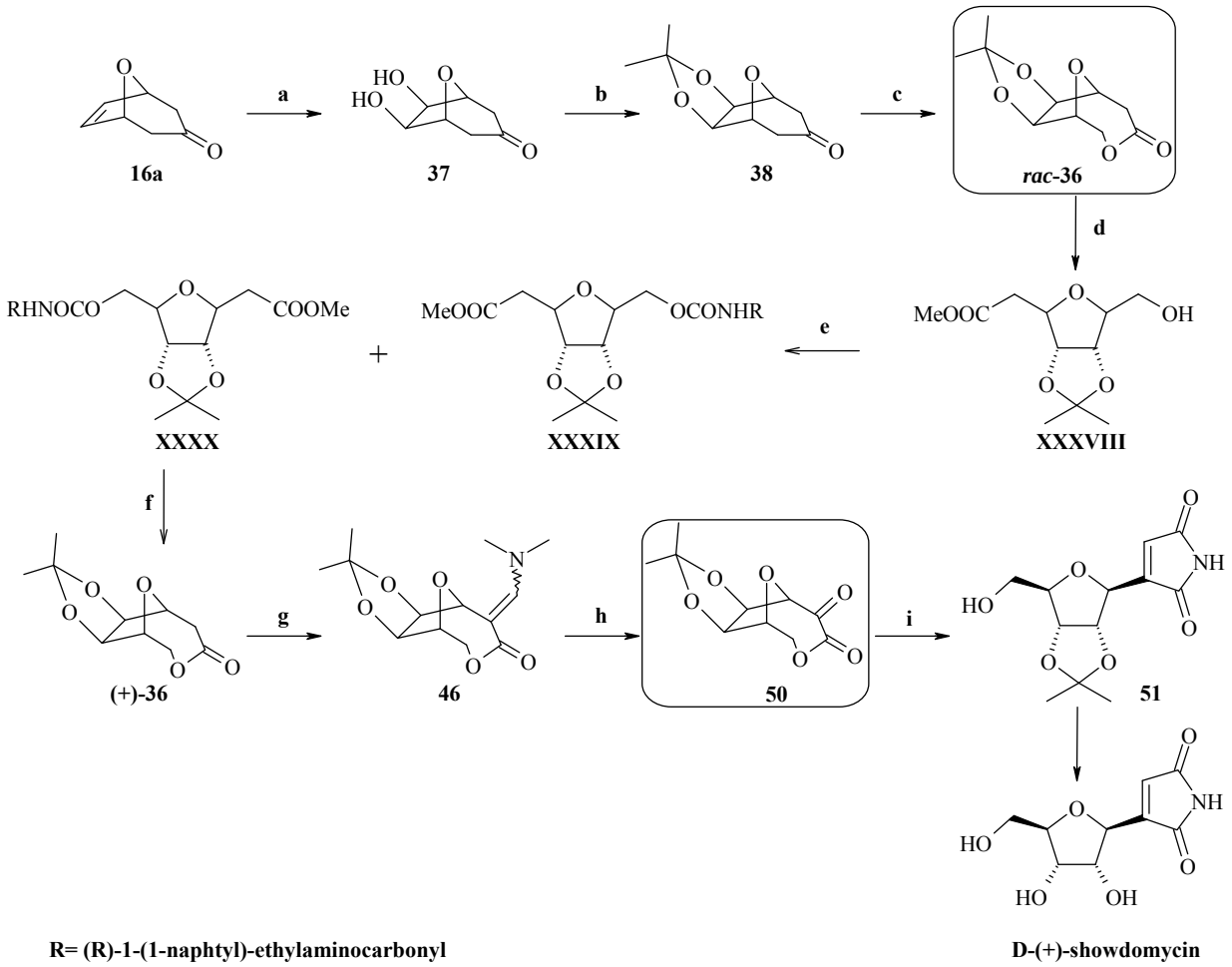


Figure 5.4 Total synthesis of D-(+)-showdomycin by Noyori *et al*.

The retro synthetic analysis of the first part of the sequence described above, starting from racemic acetonide **36** is outlined in Figure 5.5, **A**. Since it is not possible to perform the chemical Baeyer-Villiger oxidation of **38** in an enantioselective way, we envisioned to apply our expertise in microbial Baeyer-Villiger oxidations of prochiral ketones: switching the order of reaction steps would allow obtaining chiral lactone **36** *via* biooxidation product **16b** from ketone **16a** in a stereoselective manner.

The second obstacle of the presended synthetic strategy was the low yield for the introduction of a carbonyl group in the α-position of lactone **36** *via* ozonolysis. In a previously published literature reference, the modification of the α-position of compound **36** was reported *via* lithiation and introduction of an appropriate electrophile. We considered elaborating this alternative pathway to access compound **49** in a completely new approach. This concept is summarized in Figure 5.5, B. The key step of this approach is the successful introduction of a sulfur electrophile into the α-position of compound **36**, which can then be oxidized to the sulfoxide. Subsequent Pummerer rearrangemnet of this species should give the desired compound **49**, which can ultimately be converted to the protected D-(+)-showodomycin **50** according to Noyori's approach.

Finally, the formation of the maleinimide core can be envisioned by another synthetic strategy (Figure 5.5, C). The introduction of a nitrile functionality into the α-position of compound **36** should give intermediate **47** which could undergo subsequent cyclization to the protected D-(+)-showdomycin. Based on those considerations we started our synthetic elaborations.

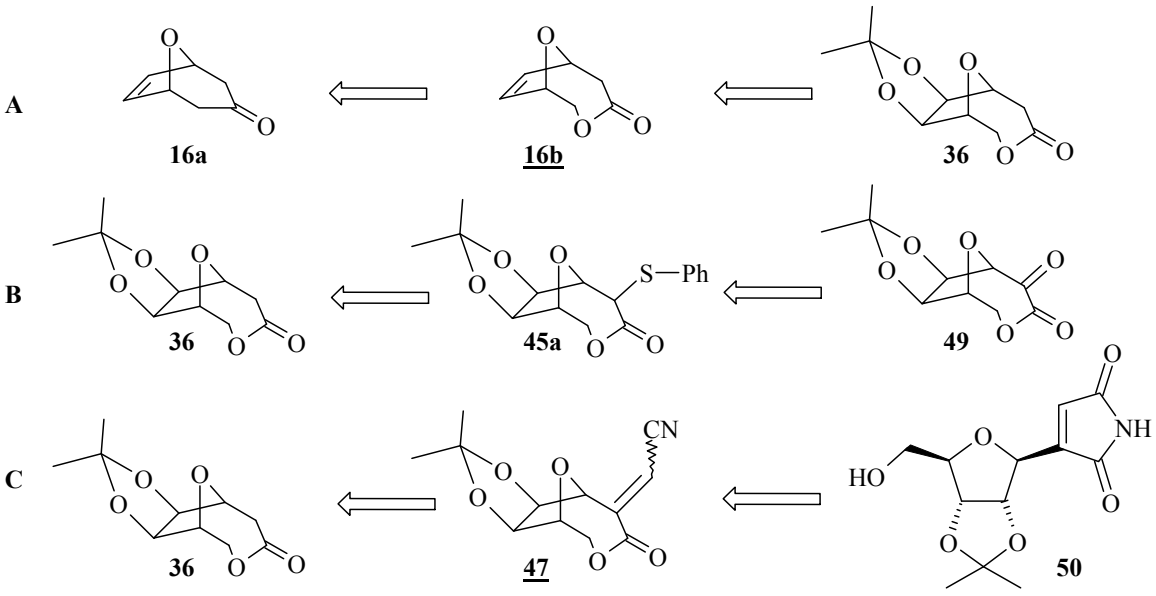


Figure 5.5 Three main strategies for the optimization of the synthetic approach of Noyori towards D-(+)-showodmycin.

Our approach started with heterobicyclic lactone **16b** (Figure 5.7) obtained by stereoselective Baeyer-Villiger biooxidation with recombinant whole-cells expressing $CPMO_{Coma}$ upon an optimized fermentation protocol as outlined before. We considered this compound as appealing platform for formal total syntheses of various natural products containing a tetrahydrofuran structural motif originating from achiral precursors. Recently, we have identified sub-clustering of several BVMOs based on homology in protein sequence.[66] These two groups of biocatalysts are capable to oxidize cyclic ketones to enantiocomplementary lactones on a significant number of substrates. In addition, CPMO from *Comamonas* sp. NCIMB 9872 (E.C.)[42,43] and a related protein from a *Brevibacterium* sp.[4f] display a certain tolerance for structurally demanding ketone precursors.[10]

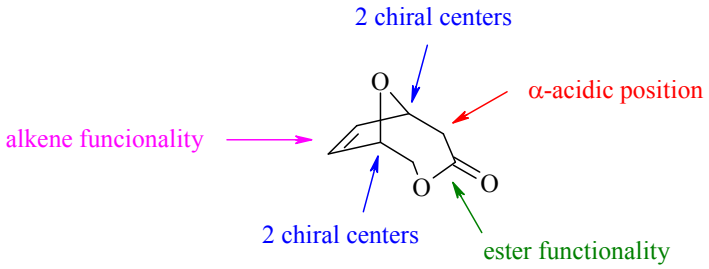


Figure 5.6 Chiral lactone **16b** – a platform for several valuable chemical transformations.

Within this contribution we present the first utilization of a chiral lactone obtained by recombinant whole-cell mediated biooxidation with CPMO in natural compound synthesis.
This key intermediate possesses an alkene functionality for electrophilic addition reactions which remains intact upon the biocatalytic Baeyer-Villiger oxygenation. The α-acidic center next to the lactone functionalty can possibly be alkylated or attacked by an appropriate electrophile. In addition, the strained bicyclic conformation of this compound should enable stereoselective transformations at those positions. Moreover, the lactone contains two already specified stereogenic centers introduced in the microbial biooxygenation with a desymmetrization step. Finally, a masked hydroxy acid functional group remains susceptible for subsequent transformations (Figure 5.6). Considerations regarding the reactivity and stereochemistry of lactone **16b** are based on the structural similarity to **16a** and the chemistry performed by Noyori *et al.* on this building block. Consequently, we were interested to investigate the potential of product **16b** for synthetic applications (Figure 5.6).

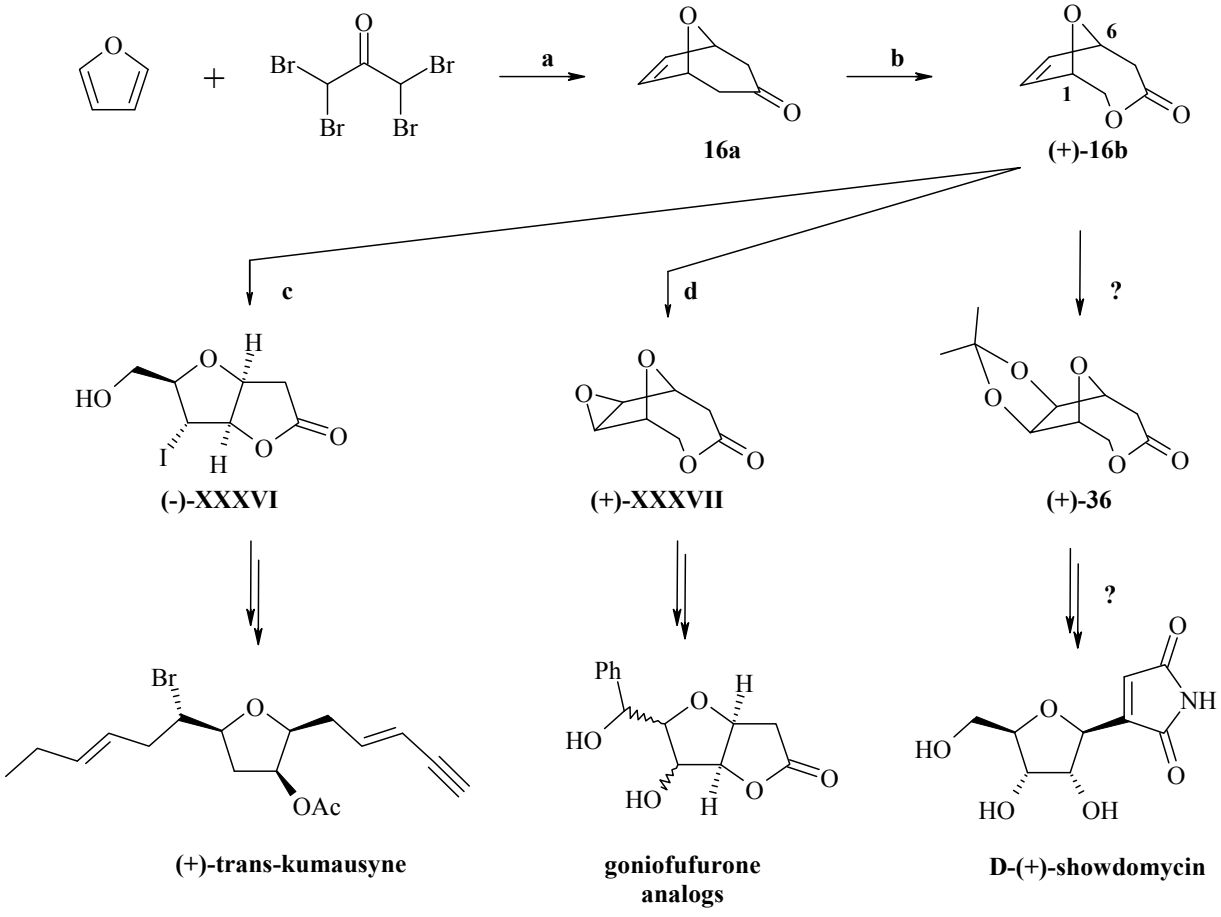


Figure 5.7 a) Cu/Zn-couple, ultrasound, MeCN, 10°C, then Cu/Zn-couple, NH_4Cl, EtOH/MeCN, -78°C to rt, 72%; b) *E.coli* expressing CPMO, IPTG, lab-bench fermenter, 25°C, 70%, 95% ee; c) MeCN/H_2O, KOH, rt, then I_2/KI, 40°C, dark, 70%; d) mCPBA, $CHCl_3$, 40°C, 79%.

First applications of this lactone in formal natural product synthesis by our group outlined access to tetrahydrofuran containing compounds.[52b] Hydrolysis of lactone (+)-**16b** gave the corresponding hydroxy-acid, which was directly submitted to a halolactonization reaction in a

single operation. Using KI/I_2 iodolactone (-)-**XXXVI** was obtained in excellent diastereoselectivity, which is a pivotal precursor for the synthesis of *trans*-kumausyne.[123] Applying chemical oxygenation strategy, epoxidation of lactone (+)-**16b** gave epoxide (+)-**XXXVII** in excellent diastereoselectivity, and subsequent transformation leads to a valuable precursor for structural analogs of the cytotoxic metabolite goniofufurone.[124] The main emphasis of this part of the thesis was the development of a new synthetic route for the total synthesis of D-(+)-showdomycin.

A key step in the whole synthetic route was the dihydroxylation of the unsaturated latctone (+)-**16b**. This step was envisioned to be readily realized by the osmium(VIII)-catalized dihydroxylation[125] which converts olefins into vicinal diols. The overall catalytic cycle as outlined in Figure 5.8 is initiated by reaction of the catalyst OsO_4 with the oelfinic substrate to generate osma(VI) glycolate. Re-oxidation of the Os center is accomplished by an auxiliary oxydans, for example NMO, which yields in a trioxo osmium(VIII)glycolate. The free diol is obtained upon hydrolytic cleavage and OsO_4 is regenerated.

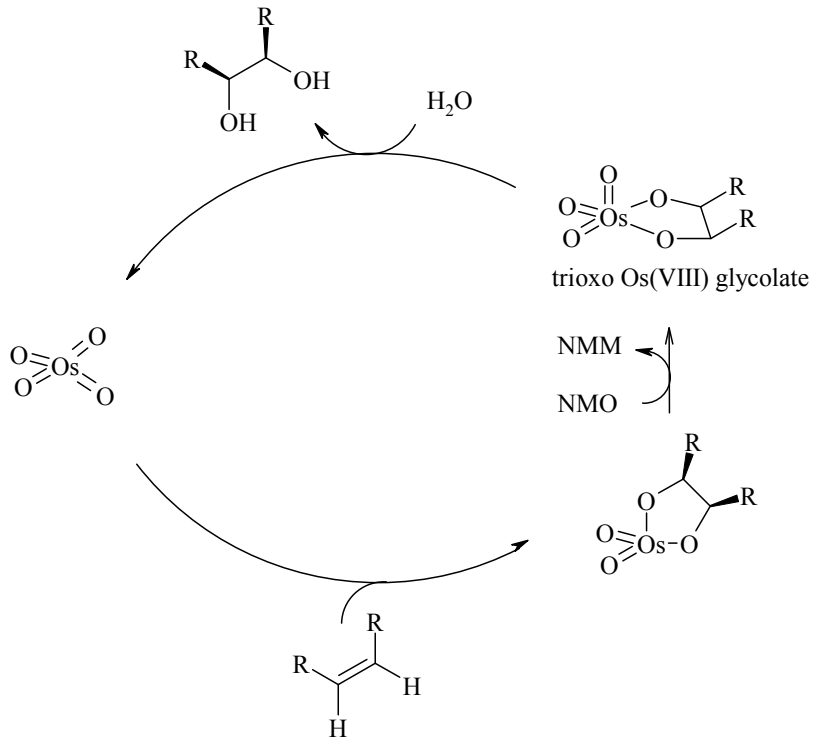


Figure 5.8 Current understanding of the catalytic cycle of OsO_4 mediated dihydroxylation.

[123] a) Faulkner, D.J. *Nat. Prod. Rep*. **1994**, *1*, 251-264; b) Suzuki, T.; Koizumi, K., Suzuki, H.; Kurosawa, E. *Chem. Lett*. **1983**, 1643-1644.

[124] a) Murphy, P.J.; Dennison, S.T. *Tetrahedron* **1993**, *49*, 6695-6700; b) Murphy, P.J. *J. Chem. Soc., Chem. Commun*. **1992**, 1096.

[125] a) Zaitsev, A.B.; Adolfsson, H. *Synthesis* **2006**, *11*, 1725-1756; b) Cha, J.K.; Kim, N.-S. *Chem. Rev*. **1995**, *95*, 1761-1795; c) Kolb, H.C.; Van Nieuwenhze, M.S.; Sharpless, K.B. *Chem. Rev*. **1994**, *94*, 2483-2547.

5.2 Synthetic Elaboration

Applying a recently outlined sonochemical protocol for a [4+3] cycloaddition[126] of furan and tetrabromoacetone followed by reductive dehalogenation, we were able to optimize the synthesis of oxabicycloketone **16a** in a single chemical operation and an overall yield of 72% (Figure 5.9). This prochiral compound is an excellent substrate for CPMO and the corresponding lactone (+)-**16b** was obtained in good optical purity (95% ee/chiral phase GC, $[\alpha]_D^{20}$ = +85.2, *c* 0.2, $CHCl_3$) in a desymmetrization step[51] introducing two new stereogenic centers in a single biotransformation step. Optimization of the fermentation conditions was performed taking advantage of the SFPR (substrate feed – product removal) methodology presented in this thesis in chapter 4.[108,127] Compound (+)-**16b** was obtained in 79% isolated yield from biotransformations using a precursor concentration of 5g/L fermentation broth.

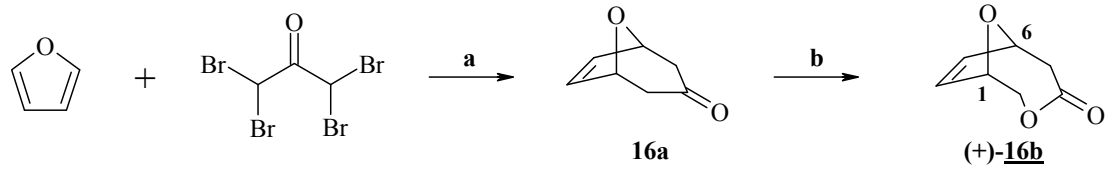


Figure 5.9 Synthesis of biooxidation product **16b**. a) Cu/Zn-couple, ultrasound, MeCN, 10°C, then Cu/Zn-couple, NH_4Cl, EtOH/MeCN, -78°C to r.t., 72%; b) *E.coli* expressing CPMO, IPTG, lab-bench fermenter, 25°C, 79%, 95% ee.

Having established facile access to intermediate (+)-**16b** on gram scale, we embarked on a study to utilize the functional diversity and structural rigidity of the compound within subsequent chemical transformations. Based on previous work by Noyori on a similar structural core, we expected a high degree of diastereoselectivity for following reactions. Since the chiral unsaturated lactone **16b** was not known in the literature we were additionally interested in the determination of the absolute configuration. We planned to establish this by transformation of lactone **16b** into a literature known compound. According to the literature the absolute configuration of one enantiomer of the dihydroxylated acetonide protected compound **(+)-36** was already published.

Consequently, we tried to develop a stereoselective synthetic route towards this compound **36** (Figure 5.10). Upon dihydroxylation of (+)-**16b** *via* osmylation we observed rapid conversion of starting material to a new compound under standard Upjohn conditions using NMO as oxidant to recycle OsO_4. However, the corresponding diol **35** was very difficult to isolate in satisfactory yields due to its excellent water solubility. Subsequent protection of diol **35** to the corresponding acetonide **36** failed whenever crude diol was applied in the reaction. Purification of diol **35** *via* column chromatography gave the desired compound **35** in only 25% yield. However, protection of pure diol **35** gave the desired product **36** quantitatively

[126] Mihovilovic, M.D.; Grötzl, B.; Kandioller, W.; Snajdrova, R.; Muskotal, A.; Bianchi, D.A.; Stanetty, P. *Adv. Synth. Catal*. **2006**, *348*, 463-470; b) Montana, A.M.; Grima, P.M. *Tetrahedron Lett*. **2001**, *42*, 7809-7813.

[127] Rudroff, F.; Alphand, V.; Furstoss, R.; Mihovilovic, M.D. *Org. Process Res. Dev*. **2006**, *10*, 599-604.

under classical ketalization conditions in the presence of diemthoxypropane (DMP), acetone and p-TSA. Within the synthesis of protected compound **36** in optical pure form we were able to conclude the formal total synthesis of D-(+)-showdomycin *via* a new stereoselective microbial Baeyer-Villiger approach. Hence, absolute configuration of biooxidation product **16b** was determined as 1*S*, 6*S* upon comparison to data reported for acetonide **(+)-36** in the literature.

Problems to reproduce the described procedure, rather lower yields in up-scaling experiments, and the prospects of modifying the total synthesis of D-(+)-showdomycin prompted us to investigate different synthetic approaches for the synthesis of the desired protected lactone **36**.

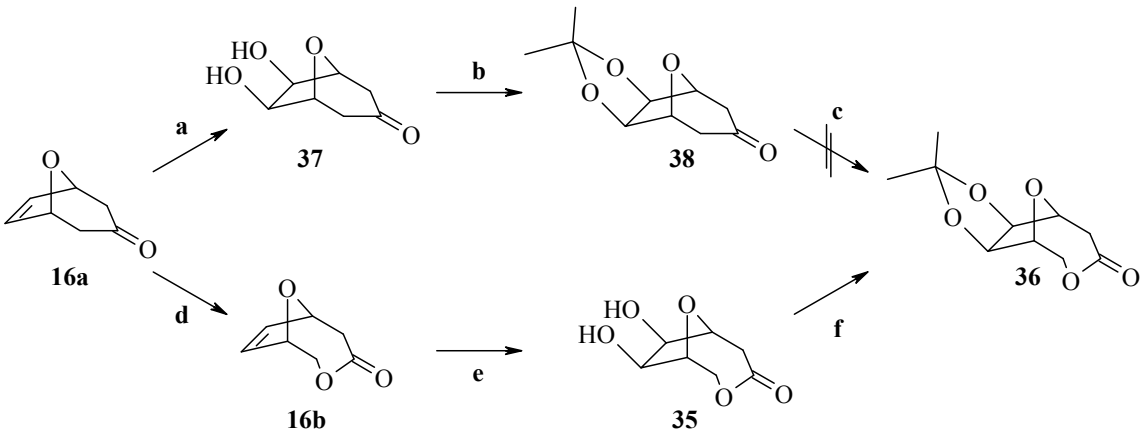


Figure 5.10 Synthesis of lactone **36**; a) OsO_4/NMO, acetone/H_2O, rt, 60%; b) DMP/acetone, p-TSA, rt, 30min, quant.; d) *E.coli* expressing CPMO, IPTG, lab-bench fermenter, 25°C, 79%, 95% ee; e) OsO_4/NMO, acetone/H_2O , rt, quant.; f) DMP/acetone, p-TSA, rt, 24h, 25%.

Since it is known that recombinant whole-cells expressing $CPMO_{Coma}$ are able to transform structurally more demanding prochiral ketones, the synthetic strategy was modified by switching the order of steps. Initial dihydroxylation and protection was expected to enable access to prochiral ketone **38** followed by microbial Baeyer-Villiger oxidation towards compound **36**. The synthetic approach towards ketone **38** was identical to the described methods above. The first step was the dihydroxylation of ketone **16a** to the corresponding diol **37**. Due to the much lower polarity of ketone **37** compared to lactone **35** extraction and purification of diol **37** was performed without any problems. The desired compound was isolated in 60% yield (not optimized). Subsequent protection with p-TSA and DMP/acetone gave the acetonide **38** in quantitative yield. However, biotransformation of ketone **38** with $CPMO_{Coma}$ as well as with $CHMO_{Brevi2}$ did not yield any detectable amounts of desired lactone **36**.

As we did not identify a suitable enzyme to accept acetonide **38**, we decided to revisit the isolation and protection of diol **35** (Figure 5.11). Therefore, we tried to find a dihydroxylation and subsequent protection method without aqueous work up and isolation step on the stage of diol **35**. Dihydroxylation of lactone **16b** under Upjohn conditions and treatment with acetic anhydrid in the presence of pyridine gave compound **39** in moderate yield (crude 44%). Since

we observed complete consumption of olefine **16b** in all dihydroxylation steps (TLC control) it seems to be obvious, that the critical parameter in this synthetic approach was neither the dihydroxylation nor the protection step, but the instability of diol **35**. Variation of the conditions and nature of the protecting group should consequently lead to a suitably protected diol **35** for subsequent synthetic transformations. As the acetyl protecting group did not seem to be the proper choice, we decided to change to the more stable TBDMS group. Again, classical Upjohn conditions were applied and subsequent treatment of diol **35** with imidazole and TBDMSCl in DMF at 0°C[128] gave the desired compound **40** in moderate yield (50% over 2 steps). Subsuming we were able to increase the yield, starting from acetonide **36** (25%), to diacetate **39** (44% crude) and finally to the TBDMS protected product **40** (50% pure). However, the obtained results for the dihydroxylation and protection of lactone **16b** were not satisfactory enough. At this stage of the project we hesitated to complete a total synthesis of showdomycin with a TBDMS intermediate, as the feasibility of the subsequent steps from the Noyori sequence was unclear and the additional synthetic efforts were considered to go well beyond the scope of this thesis.

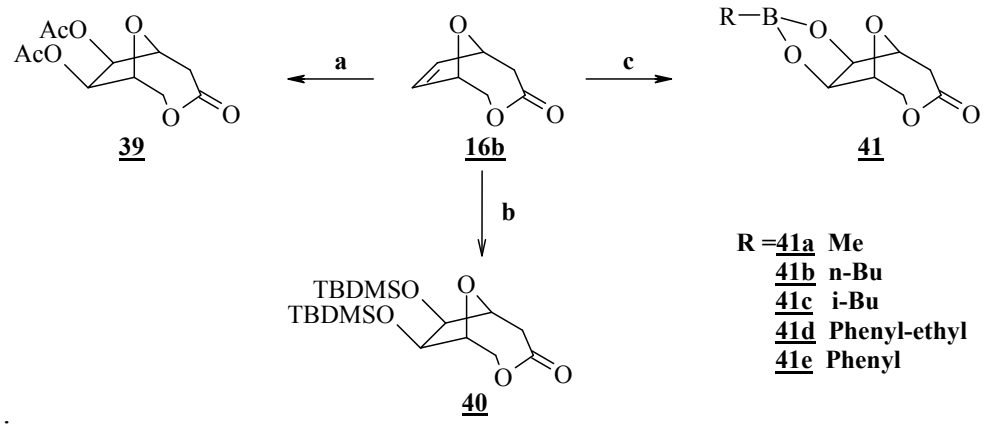


Figure 5.11 Dihydroxylation of lactone **16b** and variation of different protecting groups; a) I: OsO_4 (cat.), NMO (1.2 euqiv.),CH_2Cl_2, rt, II: pyridine/Ac_2O=1/1, rt, 44% crude; b) I: OsO_4 (cat.), NMO (1.2 euqiv.),CH_2Cl_2, rt, II: TBDMSCl (2.3equiv), imidazole (5equiv.), DMF, 0°C-rt, 50%; c) OsO_4 (cat.), NMO (1.2 equiv.), $RB(OH)_2$ (1.1 equiv.), rt, 72-96% depending on R (**41a** = 96%, **41b** = 72%, **41c** = 83%, **41d** = 73%, **41e** = 80%).

After intensive literature research we found another dihydroxylation methodology under water free conditions in the presence of a boronic acid. The so called Narasaka dihydroxylation was performed with boronic acid, catalytic amount of OsO_4 and again NMO as co-oxidant. CH_2Cl_2 was the appropriate solvent for this reaction.[129] This methodology was used by the group of Sharpless for the synthesis of polyols, whereas a convenient oxidative deprotection of the boronic esters was described.[130] A similar reaction mechanism as proposed for the classical dihydroxylation of olefins[125] was described for the catalytic cycle

[128] Feldman, K.S.; Sambandam, A., Bowers, K.E., Appel, H.M. *J. Org. Chem*. **1999**, *64*, 5794-5803.

[129] a) Sakurai, H.; Iwasawa, N.; Narasaka, K. *Bull. Chem. Soc. Jpn.* **1996**, *69*, 2585-2594; b) Iwasawa, N.; Kato, T.; Narasaka, K. *Chem. Lett*. **1988**, 1721-1724.

[130] Gypser, A.; Michel, D.; Nirschl, D.S.; Sharpless, K.B. *J. Org. Chem*. **1998**, *63*, 7322-7327.

for phenyl boronic acid initiated dihydroxylation (Figure 5.12 left part). Thus dihydroxylation leads to the glycolate osmium(VI)ester, which is reoxidized to the corresponding glycolate osmium(VIII)ester. At this stage, the osmiumdiolate entity is cleaved by electrophilic cleavage (Figure 6.12 right part). In such a case, interaction between the electrophilic boron center and the basic oxygen atom of the glycol entity weakens the osmium-oxygen bond and ultimately leads to transesterification from osmium to boron. This releases boronic esters and regenerates the OsO_4 catalyst.

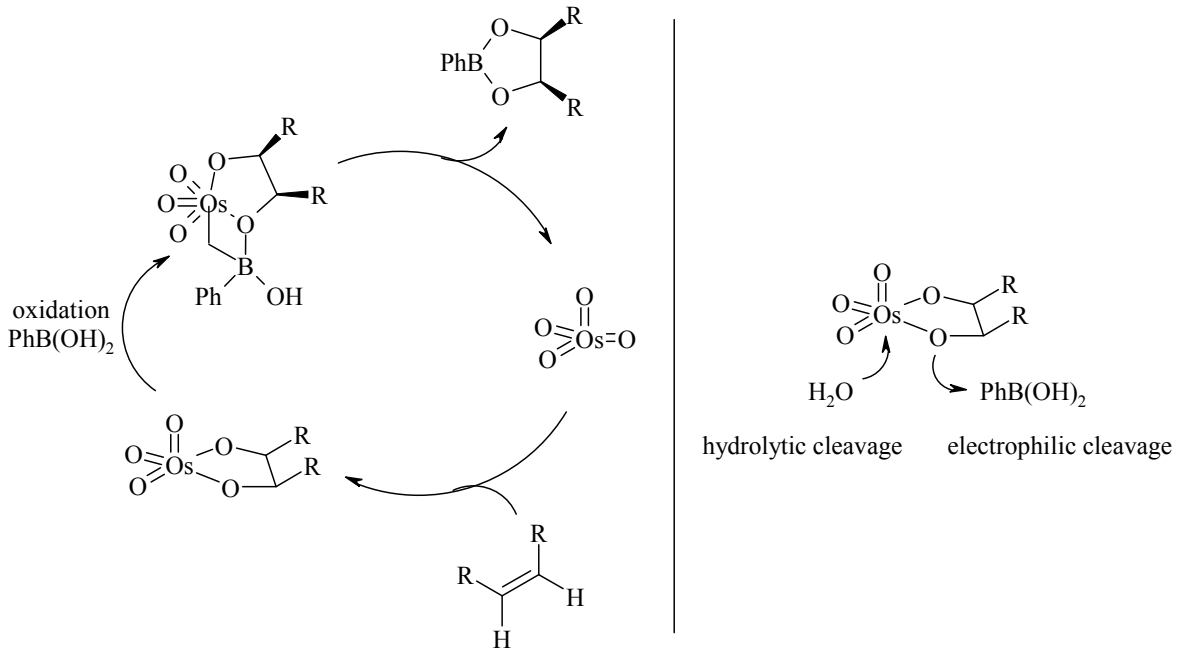


Figure 5.12 Left part: Catalytic cycle for phenyl boronic acid-initiated turnover in enantioselective olefin dihydroxylation (Narasaki-dihydroxylation). Right part: Hydrolytic cleavage *vs.* electrophilic cleavage of osmaglycolates.

We applied the Narasaki hydroxylation to the conversion of **16b** towards five different boronic esters (**41a-e**). This reaction worked quite smoothly and gave all chiral boronic esters (**41a-e**) in very good to excellent yields without any isolation and purification problems (Figure 5.11). This method turned out to be the best for the dioxygenation of the lactone core with respect to both diastereoselectivity and isolated yield. The next step in our synthetic plan was then the introduction of a sulfur electrophile in the α-position of the lactone functionality. However, based on the literature, the application of the boronic ester functionality as protective group under lithiation conditions turned out to be problematic. Several control experiments with **41e** confirmed the instability of the boronic esters and only decomposition of the starting material was observed. While the conversion to boronic esters **41a-e** solved the isolation problems after the dihydroxylation step, the subsequent transformation to a more convenient protecting group (Figure 5.13) became an issue at stake. In this context, the most appropriate protecting group for our purpose was again the acetonide according to the literature. Therefore, our next attempt was a "transprotection" reaction of the obtained boronic esters.

Three completely different synthetic strategies were tested to obtain the desired compound **36**. It is known from the literature, that boronic esters can be easily cleaved by acidic or basic hydrolysis.[131] Therefore we tried different hydrolytic cleavage conditions (Table 5.1, entries **a-d**). We supposed that the cleavage of alkyl-boronic esters (**41a-d**) could be performed in a faster and more efficient way than phenyl boronic ester **41e**.
Neither hydrolysis in the presence of 1M hydrochloric acid (entry **a**) nor triflouroacetic acid (entry **b**) gave the desired diol **35**. Even upon reaction times up to 24 hours and increased temperature (rt – reflux) no hydrolysis of the boronic ester was observed.

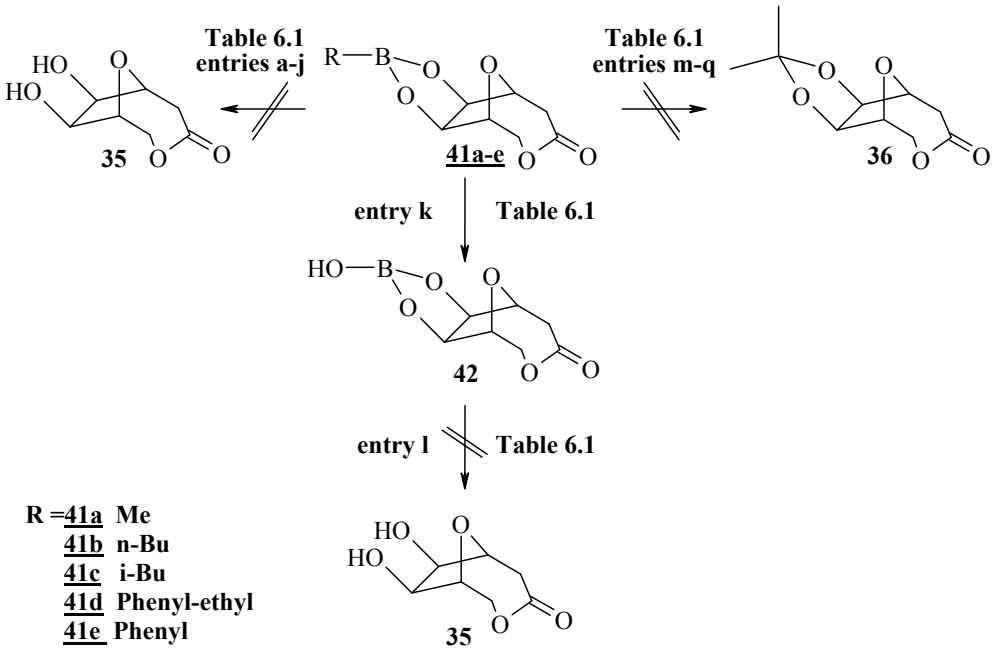


Figure 5.13 Synthetic strategies for the "transprotection" of boronic esters **41a-e**.

Recent publications described the transesterification of phenylboronic esters in the presence of pinacol under acidic conditions (entries **c**, **d**).[131] Again, no conversion of the starting material was observed.
Another approach described by Jung and Lazarova was performed (entries **e**, **f**),[132] which is based on the nucleophilic attack of nitrogen and subsequent complexation of the free boronic acid. The boronic ester was dissolved in 2-propanol and diethanolamine/ N-methyldiethanolamine was added. The reaction mixture was stirred at rt for 24 hours but again, no conversion was observed.
Boronic esters are well known compounds in palladium catalyzed Suzuki-coupling chemistry. In addition, the lability of boronic esters during such coupling reactions in the presence of traces of water and bases like $CsCO_3$ is also known in the scientific community. Consequently, Suzuki reactions were attempted with **41e** in the presence of iodobenzene as

[131] Pennington, T.E.; Kardiman, C.; Hutton, C.A. *Tetrahedron Lett*. **2004**, 6657-6660.

[132] Jung, M.E.; Lazarova, T.I. *J. Org. Chem*. **1999**, *64*, 2976-2977.

coupling partner (entries **g**, **h**). However, neither coupling product (biphenyl) nor deprotected starting material **35** nor boronic ester **41e** was detected by GC-MS analysis.
Also, no cleavage of boronic esters in the presence of a strong Lewis acid like BCl_3 was observed (entry **k**).[133]
A completely different approach was the oxidation of boronic acids with hydrogen peroxide or m-CPBA (entries **j**, **k**). No reaction was observed for ester **41a** in the presence hydrogen peroxide, whereas the organic reagent (m-CPBA) oxidized esters **41a** and **41d** quantitatively (in CH_2Cl_2, rt). Based on ^1H and ^{13}C-NMR analysis, complete conversion of the boronic esters **41a** and **41d** into the corresponding boric ester **42** could be observed. In the case of the oxidation of boronic ester **41a**, the signal for the methyl group disappeared in the ^1H-NMR (forming MeOH which is lost upon evaporation during work up) and in the case of crude ester **41d** a significant shift of the CH_2-group next to the boron from about 1 to 4ppm because of the formation of phenylethanol, was observed. However, subsequent hydrolysis of compound **42** did not give any satisfactory results (entry **l**).

Entry	Solvent	Reactant	Reaction Time	Temp. [°C]	Conv. [%]
a	acetonitril	1M HCl	1h-24h	rt/rf	n.c.
b	acetonitril	1M HCl / 5 equiv. pinacol	24h	rt	n.c.
c	acetonitril	CF_3COOH	1h-24h	rt/rf	n.c..
d	acetonitril	CF_3COOH / 5 equiv. pinacol	24h	rt	n.c.
e	i-propanol	CH_3-$N(CH_3CH_2OH)_2$	24h	rt	n.c.
f	i-propanol	H-$N(CH_3CH_2OH)_2$	24h	rt	n.c.
g	dioxan	Na_2CO_3 / 2equiv.	20min	MW/150	decomposition
h	dioxan	Cs_2CO_3 / 2equiv.	20min	MW/150	decomposition
i	CH_2Cl_2	BCl_3	24h	-78/rt	n.c.
j	EtOAc/acetone	30% H_2O_2	24h	rt	n.c.
k	CH_2Cl_2	m-CPBA	24h	rt	100
l	H_2O	2N NaOH	24h	rt	-
m	acetone/dry	CF_3COOH	24h	rt	n.c./s.p.
n	acetone/dry	CF_3COOH	24h	reflux	decomposition
o	acetone/DMP	CF_3COOH / 1equiv. H_2O	20min	MW	decomposition
p	acetone	Amberlyst 15 (H^+)	24h	rt	n.c./s.p.
q	acetone	Amberlyst 15 (H^+) + $(CH_2OH)_2$	24h	rt	n.c./s.p.

Table 5.1 Attempts for the deprotection of boronic esters **41a-d**.

[133] Martichonok, V.; Jones, J.B. *J. Am. Chem. Soc*. **1996**, *118*, 950-958.

Finally we tried to perform a direct transprotection of boronic esters **41a-e** to lactone **36** (entries **m-q**). But again, all attempts failed and the desired compound **36** was not obtained under any conditions described in Table 5.1.
Surprisingly, all boronic esters (alkyl as well as aryl residues) seemed to be much more stable to hydrolytic and electrophilic cleavage conditions than expected. Hence, the advantage of high yields and easy purification within the Narasaki dihydroxylation was compensated by the fact that no further useful chemical transformation could be performed. Therefore, alternative methods for the synthesis of key intermediate **36** had to be investigated.

While reaction of lactone **16b** to protected diols **39-41** (Figure 5.11) did not give the desired results, we focused our ongoing investigations again on the optimization of the synthesis of lactone **36**. After intensive literature search for such ketalization reactions (Figure 5.14), several different new approaches were identified and attempted. A summary of all reaction conditions is given in Table 5.2.

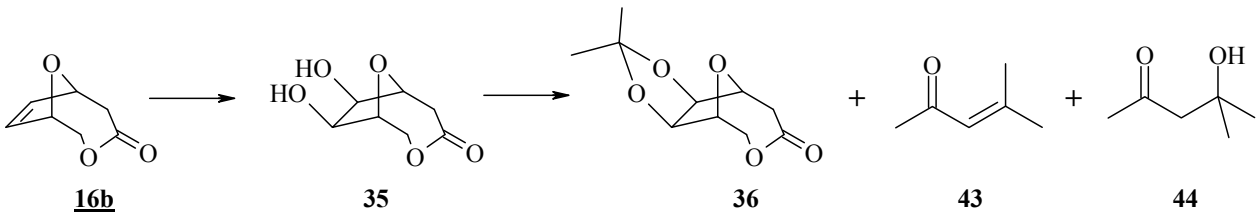


Figure 5.14 Synthetic elaborations of osmylation and subsequent diol protection to the desired lactone **36**.

Considering the previous optimization work for the dihydroxylation step, osmylation of lactone **16b** was performed under Narasaka waterfree conditions in the absence of boronic acids. Evaporation of all volatiles gave the crude diol, which was used without further purification in the following ketalization step.
In a first approach ketalization of diol **35** was carried out with p-TSA in the presence of DMP and dry acetone. Again, no reproducible results especially in the up scaling of the reaction were obtained. Solubility problems of diol **35** and impurities from the dihydroxylation step might interfere and inhibit a continuous transformation (entry **a**).
Variation of different solvents (entries **b, c**) to circumvent solubility problems did not give the desired protected diol **36**. Identification of two new spots (first observed on TLC) by GC-MS analysis strongly indicated formation of the aldol-products of acetone (**43**, **44**). Attempts to use 2-methoxypropene and p-TSA (entry **d**) in the presence of THF failed and, again, no conversion was observed. Exchange of the proton donor (entry **e**) to CSA gave similar results. Entries **f** and **g** represent repeating experiments utilizing methoxy propene instead of DMP/acetone for the formation of the desired ketal **36**. Again, no conversion of the diol **35** was observed and only by-products (**43**, **44**) were formed. According to the literature, the addition of iodine dissolved in acetone promotes formation of acetonides in various carbohydrates. However predominant formation of unwanted by-products and no transformation to the desired acetonide were observed (entry **h**).

Acetonide formation in the presence of Lewis acids is a well known and widely used methodology. Five different Lewis acids (entries **i-m**) were investigated and, interestingly, conversions to the desired protected diol **36** were obtained in all cases. Using $TiCl_4$, at -60°C in the presence of diol **35** (dissolved in acetone) gave by-products **43** and **44**, exclusively (entry **i**). While increasing the temperature to rt and then to 40°C a continuous transformation of the starting material was achieved. Nevertheless, predominant side product formation resulted in difficulties in the purification of the desired product *via* column chromatography due to very similar R_f values of **36** and **44**. Similar results were obtained with Lewis acids $SnCl_4$ and BF_3*Et_2O (entries **j**, **k**). The reaction of methansulfonic acid and diol **35** in acetone was described in entry **l**. In this case complete decomposition of compound **35** was observed.

Entry	Additive	Solvent	Reactant	Temp [°C]	Yield [%]
a[134]	p-TSA	acetone	DMP	rt/40	25[b]
b[135]		DMF	DMP/acetone	rt/40	s.p.
c[136]		CH_2Cl_2	DMP/acetone	rt/40	s.p.
d[137]		THF	methoxy propen	rt/40	s.p.
e	CSA	DMF	DMP/acetone	rt/40	s.p.
f		DMF	methoxy propene	rt/40	s.p.
g		THF	methoxy propene	rt/40	s.p.
h[138]	I_2	acetone	-	rt/40	s.p.[a]
i	$TiCl_4$	acetone	-	-60/rt/40	traces/s.p.[a]
j	$SnCl_4$	acetone	-	-60/rt/40	traces/s.p.[a]
k	BF_3*Et_2O	acetone	-	rt/40	traces/s.p.[a]
l	MSA	acetone	-	rt/40	decomposition
m[139]	**$AlCl_3$**	**acetone**	**-**	**0/rt/40**	**48[b]/s.p.**

[a] predominant formation of unwanted side product **43, 44** and no complete conversion to the desired compound **36**; [b] complete conversion of lactone **16b** and isolated yield of product **36** after column chromatography;

Tabel 5.2 Optimization of acetonide protection to the desired lactone **36**.

Finally, the reaction of freshly sublimed $AlCl_3$, acetone and crude diol **35** was investigated. Again, low temperatures favor the formation of by-products **43** and **44**, while increased temperature (40°C) enabled formation of the desired ketal **36** (entry **m**). Within this experiment complete conversion was obtained and the desired acetonide **36** was isolated in 48% yield over 2 steps after aqueous basic work up (sodium bicarbonate), extraction with CH_2Cl_2 and purification *via* column chromatography. Moreover, up-scaling of the reaction to gram quantities of starting material (+)-**16b** turned out to be successful and the yield of 48%

[134] Trost, B.; Dudash jr., J.; Dirat, O. *Chem. Eur. J.* **2002**, *8*, 259-268.

[135] Mazitschek, R.; Huwe, A.; Giannis, A. *Org. Biomol. Chem.* **2005**, *3*, 2150-2154.

[136] Kumareswaran, R.; Hassner, A. *Tetrahedron Asymm.* **2001**, *12*, 3409-3415.

[137] Cai, J.; Davison, B.E.; Ganellin, C.R.; Thaisrivongs, S. *Tetrahedron Lett.* **1995**, *36*, 6535-6536.

[138] Kartha, K.P.R. *Tetrahedron Lett.* **1986**, *27*, 3415-3416.

[139] Lal, B.; Gidwani, R.M.; Rupp, R.H. *Synthetic Comm.* **1989**, 711-715.

was highly reproducible. Consequently, we were able to circumvent aqueous reaction conditions for the dihydroxylation step and enabled access to acetonide (+)-**36** in a single operation as pivotal precursor for the total synthesis of the cytostatic D-(+)-showdomycin.[16]
Since we were able to upscale the synthesis of acetonide **36** we focused our final investigations on the introduction of appropriate functional groups for the completion of the total synthesis for D-(+)-showdomycin.
The main emphasis was now, as described above, the introduction of a carbonyl group into the α-position of the lactone **36** *via* an alternative strategy.

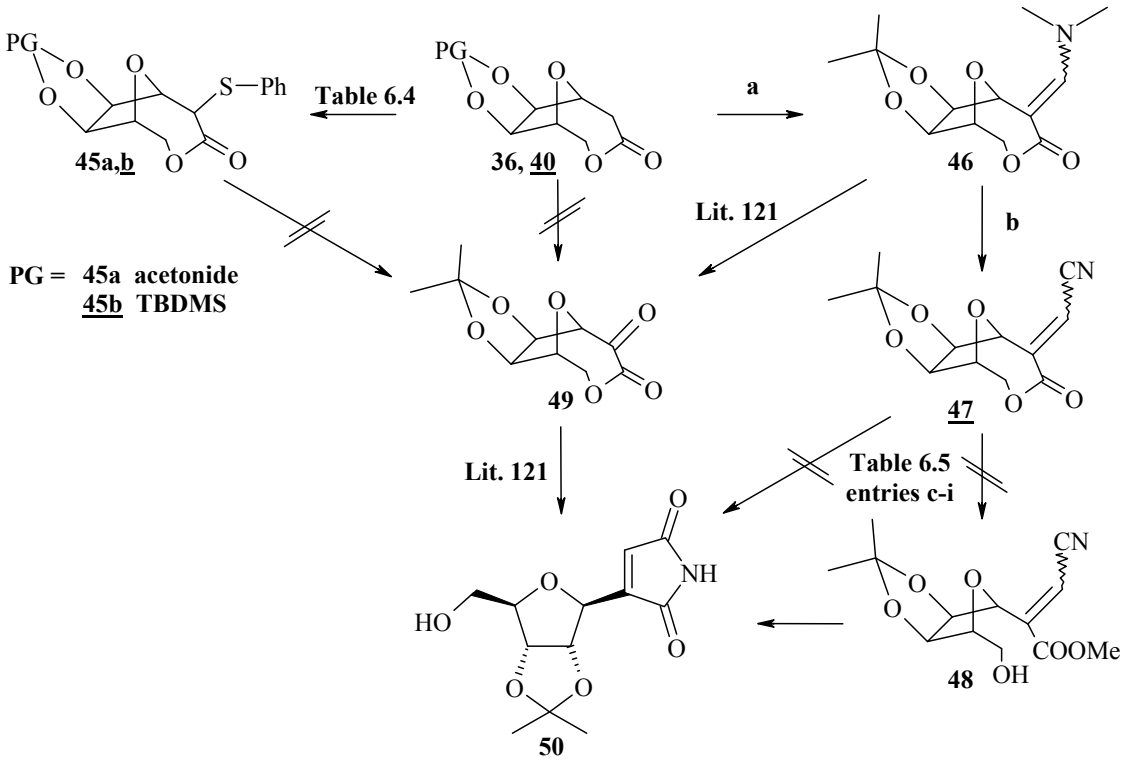


Figure 5.15 Alternative strategies towards the synthesis of D-(+)-showdomycin. a) t-$C_4H_9OCH[N(CH_3)_2]_2$, DMF, 60°C, 4h, 89%; b) KCN, toluene/AcOH, 40°C, 3h, 77%.

Thus, we tried to exploit the acidity of the α-position next to the ester group. First attempts were applied on the formation of the lithium enolate species with subsequent electrophilic attack of an appropriate electrophile. According to the literature best deprotonation conditions were applied within the presence of *tert*.-BuLi and HMPTA at -78°C in THF.[140] Based on this previously published protocol, we tried exactly the same reaction conditions. However, in our hands we did not observe deprotonation at the α-position (Table 5.3). Quenching of the reaction mixture at -15°C with a fast and appropriate electrophile like diphenyldisulfide did not give the desired compound **45a**.
After many different attempts, carefully drying all solvents, using only freshly titrated *tert*.-BuLi, modifying reaction temperatures and performing the reaction under argon atmosphere

[140] Gensler, W.; Chan, S.; Ball, D.B.; *J. Org. Chem.* **1981**, *46*, 3407-3415

only traces of the desired product could be detected (entry **a**). Increasing the temperature during the deprotonation step did not give better results but rather resulted in increasing decomposition of starting material (entries **b**, **c**). Hence, we changed the electrophile to dimethyldisulfide and tried the reaction again with *tert*.-BuLi and HMPTA under different reaction temperatures (entries **d, e**). As a result, *tert*.-BuLi did not seem to be the appropriate base for the introduction of an electrophile at this specific position.

Therefore, we carried out a new experimental set up by variation of bases with different properties. At the beginning LDA (entry **f**) as much less nucleophilic base compared to *tert*.-BuLi was used in the following reactions. Again, no reaction was observed when the deprotonation was performed at -78°C and the electrophile could not be introduced, properly. In this case, starting material was recovered, quantitatively. Complete decomposition of lactone **36** was observed when increasing the reaction temperature to -15°C under deprotonation conditions (entry **g**).

Compound	Entry	Base	Electrophile	Solvent	Temp [°C]	Yield [%]
36	**a**	*tert*.-BuLi	Ph-S-S-Ph	THF	-78 / -15	n.c.
	b			THF	-60	decomposition
	c			THF	-15	decomposition
	d		Me-S-S-Me	THF	-78 / -15	decomposition
	e				-15	decomposition
	f	LDA	Ph-S-S-Ph	THF	-78 / 0	n.c.
	g				-15 / 0	decomposition
	h	LCIA	Ph-S-S-Ph	THF	-78 / -15	n.c.
	i	LHMDS	Ph-S-S-Ph	THF	-78 / 0	n.c.
	j	LTMP	Ph-S-S-Ph	THF	-78 / -15	n.c.
40	**k**	*tert*.-BuLi	Ph-S-S-Ph	THF	-78 / -15	**7**
	l			THF	-60 / -15	decomposition
	m			THF	-15	decomposition
	n	LDA	Ph-S-S-Ph	THF	-78 / 0	n.c.

Table 5.3 Lithiation conditions for the introduction of different electrophiles into compounds **36 and 40**.

According to the literature[121] aldol addition of acetonide **36** and benzaldehyde was performed in the presence of lithium cyclohexyl isopropylamid (LCIA). Formation of the lithium enolate should also allow introduction of a sulfur electrophile. However, neither LCIA nor LHMDS nor LTMP (entries **h-j**) gave satisfactory results and all attempts to introduce an electrophile failed. Finally, since we had access to the TBDMS-protected diol **40**, within a last attempt we tried to introduce the sulfur electrophile according to the lithiation conditions described in Table 5.3 entry **a**. Surprisingly, the desired compound **45b** was obtained in traces and was isolated in about 7% yield after column chromatography with minor impurities. Based on this somewhat promising result we expand our investigations for the introduction of the appropriate electrophile and tested several lithiation conditions on compound **45b** to improve the yield of the previous experiment. We performed the lithiation under the same conditions as described for the acetonide protected diol **36** and the results were compiled in table 5.3, entries **k-n**. Unfortunately, no improvement of the obtained yield was performed.

In a second approach direct oxidation of the α-position in **36** was attempted to give the desired α-carbonyl compound **49**. Oxidation in the presence of SeO_2 and acetic anhydride did not give the desired product **49** (Figure 5.15) and unreacted starting material was recovered quantitatively.

Since we were not able to introduce a carbonyl group by SeO_2 directly, we tried to implement our third synthetic strategy (outlined in Figure 5.5, E). Another synthetic approach, published by Noyori et *al*. described the introduction of a formyl group or its synthetic equivalent at α-position in the lactone *via* a Knoevenagel type condensation with the Bredereck reagent[122] *tert*.-butoxybis(dimethylamino)methane. Thus treatment of **36** with t-$C_4H_9OCH[N(CH_3)_2]_2$ in DMF at 60°C gave the dimethylamino methylene lactone **46** in 89% yield (Figure 5.15 (a)). We observed the formation of both olefinic isomers (E and Z) and for further investigations we perfomed a complete chararterization of both isomers *via* one and two dimensional NMR spectroscopy. A detailed discussion of the spectral data was given in chapter 5.3.3.

This key intermediate is a very valuable platform for the synthesis of various pyrimidine C-nuleosides. According to the literature, subsequent ozonolysis of dimethylaminomethylene lactone **46** gave the desired and rather unstable α-keto lactone **49** which can be directly transformed to the protected D-(+)-showdomycin *via* Wittig reaction in quite low yield (30%).[121]

However, our synthetic approach towards construction of the maleimide ring system envisioned the introduction of a masked carboxylic acid functionality like a nitrile, which could be cyclized to the desired maleimide core under suitable conditions. Compound **47** was synthesized by nucleophilic substitution reaction which was performed in the presence of potassium cyanide toluene and acetic acid at 40°C in analogy to literature protocols.[141] The desired compound **47** was afforded in good yield and purity (Figure 5.15 (b)).

Entry	Solvent	Temp. [°C]	Reaction time	Additive	Conv. [%]
a	TFA/$(CF_3CO)_2O$	60	3d	-	n.c.
b	TFA/$(CF_3CO)_2O$	60	3d	PPh_3	n.c.
c	TFA/$(CF_3CO)_2O$	60	16d	PPh_3	decomposition
d	formamide/DMF	MW; 150	60min	-	decomposition
e	formamide	MW; 150	90min	-	decomposition
f	MeOH/THF	rt-reflux	2h	K_2CO_3	decomposition
g	t-BuOH	reflux	2h	KOH	decomposition

Table 5.4 Synthetic approaches towards formation of maleimide core for the synthesis of D-(+)-showdomycin.

The reaction mixture was cooled while extractive work up with sodium bicarbonate and after removal of all volatiles almost pure compound **47** (77%, 3/1 of E/Z) was obtained as colorless oil. No further purification was performed due to the instability of the product against silica gel. Based on the structural refinement for compound **46** we were able to identify both olefinic isomers and obtain a mixture of E and Z of 3/1. Further information for the

[141] Bratusek, U.; Meden, A.; Scete, J.; Stanovnik, B. *Arkivoc* **2003**, 77-86.

characterization of the olefinic isomers is given in section 5.3.3. We then investigated different reaction conditions for a successful transformation of compound **47** into the desired product **50**. Exposure of the E/Z mixture of compound **47** to trifluoroacetic acid and trifluoroacetic anhydride in a sealed tube at 60°C did not afford the cyclized product **50** (entries **a-c**).[119] The reaction mixture was stirred for 3 days and no conversion was observed (entry **a**). Further attempts for the isomerization of E/Z **47** in the presence of triphenylphosphine were performed, but only decomposition of the starting material was observed upon extended reaction times (entries **b**, **c**).[142]

Cyclization in the presence of formamide and/or DMF (entries **d**, **e**) under microwave irradiation gave only decomposition of starting material. Finally, we tried to open lactone **47** *via* a transesterification reaction in the presence of MeOH, THF and solid K_2CO_3 (entry **f**) as well as *via* basic hydrolysis in the presence of solid KOH and *tert.*-butanol (entry **g**).[143] Again, only decomposition of **47** was observed.

So far, our attempts for the elaboration of a new total synthesis of D-(+)-showdomycin failed. However, our approach to optimize the formal entry to this target allows the generation of up to four chiral centers from achiral and commercially available precursors in as few as three chemical operations. Hence, we consider the outlined synthetic route to a crucial precursor of this natural product as efficient shortcut to previous sequences taking advantage of a sustainable biooxygenation technique.

Summarizing, we have developed access to several pivotal precursors for natural products containing a tetrahydrofuran structural core utilizing biooxidative desymmetrization. The combined chemo-enzymatic oxidation strategies displayed excellent enantio- and diastereoselective control for the synthetic elaboration of key intermediate (+)-**16b** involving diverse subsequent transformations.

[142] Stanetty, P.; Kremslehner, M. *Tetrahedron Lett*. **1998**, *39*, 811-812.

[143] Hall, H.J.; Gisler, M. *J. Org. Chem*. **1976**, *41*, 3769-3770.

5.3 Crystal Data and Structure Refinement

5.3.1 Crystal Data for (1S,6S)-3,9-Dioxabicyclo[4.2.1]non-7-en-4-one (16b)

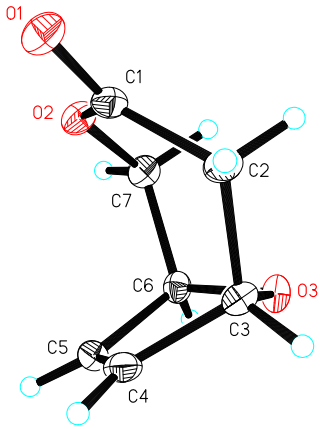


Figure 5.16 X-ray structure for (1S,6S)-3,9-Dioxabicyclo[4.2.1]non-7-en-4-one **16b**

With the synthesis of lactone (+)-**36** *via* microbial Baeyer-Villiger biooxidation and comparison with the precursor for D-(+)-showdomycin as characterized in the literature[121] we were also able to establish the absolute configuration of biooxidation product (+)-**16b** as (1*S*,6*S*). The structure of **16b** was reconfirmed by single-crystal X-ray diffraction by crystallization from a mixture of EtOH and CH_2Cl_2 (reflexes were collected with a Bruker Smart APEX CCD 3-circle diffractometer).

The crystal system turned out to be monoclinic. Within the conformation of the 8-membered lactone ring we were able to explain the high degree of diastereoselectivity observed to our synthetic transformations. In the case of the dihydroxylation of (+)-**16b** complexation of OsO_4 and the bridging oxygen atom strongly affects the stereochemistry of the reaction. The *endo*-face is shielded by the lactone group, therefore formation of only one diastereomer was observed.

5.3.2 Crystal Data for ζ-lactone-3,6-anhydro-2-deoxy-4,5-O-(phenylborylene)-D-allo-Heptonic acid (41e)

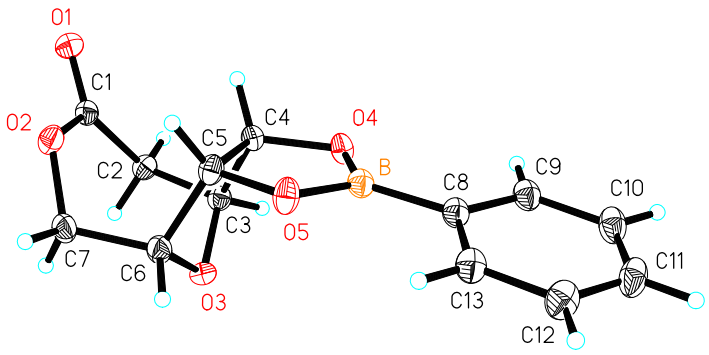


Figure 5.17 X-ray structure for ζ-lactone-3,6-anhydro-2-deoxy-4,5-O-(phenylborylene)-D-allo-Heptonic acid **41e**.

Single-crystal X-ray diffraction data for compound **41e** was collected with a Bruker Smart APEX CCD 3-circle diffractometer operating with graphite monochromatized Mo-Kα radiation. Structure solution and refinement was performed with Bruker AXS SHELXTL software. Compound **41e** was crystallized in a mixture of EtOH and CH_2Cl_2 and gave an orthorhombic crystal system. Dihydroxylation and subsequent formation of the boronic ester **41e** was strictly influenced by the conformation of the 8-membered lactone ring and the coordination between the bridged oxygen and the boron-atom. Again, formation of only one diastereomer was observed.

5.3.3 E,Z Assignment of 3,6-Anhydro-2-deoxy-2-[(dimethylamino) methylene]-4,5-O-(1-methylethylidene)-D-allo-heptonic Acid ζ-Lactone 46 *via* NOE-experiments

Due to the fact, that compound **46** represented one of the key intermediates for our synthetic approach towards D-(+)-showdomycin we were interested in the full characterization of this substance. Main emphasis on the spectral assignment was the identification of the olefinic E and Z isomers. According to the literature Noyori *et al.* presented that the product was a 1/2 mixture of E and Z-olefinic isomers based on ^{1}H-NMR spectral data, whereas two sets of singlets, δ 2.94 and 3.14 [$N(CH_3)_2$] and 6.70 and 7.34 (C=C<u>H</u>-$N(CH_3)_2$) were reported,. However, due to the results of our NOE experiments we disagree with this already published data. Figure 5.18 displays the main NOE experiments that are important for a correct assingmnet of both olefinic isomers.

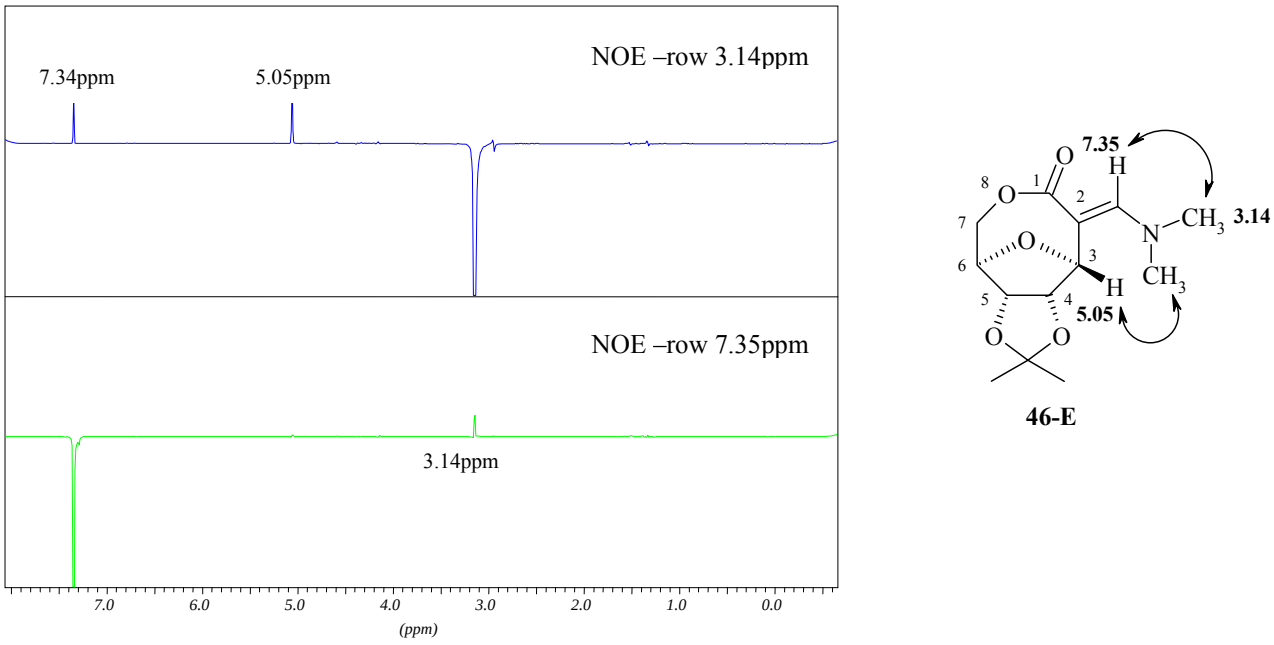
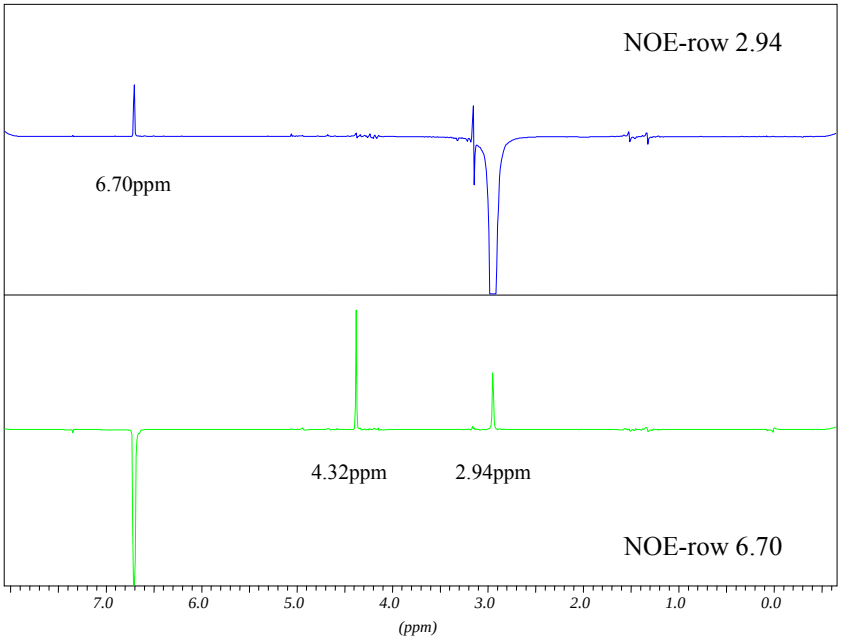
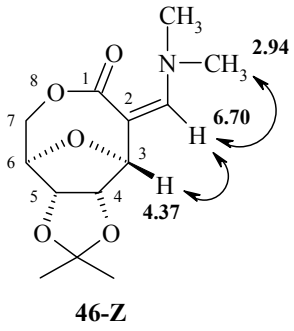


Figure 5.18 NOE spectral data for of E-3,6-Anhydro-2-deoxy-2-[(dimethylamino) methylene]-4,5-O-(1-methylethylidene)-D-allo-heptonic Acid ζ-Lactone **46**.

The correlation of H-3 (5.05ppm) with one of the N-methyl groups (3.14ppm) is only possible, when the configuration of **46** is E. A second characteristic correlation was observed between and the olefinic H (7.34ppm) and again one of the N-methyl groups (3.14ppm). This data strongly indicates that the olefinic singlet at 7.34ppm is the main characteristic signal for the **E-46** configuration.





46-Z

Figure 5.18 NOE spectral data for of Z-3,6-Anhydro-2-deoxy-2-[(dimethylamino) methylene]-4,5-O-(1-methylethylidene)-D-allo-heptonic Acid ζ-Lactone **46**.

Hence we took a closer look at the the second isomer. In this case we observed a correlation between the olefinc H (6.70ppm) and two other protons at one hand with the N-methyl group (2.94ppm) and on the other hand with H-3 (4.32ppm). Furthermore, we obtained only one signal for the N-methyl group (2.94ppm), because of the correlation with the olefinic H (6.70ppm), and no further interaction with H-3. Again, this is very strong evidence that the olefinic proton at 6.70ppm corresponds to the Z-isomer of **46**.

Conclusively, our investigations for the structural refinement of compound **46** indicate that we obtained a mixture of both olefinic isomers from E and Z in a ratio of 2/1, with the distribution of isomers switched as compared to previously published literature.

Based on this structural assignment we were also able to assign E and Z isomers in compound **47**.

Experimental Part

6.1 General

Unless otherwise noted, chemicals and microbial growth media were purchased from commercial suppliers and used without further purification. All solvents were distilled prior to use. Flash column chromatography was performed on silica gel 60 from Merck (40-63μm). Basic silica gel was obtained by mixing NEt_3 (5%), the desired solvent mixture and silica gel. This suspension was stirred for 5 minutes and was used for column chromatography. Medium pressure column chromatorgaphy was performed on a regular silica gel column with a Büchi 681 Chromatography Pump with Automatic Fraction Collector.
Melting points were determined using a Kofler-type Leica Galen III micro hot stage microscope and are uncorrected. Microwave reactions were performed on a CEM Explorer PLS^{TM} microwave unit. Biotransformations were carried out in a New Brunswick Bioflow 110 fermenter equipped with pH probe, oxygen probe, flow controller and temperature control was used. Monitoring of all fermentation parameters was performed using the Biocommand Plus 3.30 software by New Brunswick. Glucose concentrations were determined with *Roche Accu Chek-go*.

NMR-spectra were recorded from $CDCl_3$ or DMSO-d_6 solutions on a Bruker AC 200 (200 MHz) or Bruker Advance UltraShield 400 (400 MHz) spectrometer 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.
Combustion analysis was carried out in the Microanalytic Laboratory, University of Vienna.
General conversion control and examination of purified products were performed with GC Top 8000 / MS Voyager (quadropol, EI+) using a standard capillary column BGB5 (30mx0.32mm ID). Enantiomeric excess was determined *via* GC using a BGB 175 column (30mx0.25mm ID, 0.25μm film) and a BGB 173 column (30mx0.25mm ID, 0.25μm film) on a ThermoQuest Trace GC 2000 and a Thermo Focus GC.

Specific rotation $[\alpha]_D^{20}$ was determined using a Perkin Elmer Polarimeter 241 by the following equation: $[\alpha]_D^{20} = 100*\alpha / [c]*l$; c[g/100mL], l[dm]

Dip reagent: I) 13.2g conc. sulfuric acid
0.80g cerium(IV)-ammonium nitrate
10.0g phosphor molybdate
150mL ethanol

II) 1.00g $KMnO_4$
20.0g K_2CO_3
10.0mL NaOH / 5%
150mL H_2O

Abbreviations:

amp.	ampicilin
b.p.	boiling point
CSA	camphor sulfonic acid
DMSO	dimethylsulfoxide
DMF	dimethylformamide
DMP	1,1-dimethoxypropane
EtOAc	ethyl acetate
Et_2O	diethyl ether
EtOH	ethanol
KRD	*Kugelrohr* distillation
LB	Luria-Bertani media
LP	light petroleum (b.p. 40-60°C)
MeOH	methanol
m.p.	melting point
m-CPBA	m-chloroperbenzoic acid
NMO	N-methylmorpholinoxide
Rf	reflux
rt	room temperature
TB	terrific broth media
THF	tetrahydrofuran
TLC	thin layer chromatography
p-TSA	p-toluenesulfonic acid

6.1.1 Biotransformation

Media for biotransformation:

LB_{amp}		**TB_{amp}**	
10.0g	bacto-peptone	12.0g	bacto-tryptone
5.00g	yeast extract	24.0g	bacto-yeast
10.0	sodium chloride	4.00ml	glycerol
		16.4g	$K_2HPO_4*3H_2O$
		2.30g	KH_2PO_4
1000mL	deion. H_2O	1000mL	deion. H_2O
4.00mL	ampicilline stock solution autoclaved at 121°C for 20 minutes, then add ampicilline stock solution	4.00mL	ampicilline stock solution autoclaved at 121°C for 20 minutes, then add ampicilline stock solution

Ampicilline – stock solution

50.0mg/mL dissolved in deion. water, sterilization by filtration (0.2µm)

IPTG – stock solution

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

Glucsoe – solution (20%)

100g glucose were dissolved in 400mL deion. water, sterilization by autoclaving at 121°C for 20 minutes

Preparation of frozen stocks

E.coli strain was incubated at 37°C on LB_{amp} plates for 12-15 hours. A single colony was selected and a 10mL pre-culture was inoculated and incubated (10mL LB_{amp}, shake flask, 120rpm for 12-15hours at 37°C). After addition of 2mL glycerol, the mixture was vortexed, transferred in 1mL aliquots into Eppendorf vials and stored at -80°C.

6.2 Parallel Screening Methodology

Procedure: Fresh LB_{amp} medium (1-2mL) was inoculated with 1% (10-20µL) of an overnight preculture of recombinant *E. coli* strains: $CHMO_{Acineto}$, $CPMO_{Pseudo}$, $CHMO_{Brevi\ I}$, $CHMO_{Brevi\ II}$, $CHMO_{Rhodo\ I}$, $CHMO_{Rhodo\ II}$, $CHMO_{Brachy}$, $CHMO_{Arthro}$ and $CHMO_{Acineto}$-mutants (Table 6.1.) in 12- or 24-well dishes (supplied by *Greiner Bio-One*). The culture was incubated at 120 rpm at 37°C on an orbital shaker for 3 hours, and then 1-2µl IPTG stock solution (200mg/mL) was added. The ketone (0.5mg/mL) was added neat along with β-cyclodextrin (1 equiv.) to facilitate biooxidation of slowly converted substrates. The culture was incubated for 24 hours at 120 rpm at 25°C. Samples (500µL) were taken, mixed with standard solution of methyl benzoate in EtOAc or dichlormethane (500µL) and GC analysis was performed.

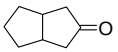
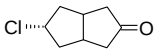
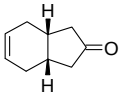
Mutant	AA-exchanges
1-C2-B7	F432Y, K500R
1-F1-F5	L143F
1-E12-B5	F432I
1-H7-F4	L426P, A541V
1-H3-C9	L220Q, P428S, T433A
1-F4-B9	D41N, F505Y
1-K6-G2	K78E, F432S
1-K2-F5	F432S
2-D19-E6	E292G, T433I, L435Q, T464A, L143F

Table 6.1. $CHMO_{Acineto}$-mutants and their corresponding AA-exchanges

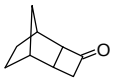
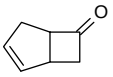
GC-analysis: Chemical oxidation of ketones was mandatory for the preparation of racemic reference material. Due to calculation and description of enantioselectivity of the obtained lactones, baseline separation of racemic reference material performed by chiral GC analysis was necessary. All GC analyses were performed on either BGB 175 or BGB 173 chiral phase GC columns.

Method A (biotrans-STD)	80°C-2min→80-160°C, 5°C/min, 160°C-1min→160-220°C, 10°C/min, 220°C-8min
Method B (enantiomerentrennung_2°C)	80°C-2min→80-220°C, 2°C/min, 220°C-8min
Method C (enantiomerentrennung_1°C_opt)	80°C-2min→80-220°C, 1°C/min, 220°C-8min
Method D (trimethoxytrennnug_2°min)	160°C-2min→160-230°C, 2°C/min, 230°C-15min

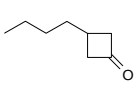
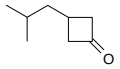
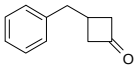
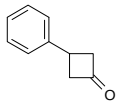
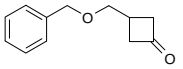
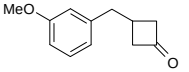
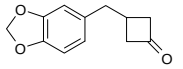
6.3 Original Data of Mutant Screening

							
		22a		**23a**		**15a**	
Strain		**Exp1**	**Exp2**	**Exp1**	**Exp2**	**Exp1**	**Exp2**
1-C2-B7 (R)	***Conv.***	50%	45%	100%	100%	n.c[b].	n.c.
	ee, (+/-)[a]	91%,(-)	90%, (-)	99%, (-)	99%, (-)	n.a.[c]	n.a.
1-F1-F5 (R)	***Conv.***	-[d]	100%	100%	100%	n.c.	n.c.
	ee, (+/-)	-	94%, (-)	99%, (-)	99%, (-)	n.a.	n.a.
1-E12-B5 (R)	***Conv.***	86%	86%	100%	100%	n.c.	n.c.
	ee, (+/-)	87%, (-)	87%, (-)	88%, (-)	87%, (-)	n.a.	n.a.
1-H7-F4 (R)	***Conv.***	75%	80%	100%	100%	n.c.	n.c.
	ee, (+/-)	17%, (-)	17%, (-)	57%, (+)	57%, (+)	n.a.	n.a.
1-H3-C9 (S)	***Conv.***	51%	64%	90%-	72%	n.c.	n.c.
	ee, (+/-)	56%, (-)	56%, (-)	96%, (-)	96%, (-)	n.a.	n.a.
1-F4-B9 (S)	***Conv.***	85%	77%	100%	100%	n.c.	n.c.
	ee, (+/-)	87%, (-)	87%, (-)	82%, (-)	83%, (-)	n.a.	n.a.
1-K6-G2 (S)	***Conv.***	100%	100%	100%	100%	n.c.	n.c.
	ee, (+/-)	94%, (-)	94%, (-)	99%, (-)	99%, (-)	n.a.	n.a.
1-K2-F5 (S)	***Conv.***	81%	81%	100%	100%	n.c.	n.c.
	ee, (+/-)	92%, (-)	94%, (-)	99%, (-)	99%, (-)	n.a.	n.a.
2-D19-E6 (R)	***Conv.***	-	70%	65%	56%	n.c.	n.c.
	ee, (+/-)	-	92%, (-)	99%, (-)	99%, (-)	n.a.	n.a.
GC-method		BGB 173, Method A		BGB 173, Method A		BGB 173, Method B	

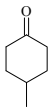
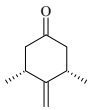
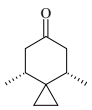
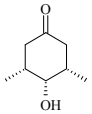
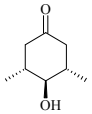
a) sign of optical rotation; b) no conversion c) not applicable, d) no result

		 **24a**		 **25a**	
Strain		**Exp1**	**Exp2**	**Exp1**	**Exp2**
1-C2-B7 (R)	***Conv., n/a*** ***ee, (+/-)***[a]	100%, 69/31 38%/99%, (-)/(-)	100%, 69/31 38%/99%, (-)/(-)	100%, 36/64 99%/57%, (-)/(-)	100%, 36/64 99%/57%, (-)/(-)
1-F1-F5 (R)	***Conv.*** ***ee, (+/-)***	100%, 80/20 29%/99%, (-)/(-)	100%, 81/19 30%/99%, (-)/(-)	100%, 41/59 99%/76%, (-)/(-)	100%, 41/59 99%/74%, (-)/(-)
1-E12-B5 (R)	***Conv.*** ***ee, (+/-)***	100%, 49/51 90%/99%, (-)/(-)	100%, 49/51 89%/99%, (-)/(-)	100%, 47/53 99%/72%, (-)/(-)	100%, 46/54 99%/70%, (-)/(-)
1-H7-F4 (R)	***Conv.*** ***ee, (+/-)***	100%, 47/53 94%/97%, (-)/(-)	100%, 47/53 94%/97%, (-)/(-)	100%, 45/55 99%/89%, (-)/(-)	100%, 47/53 99%/87%, (-)/(-)
1-H3-C9 (S)	***Conv.*** ***ee, (+/-)***	100%, 69/31 38%/99%, (-)/(-)	100%, 69/31 39%/99%, (-)/(-)	100%, 29/71 99%/42%, (-)/(-)	100%, 29/71 99%/35%, (-)/(-)
1-F4-B9 (S)	***Conv.*** ***ee, (+/-)***	100%, 72/28 40%/99%, (-)/(-)	100%, 71/29 40%/99%, (-)/(-)	100%, 43/57 99%/69%, (-)/(-)	100%, 43/57 99%/68%, (-)/(-)
1-K6-G2 (S)	***Conv.*** ***ee, (+/-)***	100%, 59/41 63%/99%, (-)/(-)	100%, 59/41 63%/99%, (-)/(-)	100%, 44/56 99%/78%, (-)/(-)	100%, 43/57 99%/75%, (-)/(-)
1-K2-F5 (S)	***Conv.*** ***ee, (+/-)***	100%, 58/42 65%/99%, (-)/(-)	100%, 58/42 65%/99%, (-)/(-)	100%, 44/56 99%/80%, (-)/(-)	100%, 43/57 99%/74%, (-)/(-)
2-D19-E6 (R)	***Conv.*** ***ee, (+/-)***	100%, 84/16 19%/99%, (-)/(-)	100%, 84/16 20%/99%, (-)/(-)	100%, 45/55 99%/65%, (-)/(-)	100%, 45/55 99%/66%, (-)/(-)
GC-method		BGB 175, Method A		BGB 173, Method A or B	

a) sign of optical rotation

		 **5a**		 **6a**		 **7a**		 **8a**		 **9a**		 **12a**		 **13a**	
Strain		**Exp1**	**Exp2**	**Exp1**	**Exp2**	**Exp1**	**Exp2**	**Exp1**	**Exp2**	**Exp1**	**Exp2**	**Exp1**	**Exp2**	**Exp1**	**Exp2**
1-C2-B7 (R)	***Conv.*** ***ee, (+/-)***[a]	100% 16%, (-)	100% 16%, (-)	100% 60%, n.a.	100% 63%, n.a.	100% 76%, (-)	100% 75%, (-)	19% 20%, (-)	100% 48%, (-)	100% 41%, (+)	100% 42%, (+)	n.c.[b] n.a.[c]	n.c. n.a.	28% 89%, (-)	28% 86%, (-)
1-F1-F5 (R)	***Conv.*** ***ee, (+/-)***	100% 9%, (-)	100% 8%, (-)	100% 55%, n.a.	100% 57%, n.a.	100% 76%, (-)	100% 76%, (-)	100% 93%, (-)	100% 93%, (-)	100% 59%, (+)	-[d] -	100% 90%, (-)	100% 91%, (-)	100% 92%, (-)	43% 87%, (-)
1-E12-B5 (R)	***Conv.*** ***ee, (+/-)***	100% 24%, (+)	100% 24%, (+)	100% 48%, n.a	100% 48%, n.a	100% 86%, (-)	100% 86%, (-)	100% 8%, (-)	100% 10%, (-)	100% 66%, (+)	100% 68%, (+)	100% 94%, (-)	100% 95%, (-)	100% 96%, (-)	100% 96%, (-)
1-H7-F4 (R)	***Conv.*** ***ee, (+/-)***	100% 97%, (-)	100% 97%, (-)	100% 96%, n.a	100% 96%, n.a	100% 90%, (-)	100% 90%, (-)	100% 95%, (-)	100% 95%, (-)	100% 70%, (-)	- -	100% 95%, (-)	100% 94%, (-)	100% 97%, (-)	77% 95%, (-)
1-H3-C9 (S)	***Conv.*** ***ee, (+/-)***	100% 18%, (-)	100% 20%, (-)	100% 60%, n.a	100% 59%, n.a	100% 76%, (-)	100% 76%, (-)	100% 72%, (-)	100% 76%, (-)	100% 40%, (+)	100% 40%, (+)	n.c. n.a.	n.c. n.a.	n.c. n.a.	36% 41%, (-)
1-F4-B9 (S)	***Conv.*** ***ee, (+/-)***	100% 19%, (-)	100% 19%, (-)	100% 63%, n.a	100% 65%, n.a	100% 54%, (-)	100% 59%, (-)	100% 10%, (-)	100% 10%, (-)	100% 24%, (+)	100% 26%, (+)	100% 95%, (-)	100% 94%, (-)	66% 95%, (-)	57% 94%, (-)
1-K6-G2 (S)	***Conv.*** ***ee, (+/-)***	100% 92%, (-)	100% 93%, (-)	100% 87%, n.a	100% 84%, n.a	100% 77%, (-)	100% 77%, (-)	100% 94%, (-)	100% 94%, (-)	100% 75%, (-)	100% 75%, (-)	100% 91%, (-)	100% 91%, (-)	100% 91%, (-)	100% 89%, (-)
1-K2-F5 (S)	***Conv.*** ***ee, (+/-)***	100% 97%, (-)	100% 96%, (-)	100% 91%, n.a	100% 91%, n.a	100% 78%, (-)	100% 79%, (-)	100 96%, (-)	- -	100% 83%, (-)	100% 84%, (-)	100% 91%, (-)	100% 90%, (-)	100% 91%, (-)	100% 91%, (-)
2-D19-E6 (R)	***Conv.*** ***ee, (+/-)***	100% rac, n.a.	100% rac, n.a.	100% 57%, n.a	100% 57%, n.a	100% 76%, (-)	100% 76%, (-)	100% 82%, (-)	100% 82%, (-)	100% 7%, (+)	100% 7%, (+)	54% 92%, (-)	48% 94%, (-)	66% 95%, (-)	66% 95%, (-)
GC-method		BGB 175, Method B		BGB 175, Method B		BGB 175, Method A		BGB 173, Method A		BGB 175, Method B		BGB 173, Method B		BGB 173, Method B	

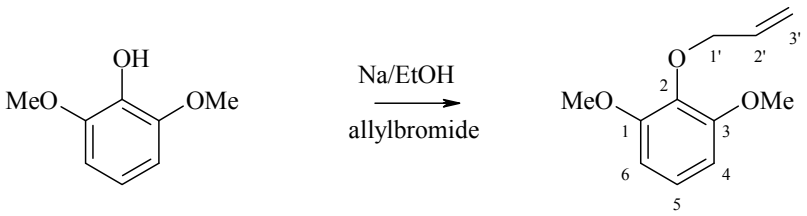
a) sign of optical rotation; b) no conversion c) not applicable, d) no result

		 **20a**		 **21a**		 **26a**		 **27a**		 **28a**		 **29a**	
Strain		**Exp1**	**Exp2**	**Exp1**	**Exp2**	**Exp1**	**Exp2**	**Exp1**	**Exp2**	**Exp1**	**Exp2**	**Exp1**	**Exp2**
1-C2-B7 (R)	***Conv.***	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	49%	53%
	ee, (+/-)[a]	99%, (-)	99%, (-)	99%, (-)	99%, (-)	99%, (+)	99%, (+)	99%, (+)	99%, (+)	99%, (-)	99%, (-)	96%, (+)	98%, (+)
1-F1-F5 (R)	***Conv.***	100%	100%	100%	100%	100%	100%	45%	48%	100%	100%	7%	10%
	ee, (+/-)	99%, (-)	99%, (-)	99%, (-)	99%, (-)	99%, (+)	99%, (+)	99%, (+)	99%, (+)	99%, (-)	99%, (-)	97%, (+)	95%, (+)
1-E12-B5 (R)	***Conv.***	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	n.c.[b]	n.c.
	ee, (+/-)	99%, (-)	99%, (-)	99%, (-)	99%, (-)	99%, (+)	99%, (+)	99%, (+)	99%, (+)	99%, (-)	99%, (-)	n.a.[c]	n.a.
1-H7-F4 (R)	***Conv.***	-[d]	-	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
	ee, (+/-)	-	-	99%, (-)	99%, (-)	99%, (+)	99%, (+)	99%, (+)	99%, (+)	99%, (-)	99%, (-)	99%, (+)	99%, (+)
1-H3-C9 (S)	***Conv.***	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	-
	ee, (+/-)	99%, (-)	99%, (-)	99%, (-)	99%, (-)	99%, (+)	99%, (+)	99%, (+)	99%, (+)	97%, (-)	97%, (-)	98%, (+)	-
1-F4-B9 (S)	***Conv.***	100%	100%	88%	90%	70%	67%	100%	100%	100%	100%	13%	17%
	ee, (+/-)	99%, (-)	99%, (-)	99%, (-)	99%, (-)	99%, (+)	99%, (+)	99%, (+)	99%, (+)	99%, (-)	99%, (-)	98%, (+)	99%, (+)
1-K6-G2 (S)	***Conv.***	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
	ee, (+/-)	99%, (-)	99%, (-)	99%, (-)	99%, (-)	99%, (+)	99%, (+)	99%, (+)	99%, (+)	99%, (-)	99%, (-)	99%, (+)	99%, (+)
1-K2-F5 (S)	***Conv.***	100%	100%	100%	100%	79%	100%	100%	100%	100%	100%	100%	100%
	ee, (+/-)	99%, (-)	99%, (-)	99%, (-)	99%, (-)	99%, (+)	99%, (+)	99%, (+)	99%, (+)	99%, (-)	99%, (-)	99%, (+)	99%, (+)
2-D19-E6 (R)	***Conv.***	100%	-	100%	100%	100%	100%	100%	100%	100%	100%	16%	14%
	ee, (+/-)	99%, (-)	-	99%, (-)	99%, (-)	99%, (+)	99%, (+)	99%, (+)	99%, (+)	99%, (-)	99%, (-)	99%, (+)	99%, (+)
GC-method		BGB 173, Method A		BGB 173, Method A		BGB 173, Method A		BGB 175, Method A		BGB 173, Method A		BGB 175, Method C	

a) sign of optical rotation; b) no conversion, c) not applicable; d) no result

6.4 Synthesis of 3-(3,4,5-Trimethoxy-benzyl)-cyclobutanone 14a

6.4.1 Preparation of 2-(Allyloxy)-1,3-dimethoxybenzene 2



1 **2**

Procedure: Allyl bromide was distilled prior to use.

Small pieces of sodium (1.79g, 78.0mmol, 1.2equiv.) were suspended in dry EtOH (150mL) and stirred under argon atmosphere until completely dissolved. Then a solution of 2,6-dimethoxyphenole **1** (10.0g, 65mmol) in dry EtOH (120mL) was added dropwise *via* a syringe and the mixture was stirred for one hour. Pure allyl bromide (9.44g, 78.0mmol, 1.2equiv.) was added dropwise and the reaction mixture was stirred overnight at rt.

The reaction mixture had turned into grey slurry. More allyl bromide (1.00g, 8.3mmol) was added and the reaction was stirred until GC/MS showed full conversion (24h).

The reaction mixture was concentrated at reduced pressure and the remaining suspension was then diluted with water (400mL). After extraction with Et_2O (approx. 800mL) at pH 12 the combined organic layers were dried over Na_2SO_4. Evaporation of the solvent provided the allyl ether **2** as golden yellow oil in very good purity (>99% according to GC/MS) with good yield (10.5g, 54.1mmol, 83%).

Yield: 10.5g (83%)

MW: 194.2; $C_{11}H_{13}O_3$

golden yellow oil (Lit.[144])

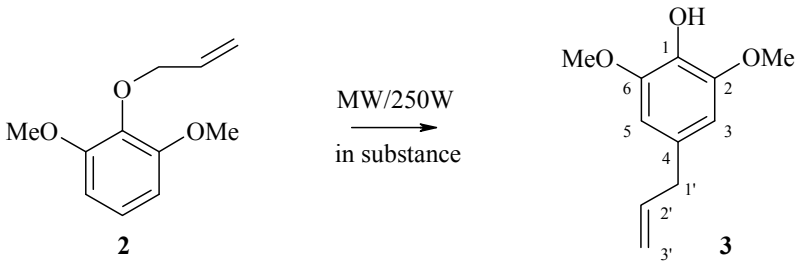
^{1}H NMR ($CDCl_3$): δ 3.84 (s, 6H, 2xO-CH_3), 4.52 (d, J=6Hz, 2H, H-1'), 5.14-5.36 (m, 2H, H-3'), 6.02-6.22 (m, 1H, H-2'), 6.57 (d, J=8Hz, 2H, H-4/H-6), 6.98 (t, J=8Hz, 1H, H-5);

^{13}C NMR ($CDCl_3$): δ 55.9 (q, 2xO-CH_3), 74.0 (t, C-1'), 105.1 (2xd,C-4/C-6), 117.5 (t, C-3'), 123.5 (d, C-5), 134.5 (d, C-2'), 136.6 (s, C-2), 153.6 (2xs, C-1/C-3)

m/z: 194 (61, M^+), 153 (100), 125 (48), 110 (56), 95 (38), 93 (40).

[144] Jing, X.; Gu, W.; Bie, P.; Ren, X.; Pan, X. *Synthetic Comm.* **2001**, *31*, 861-867.

6.4.2 Preparation of 4-Allyl-2,6-dimethoxyphenole 3



2 **3**

Procedure: Pure 2-allyloxy-1,3-dimethoxybenzene **2** (2.0g, 10.3mmol) was sealed in a microwave tube and irradiated in neat form for 75 min (250 W, 180°C). Reaction control by GC/MS indicated complete conversion >95% purity according to GC/MS analysis) and the crude product **3** was used without further purification in the next reaction step.

Yield: 2.00g (100%)

MW: 194.2; $C_{11}H_{13}O_3$

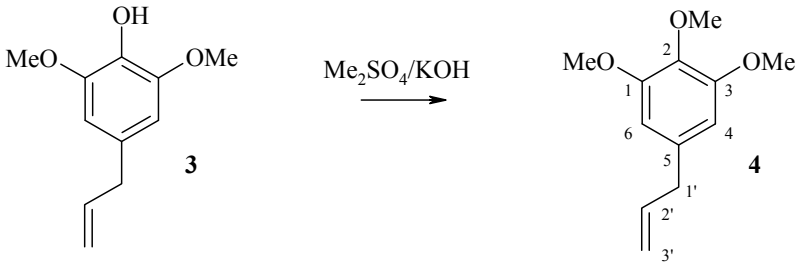
brown oil, (Lit.[144])

^{1}H NMR ($CDCl_3$): δ 3.23 (d, J=6Hz, 2H, H-1'), 3.78 (s, 6H, 2xO-CH_3), 4.96-5.05 (m, 2H, H-3'), 5.35 (s, 1H, aryl-OH), 5.77-5.97 (m, 1H, H-2'), 6.33 (s, 2H, H-3/ H-5);

^{13}C NMR ($CDCl_3$): δ 40.1 (t, C-1'), 56.0 (2xq, 2xO-CH_3), 104.9 (2xd, C-3, C-5), 115.5 (t, C-3'), 130.8 (s), 132.8 (s), 137.4 (d, C-2'), 147.8 (2xs, C-2, C-6);

m/z: 194 (100, M^+), 179 (11), 147 (10), 131 (14), 119 (20), 91 (25), 77 (13).

6.4.3 Preparation of 5-Allyl-1,2,3-trimethoxybenzene 4



Procedure: Dimethyl sulfate was distilled prior to use (73.5 °C/18mbar).

4-Allyl-2,6-dimethoxyphenole **3** (8.23g, 42.4mmol) was dissolved in 10% aqueous potassium hydroxide solution (2.97g KOH, 53.0mmol, 1.25equiv.) under vigorous magnetic stirring. The reaction mixture turned dark blue and later to a greenish yellow to become a slurry. An ice cooling bath was installed and dimethyl sulfate (5.88g, 46.7mmol, 1.1equiv.) was added slowly; no temperature change was observed. The cooling bath was removed and the mixture was stirred for 4 hours. For completion of the reaction more dimethyl sulfate was added (0.54 g, 0.1equiv.) and the reaction was continued at reflux temperature. After 30 min reaction control by TLC and GC-MS showed complete conversion.

The reaction solution was extracted with Et_2O (5 x 60mL). The combined organic layers were dried over anhydrous sodium sulfate and concentrated *in vacuo.*

Kugelrohr distillation of the crude product yielded the desired compound **4** as colorless oil in good yield (8.20g, 39.4mmol, 93%).

Yield: 8.20g (93%, Lit.[145] 47%)

MW: 208.3; $C_{12}H_{16}O_3$

colorless oil, b.p.: 106-108°C/1mbar KRD, (Lit.[146] 106-107°C/10mbar)

^{1}H NMR ($CDCl_3$): δ 3.26 (d, J=7Hz, 2H, H-1'), 3.78 (s, 9H, 3xO-CH_3), 4.99-5.09 (m, 2H, H-3'), 5.79-5.99 (m, 1H, H-2'), 6.34 (s, 2H, H-4/H-6);

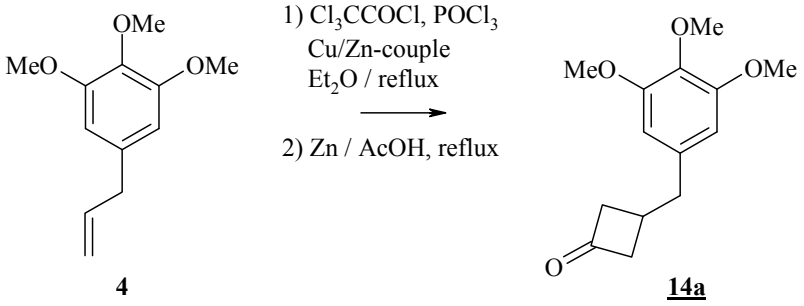
^{13}C NMR ($CDCl_3$): δ 39.4 (t, C-1'), 54.8 (2xq, C-1, C-3-OMe), 59.6 (q, C-2-OMe), 104.1 (2xd, C-4, C-6), 114.8 (t, C-3'), 134.6 (s), 135.0 (s), 151.9 (2xs, C-1, C-3);

m/z: 208 (100, M^+), 193 (62), 177 (13), 133 (17), 118 (13), 91 (15), 77 (18).

[145] Medina, A.L.; Lucero, M.E.; Holguin, F.O.; Estell, R.E.; Posakony, J.J.; Simon, J.; O'Connell, M.A. *J. Agric. Food Chem.* **2005**, *53*, 8694-8698.

[146] Gunasekaran, A.; Balasubramanian, K. *Indian J. Chem. Sect. B* **1988**, *27*, 308-310.

6.4.4 Preparation of 3-(3,4,5-Trimethoxybenzyl)-cyclobutanone 14a



4 **14a**

6.4.4.1 Synthesis of 2,2-Dichloro-3-(3,4,5-trimethoxy-benzyl)-cyclobutanone

Procedure: A stirred suspension of zinc dust (10g, 0.15mol) in water (40mL) was degassed by passing through N_2 for 15 minutes. Subsequently, $CuSO_4$ (750mg, 4.7mmol) was added at once and the black suspension was stirred while N_2 was passed through for an additional 45 minutes. The Cu/Zn-couple was collected on a sintered glass funnel and was washed successively with 100mL degassed water and acetone. The Cu/Zn-couple was dried *in vacuo* and stored (max. 2 days) under N_2.

Freshly prepared Cu/Zn-couple (0.56g, 8.64mmol, 1.2equiv.) was suspended in dry diethyl ether (20mL). 5-Allyl-3,4,5-trimethoxybenzene **4** (1.50g, 7.20mmol) was added and the reaction mixture was set under nitrogen atmosphere. A mixture of Cl_3CCOCl (1.57g, 8.64mmol, 1.2equiv.) and $POCl_3$ (1.32g, 8.64mmol, 1.2equiv.) (both distilled freshly) dissolved in dry diethyl ether (10mL) was added subsequently over a period of one hour. Finally, the reaction mixture was refluxed for six hours, cooled to rt (to avoid side reactions) and again supplemented by a mixture of Cl_3CCOCl, $POCl_3$ (1.2eq) and Cu/Zn-couple (1.2equiv.).

After 12 hours complete conversion was determined by TLC (GC/MS reaction control did not give satisfactory results; detection of the chloro compound was not efficient enough). The crude reaction mixture was diluted with CH_2Cl_2 and extracted with satd. bicarbonate solution. The aqueous phase was extracted three times with ethyl acetate. The combined organic layers were dried over sodium sulfate. After evaporation of the solvent the desired compound was isolated in quantitative yield (2.25g, 7.20mmol, quant.). The crude product (>95% according to GC/MS) was used without further purification in the next step.

6.4.4.2 Synthesis of 3-(3,4,5-Trimethoxybenzyl)-cyclobutanone 14a

Procedure: Crude 2,2-dichloro-3-(3,4,5-trimethoxybenzyl)-cyclobutanone (2.25g, 7.20mmol) was dissolved in glacial acetic acid (20mL). Then zinc dust (1.4g, 21.6mmol, 3equiv.) was added. The reaction mixture was refluxed overnight. After TLC control indicated complete conversion the reaction mixture was cooled to rt and diluted with ethyl acetate.
After extraction with saturated aqueous sodium bicarbonate solution the organic layer was dried and the solvent was evaporated. The crude compound **14a** was purified by flash column chromatography (LP/EtOAc=4/1-2/1).

Yield: 864mg (48% over 2 steps)
MW: 250.3; $C_{14}H_{18}O_4$

colorless oil

^{1}H NMR ($CDCl_3$): δ 2.67–2.86 (m, 5H), 3.08–3.24 (m, 2H), 3.83 (s, 3H p-OCH_3), 3.86 (s, 6H, 2 x m-OCH_3), 6.41 (s, 2H, H-2`/H-6`);

^{13}C NMR ($CDCl_3$): δ 25.1 (d, C-3), 42.2 (t, $\underline{C}H_2$-Ph), 52.3 (2xt, C-2/C-4), 56.1 (2xq, m-OCH_3), 60.8 (q, p-OCH_3), 105.4 (2xs, C-2`/C-6`), 135.7 (2xs, C-3`/C-5`), 136.4 (s, C-4`), 150.9 (s, C-1`), 207.7 (C=O);

Calc.:	C 67.18%,	H 7.25%
Found.:	C 67.06%,	H 7.25%

m/z: 250 (60, M^+), 208 (22), 193 (41), 181 (100), 91 (19), 77 (25).

6.5 Baeyer-Villiger Oxidation

6.5.1 Chemical Baeyer-Villiger Oxidation (Method A)

Procedure: *m*-Chloroperoxybenzoic acid (1.5 equiv., 70% purity) was added to a solution of the corresponding ketone (1 equiv.) in dry dichloromethan (10% solution) and the mixture was stirred overnight at rt. Complete conversion was determined by TLC. When the reaction was complete triethylamine (0.5 equiv.) was added and the mixture was stirred for 15 minutes. Subsequently, water was added and the organic layer was separated. The aqueous layer was extracted two times with dichloromethane. The combined organic phases were washed with satd. sodium bicarbonate solution, dried over sodium sulfate, filtered and evaporated.

GC-analysis: Chemical oxidation of ketones was mandatory for the preparation of racemic reference material. Due to calculation and description of enantioselectivity of the obtained lactone, baseline separation of the racemic reference material performed by chiral GC analysis was necessary.

Method A (biotrans-STD)	80°C-2min→80-160°C, 5°C/min, 160°C-1min→160-220°C, 10°C/min, 220°C-8min
Method B (enantiomerentrennung_2°C)	80°C-2min→80-220°C, 2°C/min, 220°C-8min
Method C (enantiomerentrennung_1°C_opt)	80°C-2min→80-220°C, 1°C/min, 220°C-8min
Method D (trimethoxytrennnug_2°min)	160°C-2min→160-230°C, 2°C/min, 230°C-15min

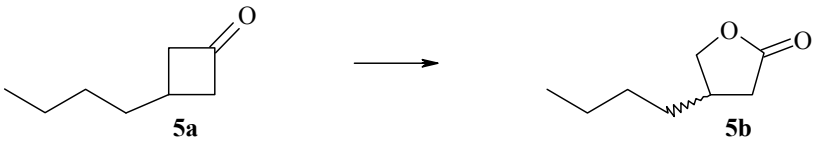
6.5.2 Microbial Baeyer-Villiger Oxidation (Method B)

Procedure: Fresh LB_{amp} medium (250mL) was inoculated with 1% (2.5mL) of an overnight preculture of recombinant *E. coli* strains: $CHMO_{Acineto}$, $CPMO_{Pseudo}$, $CHMO_{Brevi\ I}$, $CHMO_{Brevi\ II}$, $CHMO_{Rhodo\ I}$, $CHMO_{Rhodo\ II}$, $CHMO_{Brachy}$, $CHMO_{Arthro}$, in a baffled Erlenmeyer flask. The culture was incubated at 120 rpm at 37°C on an orbital shaker for 2 hours, and then 50µl IPTG stock solution was added to a final concentration of 0.004 wt/v. The substrate (100mL) was added neat along with β-cyclodextrin (1 equiv.). The culture was incubated overnight at 120 rpm at rt until GC (sample of 700µl) showed complete conversion of the ketone (24 hours).

After complete conversion the biomass was separated by centrifugation (15 min., 4000 rpm). The supernatant was filtered through a bed of Celite, which was subsequently washed with the extraction solvent.

The aqueous layer was extracted with the same solvent (2x200mL). The combined organic layers were dried over sodium sulfate, filtered, and the solvent was removed in *vacuo*. The crude lactones were purified by flash column chromatography.

6.5.3 4-Butyl-dihydro-furan-2(3H)-one 5b



Methode A: 3-Butylcyclobutanone **5a** (299mg, 2.37mmol) was oxidized to 4-butyl-dihydro-furan-2-one according to the general procedure. Purification of the product by column chromatography on silica gel (silica gel, LP/EtOAc = 7/1) yielded in 55% of the desired lactone **5b**.[147] (GC: BGB 175, method B)

Yield: 186mg (55%)

MW: 142.2, $C_8H_{14}O_2$

Method B: 3-Butylcyclobutanone **5a** (150mg, 1.18mmol) was oxidized with recombinant *E. coli* cells according to general procedure. The crude product was purified *via* column chromatography (silica gel, LP/EtOAc=7/1) and was obtained as yellow oil **5b**.

Enzyme	5a	5b	*ee*	abs. config.[70b]	$[\alpha]^D_{20}$
$CHMO_{Acineto}$	150mg	105mg (62%)	17%	(S)	-2.05 (*c* 2.10, $CHCl_3$)
$CHMO_{Arthro}$	150mg	87mg (51%)	32%	(S)	-1,84 (*c* 1.74, $CHCl_3$)
$CHMO_{Brachy}$	150mg	93mg (55%)	14%	(S)	-1,20 (*c* 1.86, $CHCl_3$)
$CHMO_{Brevi1}$	150mg	110mg (65%)	>99%	(S)	-5,95 (*c* 2.20, $CHCl_3$)
$CHMO_{Brevi2}$	150mg	125mg (74%)	69%	(S)	-3,68 (*c* 2.50, $CHCl_3$)
$CPMO_{Coma}$	150mg	121mg (72%)	76%	(S)	-4,46 (*c* 2.42, $CHCl_3$)
$CHMO_{Rhodo1}$	150mg	91mg (54%)	rac.	-	n.d.
$CHMO_{Rhodo2}$	150mg	98mg (58%)	rac.	-	n.d.

yellow oil

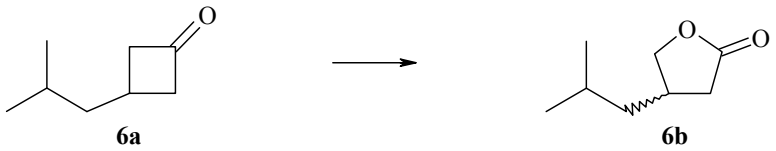
^{1}H-NMR ($CDCl_3$): δ 0.91 (t, J=5Hz, 3H, CH_3), 1.21-1.56 (m, 6H, $(CH_2)_3$), 2.11-2.28 (m, 1H, H-3), 2.46-2.68 (m, 2H, H-3), 3.92 (dd, J_1=7Hz, J_2=9Hz, 1H, H-5,), 4.40 (dd, J_1=7Hz, J_2=9Hz, 1H, H-5);

^{13}C-NMR ($CDCl_3$): δ 13.8 (q, CH_3), 22.4 (t, CH_2), 29.4 (t), 32.7 (t), 34.4 (t, C-3), 35.6 (d, CH), 73.3 (t, C-5), 177.4 (s, C=O);

m/z: 142 (1, M^+), 114 (8), 111 (8), 84 (12), 70 (15), 69 (29), 56 (100), 55 (46).

[147] Röder, E.; Krauß, H. *Liebigs Ann. Chem.* **1992**, 177-181.

6.5.4 4-(2-Methylpropyl)-dihydro-furan-2(3H)-one 6b



6a **6b**

Method A: 3-(2-Methylpropyl)cyclobutanone **6a** (305mg, 2.46mmol) was oxidized to 4-(2-methylpropyl)-dihydro-furan-2(3H)-one, according to the general procedure. After purification *via* flash column chromatography (silica gel, LP/EtOAc = 14/1) the desired lactone **6b** was isolated in 29% yield.[65] (GC: BGB 175, method B)

Yield: 103mg (29%)

MW: 142.2, $C_8H_{14}O_2$

Method B: 3-(2-Methylpropyl)cyclobutanone **6a** was oxidized with *E. coli* expressing cells according to general procedure. The crude product was purified *via* flash column chromatography (LP/EtOAc = 35/1) and the brown odorant oil **6b** was isolated in the yields and properties specified below.

Enzyme	6a	6b	*ee*	abs. config.[70a]	$[\alpha]^D_{20}$
$CHMO_{Acineto}$	172mg	103mg (53%)	rac	n.d.	n.a.
$CHMO_{Arthro}$	93mg	43mg (41%)	74%	(S)	-0.44 (*c* 0.67, $CHCl_3$)
$CHMO_{Brachy}$	93mg	25mg (24%)	77%	(S)	-1.46 (*c* 0.41, $CHCl_3$)
$CHMO_{Brevi1}$	172mg	58mg (30%)	>99%	(S)	-1.47 (*c* 1.16, $CHCl_3$)
$CHMO_{Brevi2}$	93mg	45mg (43%)	22%	(S)	-0.44 (*c* 0.67, $CHCl_3$)
$CPMO_{Coma}$	172mg	123mg (63%)	76%	(S)	-1.02 (*c* 2.46, $CHCl_3$)
$CHMO_{Rhodo1}$	93mg	38mg (35%)	49%	(S)	-2.16(*c* 0.28, $CHCl_3$)
$CHMO_{Rhodo2I}$	93mg	42mg (40%)	45%	(S)	-0.72 (*c* 0.84, $CHCl_3$)

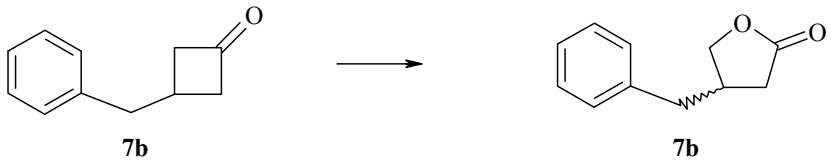
brown odorant oil

^{1}H NMR ($CDCl_3$): δ 0.92 (d, J=6Hz, 6H, 2x CH_3), 1.32-1.50 (m, 2H, CHC$\underline{H_2}$CH), 1.50-1.57 (m, 1H, C$\underline{H}(CH_3)_2$), 2.05-2.26 (m, 1H, H-3), 2.51-2.72 (m, 2H, H-3, H-4), 3.83-3.94 (m, 1H, H-5), 4.35-4.48 (m, 1H, H-5);

^{13}C NMR ($CDCl_3$): δ 22.4, 22.6 (q, 2x CH_3), 26.3 (d, $\underline{C}H(CH_3)_2$), 33.8 (d, $\underline{C}H(CH_2)_3$, 34.8 (t, CH$\underline{C}H_2$CH), 42.2 (t, C-3), 73.5 (t, C-5), 177.2 (s, C=O);

m/z: 142 (3, M^+), 112 (11), 100 (12), 84 (16), 69 (40), 56 (100), 55 (34).

6.5.5 Dihydro-4-(phenylmethyl)-furan-2(3H)-one 7b



7b **7b**

Method A: Cyclobutanone **7a** (137mg, 0.87mmol) was oxidized according to the general procedure. The product **7b** did not require additional purification. (GC: BGB 175, method A)

Yield: 133 mg (87%)

MW: 176.2, $C_{11}H_{12}O_2$

Method B: 3-(Phenylmethyl)cyclobutanone **7a** (106mg, 0.66mmol) was oxidized with all eight *E. coli* expressing cells according to general procedure. The crude product was purified *via* column chromatography (silica gel, LP/EtOAc = 10/1) and the pink odorant oil **7b** was isolated in the yields and properties specified below.

Enzyme	7a	7b	*ee*	abs. config.[70a]	$[\alpha]^D_{20}$
$CHMO_{Acineto}$	106mg	37mg (32%)	88%	(S)	-7.77 (c 0.75, $CHCl_3$)
$CHMO_{Arthro}$	106mg	65mg (56%)	93%	(S)	-5.35 (c 1.31, $CHCl_3$)
$CHMO_{Brachy}$	106mg	45mg (38%)	84%	(S)	-6.40 (c 0.89, $CHCl_3$)
$CHMO_{Brevi1}$	106mg	35mg (30%)	93%	(S)	-12.8 (c 0.70, $CHCl_3$)
$CHMO_{Brevi2}$	106mg	32mg (27%)	59%	(S)	-8.66 (c 0.36, $CHCl_3$)
$CPMO_{Coma}$	106mg	43mg (37%)	31%	(S)	-2.20 (c 0.86, $CHCl_3$)
$CHMO_{Rhodo1}$	106mg	38mg (33%)	87%	(S)	-5.60 (c 0.54, $CHCl_3$)
$CHMO_{Rhodo2}$	106mg	36mg (31%)	87%	(S)	-15.1 (c 0.48, $CHCl_3$)

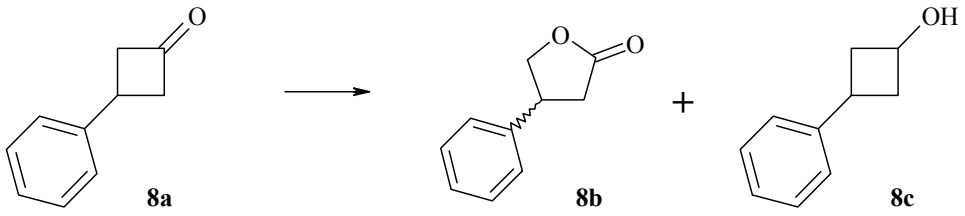
pink odorant oil

^{1}H-NMR ($CDCl_3$): δ 2.43 (dd, J_1=9Hz, J_2=17Hz, 1H, H-3), 2.72-2.93 (m, 3H, C<u>H</u>C<u>H</u>$_2$), 4.19 (dd, J_1=5Hz, J_2=9Hz, 1H, H-5), 4.32 (dd, J_1=5Hz, J_2=9Hz, 1H, H-5), 7.11-7.36 (m, 5H, Ph);

^{13}C-NMR ($CDCl_3$): δ 34.2 (t, C-3), 37.1 (d), 38.9 (t, <u>C</u>H$_2$Ph), 72.6 (t, C-5), 126.8 (d, C-4`), 128.6 (d, C-3`), 127.7 (d, C-2`), 138.3 (s, C-1`), 176.8 (s, C=O);

m/z: 176 (80, M^+), 117 (47), 115 (29), 92 (82), 91 (100), 65 (42).

6.5.6 Dihydro-4-phenyl-furan-2(3H)-one 8b



Method A: Cyclobutanone **8a** (183mg, 1.25mmol) was oxidized according to the general procedure. The product **8b** did not require additional purification. (GC: BGB 173, method A)

Yield: 243 mg (99%)

MW: 162.19, $C_{10}H_{10}O_2$

Method B: 3-Phenylcyclobutanone **8a** was oxidized with recombinant *E. coli* cells according to general procedure. After purification *via* column chromatography (silica gel, LP/EtOAc = 15/1) lactone **8b** was obtained as colorless crystals. In every case, except upon biooxidation using $CHMO_{Brevi\ I}$, reduction of ketone to alcohol **8c** was observed.

Enzyme	8a	8b	*ee*	abs. config.[70a]	$[\alpha]^D_{20}$	8c*
$CHMO_{Acineto}$	110mg	65mg (53%)	62%	(R)	-30.0 (*c* 1.20, MeOH)	4%
$CHMO_{Arthro}$	110mg	66mg (54%)	87%	(R)	-43.2 (*c* 1.20, MeOH)	4%
$CHMO_{Brachy}$	110mg	55mg (45%)	93%	(R)	-45.0 (*c* 1.00, MeOH)	13%
$CHMO_{Brevi1}$	110mg	89mg (73%)	98%	(R)	-47.3 (*c* 1.80, MeOH)	5%
$CHMO_{Brevi2}$	110mg	61mg (50%)	39%	(S)	+18.0 (*c* 1.10, MeOH)	4%
$CPMO_{Coma}$	110mg	81mg (66%)	37%	(S)	+16.9 (*c* 1.50, MeOH)	-
$CHMO_{Rhodo1}$	110mg	71mg (58%)	52%	(R)	-24.0 (*c* 1.30, MeOH)	11%
$CHMO_{Rhodo2}$	110mg	77mg (63%)	50%	(R)	-21.2 (*c* 1.50, MeOH)	15%

*calculation from GC

colorless crystals, m.p.=50-55°C, (Lit.[148]: 47-48.5°C)

^{1}H-NMR ($CDCl_3$): δ 2.65 (dd, J_1=10Hz, J_2=18Hz, 1H, H-3), 2.92 (dd, J_1=10Hz, J_2=18Hz, 1H, H-3), 3.70-3.86 (pent, J=17Hz, 1H, H-4), 4.26 (t, J=9Hz, 1H, H-5), 4.65 (t, J=9Hz, 1H, H-5), 7.19-7.41 (m, 5H,-Ph);

[148] Sato, M. *Chem. Pharma. Bull.* **1981**, *29*, 2885-2892.

^{13}C-NMR ($CDCl_3$): δ 35.6 (t, C-3), 41.0 (d, C-4), 73.9 (t, C-5), 126.6 (2xd, C-Ph), 127.6 (d, C-Ph), 129.0 (2xd, C-Ph), 139.4 (s, Ph), 176.3 (s, C=O);

m/z: 162 (29, M^+), 105 (9), 104 (100), 78 (11), 77 (11), 51 (11).

3-Phenyl-cyclobutanol 8c

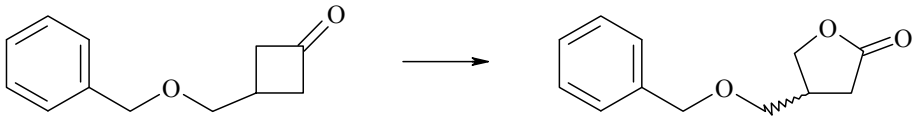
MW: 148.20, C10H12O

brown odorant oil[149]

^{1}H-NMR ($CDCl_3$): δ 1.83-2.03 (m, 3H, H-3, OH), 2.61-2.97 (m, 3H, H-3, H-4), 4.13-4.28 (m, 1H, H-1), 7.07-7.30 (m, 5H, Ph);

^{13}C-NMR ($CDCl_3$): δ 29.9 (d, C-3), 40.9 (2xt, C-2, C-4), 63.5 (d, C-1), 126.0, (d, C-4`, Ph), 126.6, (d, C-2`, Ph), 128.3, (d, C-3`, Ph), 144.6 (s, C-1`, Ph).

6.5.7 4-Benzyloxymethyl-dihydro-furan-2(3H)-one 9b



9a **9b**

Method A:3-Benzyloxymethylcyclobutanone **9a** (312mg, 1.64mmol) was oxidized to the desired lactone according to the general procedure. After purification *via* flash column chromatography (silica gel, LP/EtOAc = 2/1) the desired lactone **9b** was obtained as a yellow oil.[150] (GC: BGB 175, method B)

Yield: 178mg (53%)

MW: 206.2, $C_{12}H_{14}O_3$

Method B: 3-Benzyloxymethylcyclobutanone **9a** (116mg, 0.61mmol) was oxidized with recombinant *E. coli* cells according to general procedure. The crude product was purified *via* column chromatography (silica gel, LP/EtOAc = 2/1) and **9b** was obtained as yellow oil.

[149] Holland, H.L.; Kindermann, M.; Kumaresan, S.; Stefanac, T. *Tetrahedron Asymm.,* **1993**, *4*, 1353-1364.

[150] Hon, Y.-S.; Lee, C.-F. *Tetrahedron,* **2001**, *57*, 6181-6188.

Enzyme	9a	9b	*ee*	abs. config.[70]	$[\alpha]^{D}_{20}$
$CHMO_{Acineto}$	116mg	52mg (41%)	53%	(S)	+16.8 (*c* 1.04, $CHCl_3$)
$CHMO_{Arthro}$	116mg	23mg (18%)	58%	(R)	-19.1 (*c* 0.40, $CHCl_3$)
$CHMO_{Brachy}$	116mg	28mg (22%)	rac.	-	n.a.
$CHMO_{Brevi1}$	116mg	33mg (26%)	55%	(S)	+17.1 (*c* 0.66, $CHCl_3$)
$CHMO_{Brevi2}$	116mg	65mg (52%)	62%	(R)	-20.7 (*c* 1.30, $CHCl_3$)
$CPMO_{Coma}$	116mg	67mg (53%)	63%	(R)	-18.9 (*c* 1.34, $CHCl_3$)
$CHMO_{Rhodo1}$	116mg	25mg (45%)	6.0%	(S)	+1.80 (*c* 0.50, $CHCl_3$)
$CHMO_{Rhodo2}$	116mg	29mg (23%)	9.2%	(S)	+2.07 (*c* 0.58, $CHCl_3$)

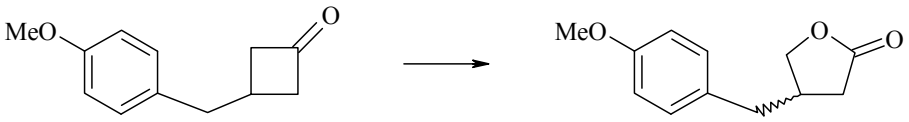
yellow oil

^{1}H-NMR (CDCl3): δ 2.36 (dd, J_1=6Hz, J_2=18Hz, 1H, H-3), 2.61 (dd, J_1=9Hz, J_2=18Hz, 1H, H-3), 2.84 (sept, J=8Hz, 1H, H-4), 3.43-3.49 (m, 2H, CH_2O), 4.17 (dd, J_1=6Hz, J_2=9Hz, 1H, H-5), 4.41 (dd, J_1=7Hz, J_2=9Hz, 1H, H-5), 4.52 (s, 2H, PhC$\underline{H_2}$O), 7.25-7.36 (m, 5H, Ph);

^{13}C-NMR ($CDCl_3$): δ 31.0 (t, C-3), 35.2 (d, C-4), 70.2 (t, CH_2O), 70.6 (t, CH_2O), 73.2 (t, CH_2O), 127.5 (2xd, C-Ph), 127.7 (d, C-Ph), 128.3 (2xd, C-Ph), 137.5 (s, C-Ph), 176.8 (s, C=O);

m/z: 206 (5, M^+), 205 (10), 177 (9), 120 (46), 105 (15), 92 (16), 91 (100), 65 (17).

6.5.8 Dihydro-4-[(4-methoxyphenyl)methyl]-2(3H)-furanone 10b



10a **10b**

Method A: 3-(4-Methoxybenzyl)-cyclobutanone **10a** (100mg, 0.53mmol) was converted according to the general procedure and gave the desired lactone as a yellow oil **10b** after 19 hours of reaction time and purification *via* column chromatography (silica gel, LP/EtOAc = 7/1). (GC: BGB 173, method B)

Yield: 50.0mg (46%)

MW: 206.2; $C_{12}H_{14}O_3$

Method B: 3-[(4-Methoxyphenyl)methyl]cyclobutanone **10a** was oxidized according to general method B. The crude product was purified *via* column chromatography (silica gel, LP/EtOAc = 10/1) and the yellow odorant oil **10b** was isolated in the yields and properties specified below.

Enzyme	10a	10b	*ee*	abs. config.[62]	$[\alpha]^D_{20}$
$CHMO_{Acineto}$	100mg	60mg (55%)	97%	(S)	-5.45 (c 0.95, $CHCl_3$)
$CHMO_{Arthro}$	69mg	69mg (89%)	97%	(S)	-6.11 (c 0.95, $CHCl_3$)
$CHMO_{Brachy}$	69mg	48mg (64%)	90%	(S)	-5.27 (c 0.74, $CHCl_3$)
$CHMO_{Brevi1}$	100mg	80mg (73%)	26%	(S)	-2.10 (c 1.60, $CHCl_3$)
$CHMO_{Brevi2}$	69mg	48mg (64%)	24%	(R)	+1.08 (c 0.74, $CHCl_3$)
$CPMO_{Coma}$	69mg	42mg (56%)	24%	(R)	+1.29 (c 0.62, $CHCl_3$)
$CHMO_{Rhodo1}$	69mg	40mg (53%)	80%	(S)	-3.97 (c 0.68, $CHCl_3$)
$CHMO_{Rhodo2}$	69mg	42mg (56%)	95%	(S)	-8.83 (c 0.60, $CHCl_3$)

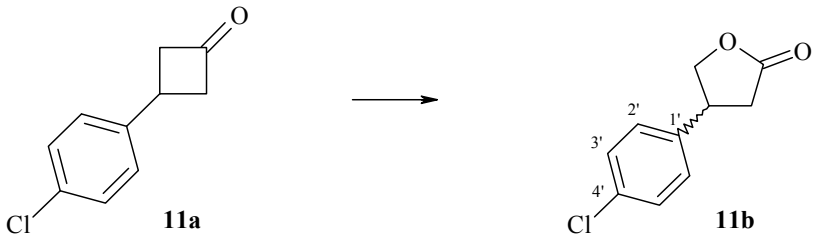
yellow odorant oil

^1H ($CDCl_3$): δ 2.27 (dd, J_1=7Hz, J_2=17Hz, 1H, H-3), 2.59 (d, J_1=8Hz, 1H, H-3,), 2.67-2.86 (m, 4H), 3.79 (s, 3H, OCH_3), 4.02 (dd, J_1=6Hz, J_2=9Hz, 1H, H-5), 4.32 (dd, J_1=9Hz, J_2=7Hz, 1H, H-5), 6.85 (d, J=9Hz, 2H, H-3', H-5'), 7.07 (d, J=9Hz, H-2', H-6');

^{13}C ($CDCl_3$): δ 34.7 (t, C-3), 37.9 (d, C-4), 38.6 (t), 55.8 (q, OCH_3), 114.7 (d, C-3'), 130.1 (d, C-2'), 130.7 (s, C-1'), 159.0 (s, C-4'), 177.4 (s, C=O);

m/z: 206 (15, M^+), 122 (9), 121 (100), 77 (8).

6.5.9 4-(4-Chlorophenyl)-dihydrofuran-2(3H)-one 11b



Method A: According to the general procedure 3-(4-chlorophenyl)-cyclobutanone **11a** (100mg, 0.55mmol) converted to the desired racemic lactone. After flash chromatography (silica gel, LP/EtOAc = 3/1) the desired product **11b** was isolated in 93% yield. (GC: BGB 173, method B)

Yield: 100mg (93%)

MW: 196.6, $C_{10}H_9ClO_2$

Method B: 3-(4-chlorophenyl)-cyclobutanone **11a** (100mg, 0.56mmol) was oxidized with recombinant *E. coli* cells according to general procedure. The crude product was purified *via* column chromatography (silica gel, LP/EtOAc = 3/1) and was obtained as yellow oil **11b**.[63]

Enzyme	11a	11b	*ee*	abs. config.[70]	$[\alpha]^D_{20}$
$CHMO_{Acineto}$	100mg	73mg (67%)	81%	(S)	+31.1 (*c 0.94*, $CHCl_3$)
$CHMO_{Arthro}$	100mg	55mg (50%)	87%	(S)	+20.2 (*c* 1.04, $CHCl_3$)
$CHMO_{Brachy}$	100mg	67mg (57%)	68%	(S)	+18.5 (*c* 1.14, $CHCl_3$)
$CHMO_{Brevi1}$	100mg	51mg (47%)	87%	(R)	-37.6 (*c* 0.54, $CHCl_3$)
$CHMO_{Brevi2}$	100mg	55mg (50%)	42%	(S)	+16.8 (*c* 1.00, $CHCl_3$)
$CPMO_{Coma}$	100mg	85mg (78%)	44%	(S)	+20.8 (*c* 1.56, $CHCl_3$)
$CHMO_{Rhodo1}$	100mg	56mg (51%)	95%	(S)	+42.1 (*c* 1.26, $CHCl_3$)
$CHMO_{Rhodo2}$	100mg	69mg (63%)	95%	(S)	+44.2 (*c* 1.02, $CHCl_3$)

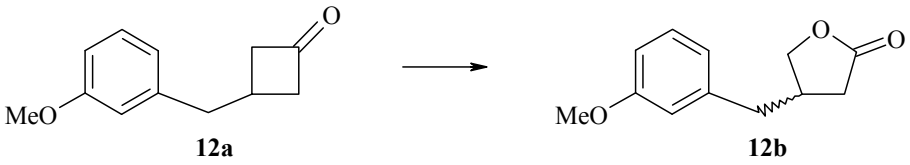
yellow oil

^{1}H-NMR ($CDCl_3$): δ 2.63 (dd, J_1=9Hz, J_2=17Hz, 1H, H-3/H-5), 2.94 (dd, J_1=9Hz, J_2=17Hz, 1H, H-3/H-5), 3.77 (quin, J=8Hz, 1H, H-4,), 4.24 (dd, J_1=8Hz, J_2=9Hz, 1H, H-3/H-5), 4.67 (dd, J_1=8Hz, J_2=9Hz, 1H, H-3/H-5), 7.18 (d, J=8Hz, 2H, Ph), 7.35 (d, J=8Hz, 2H, Ph);

^{13}C-NMR ($CDCl_3$): δ 35.6 (t, C-3), 40.5 (d, C-4), 73.8 (t, C-5), 128.0, 129.3 (2xd C-2', C-3'), 133.6, 137.9 (2xs, C-1', C-4'), 176.1 (s, C=O);

m/z: 196 (25, M^+), 140 (27), 138 (100), 103 (18).

6.5.10 Dihydro-5-(3-methoxyphenyl)-furan-2(3H)-one 12b



12a **12b**

Method A: Cyclobutanone **12a** (131mg, 0.69mmol) was oxidized according to the general procedure. The crude product **12b** was purified *via* flash column chromatography (silica gel, LP/EtOAc = 6/1). (GC: BGB 173, method B)

Yield: 32.0 mg (23%)

MW: 206.24, $C_{12}H_{14}O_3$

Mehod B: 3-[(3-Methoxyphenyl)methyl]cyclobutanone **12a** (108mg, 0.58mmol) was biooxidized according to general procedure. The crude product was purified *via* column chromatography (silica gel, LP/EtOAc = 8/1) and the yellow odorant oil **12b** was isolated.

Enzyme	12a	12b	*ee*	abs. config.[70b]	$[\alpha]^D_{20}$
$CHMO_{Acineto}$	108mg	60mg (50%)	>99%	(S)	-3.80 (c 1.20, $CHCl_3$)
$CHMO_{Arthro}$	108mg	51mg (43%)	93%	(S)	-5.85 (c 0.91, $CHCl_3$)
$CHMO_{Brachy}$	108mg	54mg (45%)	93%	(S)	-5.45 (c 0.95, $CHCl_3$)
$CHMO_{Brevi1}$	108mg	80mg (74%)	35%	(S)	-2.10 (c 1.60, $CHCl_3$)
$CHMO_{Brevi2}$	108mg	59mg (50%)	45%	(S)	-1.85 (c 0.27, $CHCl_3$)
$CPMO_{Coma}$	108mg	83mg (70%)	45%	(S)	-3.70 (c 0.98, $CHCl_3$)
$CHMO_{Rhodo1}$	108mg	72mg (60%)	98%	(S)	-6.50 (c 1.42, $CHCl_3$)
$CHMO_{Rhodo2}$	108mg	87mg (72%)	98%	(S)	-6.32 (c 1.71, $CHCl_3$)

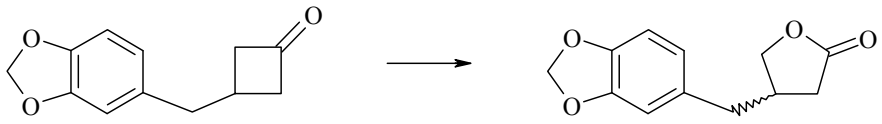
yellow odorant oil

^{1}H-NMR ($CDCl_3$): δ 2.38 (dd, J_1=9Hz, J_2=17Hz 1H, H-3), 2.54 (dd, J_1=9Hz, J_2=17Hz, 1H, H-3), 2.62-2.86 (m, 3H, C<u>H</u>C<u>H$_2$</u>), 3.72 (s, 3H, OCH_3), 3.93 (dd, J_1=6Hz, J_2=9Hz, 1H, H-5), 4.26 (dd, J_1=6Hz, J_2=9Hz, 1H, H-5), 6.62-6.74 (m, 3H, H-2`, H-4`, H-6`, Ph), 7.12-7.20 (t, J=8Hz, 1H, H-5`, Ph);

^{13}C-NMR ($CDCl_3$): δ 34.2 (t, C-3), 37.0 (d), 38.9 (t, CH_2Ph), 55.2 (q, OCH_3) 72.6 (t, C-5), 111.8 (d, C-6`), 114.6 (d, C-2`), 120.9 (d, C-4`), 129.8 (d, C-5`), 139.8 (s, C-3`), 159.9 (s, C-1`), 176.8 (s, C=O);

m/z: 206 (31, M^+), 122 (100), 121 (33), 107 (9), 91 (22), 77 (12).

6.5.11 Dihydro-4-(1,3-benzodioxol-5-ylmethyl)-furan-2(3H)-one 13b



13a **13b**

Method A: 3-(1,3-Benzodioxol-5-ylmethyl)-cyclobutanone **13a** (165mg, 0.81mmol) was oxidized according to the general procedure. The crude product was purified *via* column chromatography (LP/EtOAc = 7/1) to give a brown odorant oil **13b**. (GC: BGB 173, method B)

<u>Yield: 104 mg (59%)</u>

MW: 220.2, $C_{12}H_{12}O_4$

Method B: 3-(1,3-Benzodioxol-5-ylmethyl)-cyclobutanone **13a** (94.0mg, 0.46mmol) was oxidized with *E.coli* expressing cells according to general procedure. The crude product was purified *via* column chromatography (silica gel, LP/EtOAc = 6/1) and the brown odorant oil **13b** was isolated in the yields and properties specified below.

Enzyme	13a	13b	*ee*	abs. config.[70]	$[\alpha]^D_{20}$
$CHMO_{Acineto}$	94mg	35mg (35%)	97%	(S)	-6.16 (*c* 0.70, $CHCl_3$)
$CHMO_{Arthro}$	94mg	35mg (35%)	98%	(S)	-3.71 (*c* 0.70, $CHCl_3$)
$CHMO_{Brachy}$	94mg	61mg (60%)	98%	(S)	-4.95 (*c* 1.21, $CHCl_3$)
$CHMO_{Brevi1}$	94mg	62mg (61%)	75%	(R)	+2.38 (*c* 1.51, $CHCl_3$)
$CHMO_{Brevi2}$	94mg	53mg (53%)	37%	(S)	-2.15 (*c* 1.30, $CHCl_3$)
$CPMO_{Coma}$	94mg	57mg (56%)	40%	(S)	-2.64 (*c* 1.14, $CHCl_3$)
$CHMO_{Rhodo1}$	94mg	45mg (45%)	98%	(S)	-3.74 (*c* 0.91, $CHCl_3$)
$CHMO_{Rhodo2}$	94mg	53mg (52%)	98%	(S)	-4.24 (*c* 0.75, $CHCl_3$)

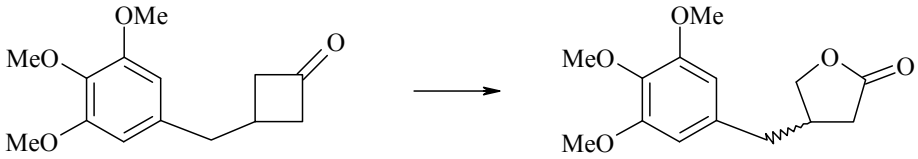
brown odorant oil

^{1}H-NMR ($CDCl_3$): δ 2.19 (dd, J_1=9Hz, J_2=17Hz, 1H, H-3), 2.46-2.80 (m, 4H, H-3, H-4, CHC<u>H</u>$_2$), 3.94 (dd, J_1=6Hz, J_2=9Hz, 1H, H-5), 4.25 (dd, J_1=6Hz, J_2=9Hz, 1H, H-5), 5.87 (s, 2H, OCH_2O), 6.50-6.56 (m, 2H, H-6`, H-2`), 6.68 (d, J=8Hz, 1H, H-5`);

^{13}C-NMR ($CDCl_3$): δ 31.7 (t, C-3), 34.9 (d), 36.2 (t, CH_2Ph), 70.1 (t, C-5), 98.6 (OCH_2O), 106.0 (d, C-2`, Ph), 106.5 (d, C-5`, Ph), 119.2 (d, C-6`, Ph), 129.5 (d, C-1`, Ph), 144.0 (s, C-4`, Ph), 145.6 (s, C-3`, Ph), 174.4 (s, C=O);

m/z: 220 (44, M^+), 136 (20), 135 (100), 105 (7), 77 (29), 51 (15).

6.5.12 Dihydro-4-(3,4,5-Trimethoxy-benzyl)-furan-2(3H)-one 14b



Method B: 3-(3,4,5-trimethoxy-benzyl)-cyclobutanone **<u>14a</u>** (65.0mg, 0.26mmol) was oxidized with *E.coli* expressing cells according to general procedure. The crude product was purified *via* column chromatography (silica gel, LP/EtOAc = 2/1) and the colorless crystals **14b** were isolated in the yields and properties specified below. [151] (GC: BGB 173, method D)

Enzyme	<u>14a</u>	14b	*ee*	abs. config.[151]	$[\alpha]^{D}_{20}$
$CHMO_{Acineto}$	50mg	48mg (90%	90%	(S)	-5.60 (c 0,96, $CHCl_3$)
$CHMO_{Arthro}$	65mg	50mg (72%)	94%	(S)	-6.10 (*c* 1.00, $CHCl_3$)
$CHMO_{Brachy}$	65mg	40mg (58%)	94%	(S)	-8.88 (*c* 0.80, $CHCl_3$)
$CHMO_{Brevi1}$	65mg	50mg (72%)	79%	(R)	+4.38 (*c* 1.00, $CHCl_3$)
$CHMO_{Brevi2}$	65mg	n.c.	n.a.	-	n.a.
$CPMO_{Coma}$	65mg	n.c.	n.a.	-	n.a.
$CHMO_{Rhodo1}$	65mg	46mg (67%)	95%	(S)	-4.75 (*c* 0.80, $CHCl_3$)
$CHMO_{Rhodo2}$	65mg	38mg (55%)	92%	(S)	-6.58 (*c* 0.76, $CHCl_3$)

[151] Tanaka, M.; Mitsuhashi, H.; Maruno, M.; Wakamatsu, T. *J. Org. Chem.* **1995**, 4339-4352.

MW: 266.3, $C_{14}H_{18}O_5$

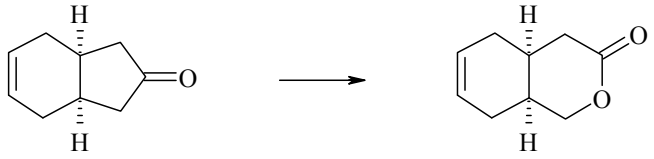
colorless crystals, m.p.: 94-96°C (Lit.[152] 98-99°C)

^{1}H-NMR ($CDCl_3$): δ 2.30 (dd, J_1=7Hz, J_2=17Hz, 1H, H-3), 2.55-2.95 (m, 4H), 3.83 (s, 3H, OCH_3), 3.85 (s, 6H, 2xOCH_3), 4.05 (dd, J_1=6Hz, J_2=9Hz, 1H, H-5), 4.36 (dd, J_1=7Hz, J_2=9Hz, 1H, H-5);

^{13}C-NMR ($CDCl_3$): δ 34.3 (t), 34.9 (d), 37.2 (d, C-4), 56.1 (2xq, OCH_3), 60.8 (q, OCH_3), 72.6 (t, C-5), 105.2 (2xd, Ph), 134.0 (s, Ph), 136.8 (s, Ph), 153.4 (s, Ph), 176.8 (s, C=O);

m/z: 266 (39, M^+), 182 (41), 181 (100), 167 (14), 151 (19).

6.5.13 Hexahydro-3H-2-benzopyran-3-one 15b



15a **15b**

Method B: Precursor **15a** was transformed according to the general biotransformation procedure. After purification by column chromatography (silica gel, LP/EtOAc = 10/1) the product **15b** was obtained as colorless oil. (GC: BGB 173, method B)

Enzyme	15a	15b	*ee*	abs. config.[95]	$[\alpha]^D_{20}$
$CHMO_{Acineto}$	106mg	40mg (33%)	5%	(4aS,8aS)	-0.70 (*c* 0.40, $CHCl_3$)
$CHMO_{Arthro}$	100mg	51mg (46%)	60%	(4aS,8aS)	-13.2 (*c*, 1.02, $CHCl_3$)
$CHMO_{Brachy}$	100mg	62mg (56%)	85%	(4aS,8aS)	-19.8 (*c* 1.24, $CHCl_3$)
$CHMO_{Brevi1}$	100mg	11mg (10%)	71%	(4aS,8aS)	n.d.
$CHMO_{Brevi2}$	184mg	103mg (92%)	94%	(4aR,8aS)	+21.0 (*c* 1.00, $CHCl_3$)
$CPMO_{Coma}$	100mg	85mg (76%)	>99%	(4aR,8aS)	+24.5 (*c* 1.00, $CHCl_3$)
$CHMO_{Rhodo1}$	100mg	52mg (47%)	73%	(4aS,8aS)	-19.7 (*c* 1.04, $CHCl_3$)
$CHMO_{Rhodo2}$	100mg	57mg (51%)	73%	(4aS,8aS)	-19.5 (*c*, 1.14 $CHCl_3$)

[152] Moritani, Y.; Fukushima, C.; Miyagishima, T.; Ohmizu, H.; Iwasaki, T. *Bull. Chem. Soc. Jpn*. **1996**, 69, *8*, 2281-2286.

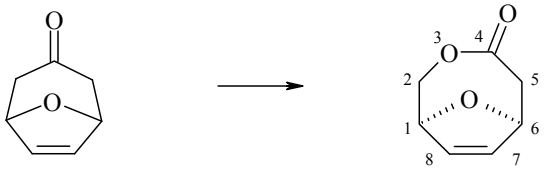
MW: 152.2, $C_9H_{12}O_2$

colorless oil (Lit.[95])

^{1}H-NMR ($CDCl_3$): δ 1.80-2.13 (m, 2H, H-5, H-8), 2.16-2.44 (m, 4H, H-4a, H-5, H-8, H-8a), 2.50-2.60 (m, 2H, H4), 4.22-4.40 (m, 2H, H-1), 5.67 (bs, 2H, H-6, H-7);

^{13}C-NMR ($CDCl_3$): δ 24.0 (t, C-8), 28.4 (t, C-5), 28.5 (d, C-4), 29.6 (d, C-8), 33.7 (t, C-4), 72.1 (t, C-1), 124.1 (d, C-7), 124.6 (d, C-6), 170.6 (s, C=O).

6.5.14 (1S,6S)-3,9-Dioxabicyclo[4.2.1]non-7-en-4-one 16b



16a **16b**

Method B: Precursor **16a** was transformed according to the general biotransformation procedure and isolated by extraction with dichloromethane (no saturation of the aqueous layer with sodium chloride). After purification *via* column chromatography (silica gel, LP/EtOAc = 1/1) **16b** was obtained as colorless crystals (m.p.: 98-100°C). (GC: BGB 175, method B)

Enzyme	16a	16b	*ee*	abs. config.	$[\alpha]^D_{20}$
$CHMO_{Acineto}$	100mg	n.c.	n.a.	-	n.a.
$CHMO_{Arthro}$	100mg	n.c.	n.a.	-	n.a.
$CHMO_{Brachy}$	100mg	n.c.	n.a.	-	n.a.
$CHMO_{Brevi1}$	100mg	n.c.	n.a.	-	n.a.
$CHMO_{Brevi2}$	119mg	26mg (19%)[a]	93%	(1S,6S)	+82.0 (*c* 0.70, $CHCl_3$)
$CPMO_{Coma}$	125mg	75mg (53%)	95%	(1S,6S)	+94.0 (*c* 0.92, $CHCl_3$)
$CHMO_{Rhodo1}$	100mg	n.c.	n.a.	-	n.a.
$CHMO_{Rhodo2}$	100mg	n.c.	n.a.	-	n.a.

[a]Biotransformation ceased after 36 hours and did not reach completion

MW: 140.1, $C_7H_8O_3$

colorless crystals, m.p.: 98-100°C

Calc.: C 60.00%, H 5.75%
Found: C 59.70% H 5.83%

^{1}H-NMR: ($CDCl_3$) δ 2.90 (dd, J_1=5Hz, J_2=16Hz, 1H, H-2), 3.20 (dd, J_1=3Hz, J_2=16Hz, 1H, H-2), 4.05 (dd, J_1=3Hz, J_2=12Hz, 1H, H-5), 4.40 (d, J=12Hz, 1H, H-5), 4.70 (d, J=3Hz, 1H, H-1), 4.85 (d, J=3Hz, 1H, H-6), 6.10 (d, J=6Hz, 1H, H-8), 6.35 (d, J=6Hz, 1H, H-7)

^{13}C-NMR ($CDCl_3$): δ 46.7 (t, C-5), 71.0 (t, C-2), 76.3 (d, C-6), 81.6 (d,C-1), 129.0 (d,C-8), 133.4 (d, C-7), 172.0 (s, C-4);

m/z: 140 (0.6, M^+), 110 (15), 98 (15), 81 (31), 68 (100).

6.6 Fermentation "Up-scaleing"

6.6.1 General Procedures

Fresh plates were streaked weekly from glycerol stocks on solid LB medium supplemented with ampicillin. A value of 0.43g/L dcw per OD_{590} unit was used to calculate biomass concentrations from optical density measurements. A New Brunswick Bioflow 110 fermenter equipped with pH probe, oxygen probe, flow controller and temperature control was used. Monitoring of all fermentation parameters was performed using the Biocommand Plus 3.30 software by New Brunswick. Glucose concentrations were determined with *Roche Accu Chek-go*. Experiments below were determined for compounds **16a, 20a, 21a, 34a (≡ S)**

Evaluation of GC Coeffizient: Due to correct GC analysis, for each compound the GC coeffizient has to be calculated. Therefore, 500µL of EtOAc solution containing a known amount of internal STD (e.g. methyl benzoate or undecan) and 500µL of EtOAc solution with a defined concentration of compounds **S** were mixed and measured by GC or GC/MS. The coeffizeint value is calculated by following equation.

$$K = A_{ref} / A * [c]/[c_{ref}]$$

K	GC coeffizent (relation of compound **S** and the internal STD)
A_{ref}	GC area of internal STD
A	GC area of compound **S**
[c]	concentration of compound **S** in water
$[c_{ref}]$	concentration of internal STD in EtOAc

Distribution of Compounds S between Water and Organic Phase: For following GC experiments it is mandatory to measure the distribution of compound **S** dissolved in water after one extraction step with the desired solvent. A known amount of compound **S** was dissolved in 1mL water and extracted with 500µL of EtOAc solution containing internal STD. The amount of compound **S** in the organic phase was determined by the following equations. If D~1, nearly complete extraction was determined.

$$m = K* A/A_{ref} *m_{ref}$$

m	amount of compound **S** in organic phase
K	GC coeffizent (relation of compound **S** and the internal STD)
A	GC area of compound **S**
A_{ref}	GC area of internal STD
m_{ref}	amount of internal STD

$$D = m_{org}/m_{aq}$$

D	distribution of compound **S** between organic and aqueous phase
m_{org}	amount of compound **S** in organic phase
m_{aq}	amount of compound **S** in aqueous phase

Water Solubility Tests: A test tube (Eppendorf tube, 1.5mL) filled with 500µL of water was saturated with organic compound **S** by vortexing for 5 min. Then, 200µl of the aqueous phase were removed by syringe upon puncturing the test tube without touching the organic layer or compound. The aqueous phase was extracted with the same amount of internal standard solution (methyl benzoate or undecan) and was determined by GC. The solubility of compound **S** in water was calculated by following equation:

$$[c_{aq}] = K*A / A_{ref}*[c_{ref}]$$

$[c_{aq}]$	concentration of compound **S** in water
K	GC coeffizent (relation of compound **S** and the internal STD)
A	GC area of compound **S**
A_{ref}	GC area of internal STD
$[c_{ref}]$	concentration of internal STD solution

GC Sample Preparation: Biotransformation samples (aqueous phase and resin) were sampled for GC/MS analysis by mixing 500µL of aqueous reaction mixture or the resin with 500µL dichloromethane containing 3mM methyl benzoate (internal standard). After vortex mixing the organic layer was analyzed by GC or GC/MS. The concentration of compound **S** was determined by following equation.

$$[c_s] = K*A / A_{ref}*[c_{ref}]$$

$[c_s]$	concentration of compound **S**
K	GC coeffizent (relation of compound **S** and the internal STD)
A	GC area of compound **S**
A_{ref}	GC area of internal STD
$[c_{ref}]$	concentration of internal STD solution

Activity Tests: These tests were performed with a model reaction using the highly reactive ketone bicyclo[3.2.0]hept-2-en-6-one **25a** known from other BVMO biotransformations in a shake flask experiment. A standard solution (100g/L) of bicyclo[3.2.0]hept-2-en-6-one **25a** in ethanol was added to the growing culture (substrate concentration of 0.5g/L). Complete conversion after 1 hour has to be achieved for cells with a high biooxidation activity.

Kinetic Studies: Fresh LB_{amp} medium (10mL) was inoculated with 1% (100µL) of an overnight preculture of recombinant *E. coli* strains in a baffled Erlenmeyer flask. The culture was incubated at 120 rpm at 37°C on an orbital shaker for 3 hours, then 2µl IPTG and the substrate (3-50mM) was added. The culture was incubated for 24 hours at 120 rpm at 25°C and GC samples were taken periodically from all experiments.

Resin Tests: The resin tests were performed in glass tubes containing 5mL of LB_{amp} media by shaking (50rpm) at room temperature. Therefore, 10 samples were prepared with a known amount of resin and substrate starting from a theoretical X^{eq} of 0.1 up to 1.5. The tubes were shaken (50rpm) for 24 hours at room temperature and the aqueous phase was determined by GC (procedure as described above). X^{eq} was calculated by following equation.

$$X = (m_{s\ total} - m_{s\ in\ H20}) / m_{wet\ resin} * F$$

X	load of compound S on the resin
$m_{s\ total}$	total amount of substrate
$m_{s\ in\ H2O}$	amount of substrate in water
$m_{wet\ resin}$	amount wet resin
F	factor of dry resin ($F = m_{resin\ dry} / m_{resin\ wet}$)

6.6.2 Method A: Standard procedure for the Baeyer-Villiger oxidation under "growing" conditions

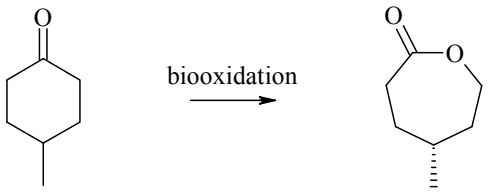
Procedure: A New Brunswick Bioflow 110 fermenter containing 1L of sterile TB medium supplemented with ampicillin (200mg/L) was inoculated with overnight culture (20mL, 2vol%) of DH5α/CPMO grown on LB medium (50mg/mL ampicillin). The 1L culture was grown at 37°C with air flow of 1L min^{-1} and stirring rates at 500rpm. A pH of 7.00±0.05 was kept constant by adding 3N NaOH or 3N H_3PO_4 automatically. When the culture density reached approx. 0.43g/L dcw the temperature was decreased to 25°C and IPTG was added to a final concentration of 0.25mM. Finally, ketone was added to the fermentation culture. The oxygen saturation was maintained by adjusting the stirring rate (250-400rpm). Levels of starting material and corresponding lactone were determined periodically by GC/MS.

6.6.3 Method B: Standard procedure for the Baeyer-Villiger oxidation under "non-growing" conditions

Procedure: A New Brunswick Bioflow 110 fermenter containing 1L of sterile TB medium supplemented with ampicillin (200mg/L) was inoculated with 20mL (2vol%) overnight culture of DH5α/CPMO grown on LB medium (50mg/mL ampicillin). The temperature was maintained at 37°C and the pH was kept constant at 7.00±0.05 by adding 3N NaOH or 3N H_3PO_4 automatically. The 1L culture was grown with an air flow of 5L min^{-1} and stirring rates at 500rpm.

The growth was continued until the culture density reached 3.01-3.44g/L dcw and the temperature was then decreased to 25°C. IPTG was added to a final concentration of 0.25mM and after an additional hour the fermentation culture was supplemented with 4g/L glucose (20% sterile solution). Two hours after induction a sample of the cell culture (20mL) was taken and activity tests were performed. The resin was suspended in sterile TB_{Amp} media (10g resin in 30mL TB-media) and was shaken (rt, 100rpm) for 20 minutes. After passing the activity tests the pre-loaded resin and any additives were added. The glucose level was measured periodically as the bioconversion progressed and the glucose adjustment (4g/L) was performed by addition of glucose solution (20%). Determination of glucose-concentration was performed by taking the crude fermentation broth and analizes it with *Roche Accu Chek-go*.

6.6.4 Biooxidation of 4-Methylcyclohexanone 20a



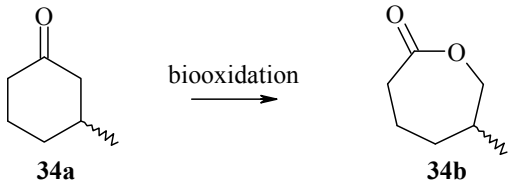
20a **20b**

Procedure I: In a New Brunswick Bioflow 110 fermenter containing 1L of sterile TB medium supplemented with ampicillin (200mg/L) cell were grown according to general method A. Finally, pure 4-methylcyclohexanone **20a** (3.36g, 30.0mM) was added to the fermentation culture. The oxygen saturation was maintained by adjusting the stirring rate (250-400rpm). Levels of 4-methylcyclohexanone **20a** and corresponding lactone **20b** were determined periodically by GC/MS (calculated yield: 84.0%, 44.0%[43] ee).

Procedure II: Cells were grown in a 1L bench top fermenter (New Brunswick Bioflow 110) according to the general method B. Pre-loaded resin (4-methylcyclohexanone **20a**, 15.0g, 133mmol / 75.0g wet Lewatit VPOC 1163 in 100mL LB_{amp} medium) adjusted to load X^{eq} = 0.40, was added to the fermentation culture. Due to differences in the water content of the resin depending on storage-time and resin batch, X^{eq} is calculated from resin dry weight otherwise reproducible results cannot be achieved. To determine the water content, the resin was dried in an oven and the loss in weight was measured.

All process conditions were maintained at the same values as those described in the general method B. GC samples were taken periodically. After nearly complete conversion (86%) or significant decrease in the activity of CPMO (20 hours) the resin was filtered off and extracted with EtOAc continuously overnight. The fermentation broth was centrifuged (4000rpm, 4°C, 10min), filtered through a bed of Celite and the aqueous solution was extracted in the same manner. The required lactone **20b** was obtained in good purity (>90% according to GC) and yield (overall yield: 80.0%, 11.7g, 106mmol, 44.0%[43] ee).

6.6.5 Biooxidation of rac-3-Methylcyclohexanone 34a



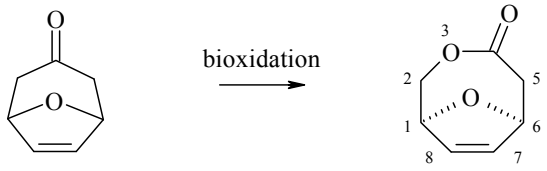
34a **34b**

Procedure I: In a New Brunswick Bioflow 110 fermenter containing 1L of sterile TB medium supplemented with ampicillin (200mg/L) cells were grown according to general method A. Exceptionally, the addition of IPTG up to a concentration of 0.25mM was performed at a cell densitiy of 0.95g/L dcw. Finally, *rac*-3-methylcyclohexanone **34a** (10.0g, 89.2mM) pre-loaded on Optipore L-493 (50g wet resin, load X^{eq} = 0.4) was added to the fermentation culture. The oxygen saturation was maintained by adjusting the stirring rate (250-400rpm). Levels of *rac*-3-methylcyclohexanone **34a** and corresponding lactone **34b** were determined periodically by GC. After 24 hours the resin was filtered off and was extracted continuously with EtOAc overnight. The fermentation broth was centrifuged (4000rpm, 4°C, 10min) and the biomass was filtered through a bed of Celite. The aqueous solution was extracted continuously with EtOAc overnight. The desired lactone **34b** was obtained in good purity (>92% according to GC) and yield (isolated yield: 6.79g, 53.0mmol, 59%, *rac*[112]).

Procedure II: In a 4L bench top fermenter (New Brunswick Bioflow 110) containing 4L of sterile TB medium supplemented with ampicillin (200mg/L) cells were grown according to general method B. Due to the high activity of obtained cells 1L was used for the subsequent transformation and the remaining broth was stored at 4°C for further usage. *Rac*-3-methylcyclohexanoe **34a** (10.0g, 89.2mmol) was pre-loaded on Optipore L-453 (50.0g wet resin; load X^{eq} = 0.4) and was added to 1L of active biocatalyst. After 27 hours of fermentation time only about 30% conversion was determined. The resin was filtered off and was re-suspended in a new solution of biocatalyst. After additional 7 hours of reaction time with a second cell cycle about 90% conversion was determined by GC (90% of **34b** *rac*[112]).

Procedure III: All process conditions (growth temperature, pH value, agitation, aeration, and glucose level) were maintained as described in method B. *Rac*-3-methylcyclohexanone **34a** (15.0g, 133mmol) was pre-loaded on Lewatit VPOC 1163 (75.0g wet resin; load X^{eq} = 0.4). After 16 hours of fermentation time nearly complete (14.7g, 102mmol, 90% of **34b**, *rac*[112]) conversion was determined. The product isolation was performed as described above.

6.6.6 Biooxidation of 8-Oxabicyclo-[3.2.1]oct-6-en-3-one 16a



16a **16b**

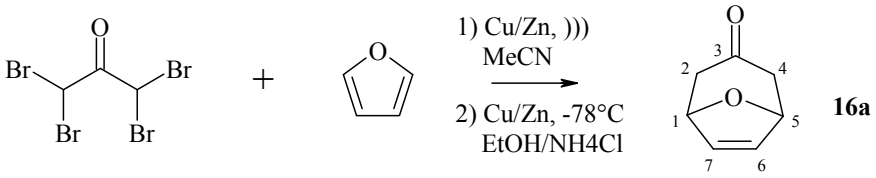
Procedure I: According to the general procedure for "non-rowing" cells (method A) 8-oxabicyclo[3.2.1]oct-6-en-3-one **16a** was biooxidized to the corresponding lactone **16b**. Ketone **16a** (0.70g, 5.60mmol) dissolved in ethanol (10ml) and β-cyclodextrin (636mg, 10mol%) were subsequently added to cells. After 24 hours of fermentation time complete conversion was determined. The fermentation broth was centrifuged (10min, 4000rpm, 4°C), passed through a bed of Celite and was extracted with dichloromethane (3 times, 300mL). The organic layer was dried with sodium sulfate and the solvent was evaporated. Purification by column chromatography (LP/EtOAc = 2/1; 50g SiO_2) gave the desired lactone **16b** in 81% (635mg, 4.54mmol) yield and 95% optical purity.

Procedure II: All process conditions- growing temperature, pH value, agitation, aeration, and glucose level were maintained as described in method B (reaction was performed in a New Brunswick Bioflow 110 fermenter). Ketone **16a** (2.00g, 16.1mmol) dissolved in ethanol (10ml) and β-cyclodextin (1.83g, 10mol%) were subsequently added to active cells. After 24 hours of fermentation time complete conversion was determined. The fermentation broth was centrifuged (10min, 4000rpm, 4°C), passed through a bed of Celite and was extracted continuously with dichloromethane overnight. The organic layer was dried with sodium sulfate and the solvent was evaporated. Purification by column chromatography (LP/EtOAc = 2/1; 100g SiO_2) gave the desired lactone **16b** in 72% (1.63g, 11.6mmol) yield and 95% optical purity.

Procedure III: According to the general procedure for "non–growing" cells (method B) 8-oxabicyclo[3.2.1]oct-6-en-3-one **16a** was biooxidized to the corresponding lactone. Ketone **16a** (5.00g, 40.0mmol) was dissolved in ethanol (10ml) and was subsequently added to the resin (50.0g wet resin, load X^{eq} = 0.2) and 100mL of LB_{amp} β-cyclodextrin (4.58g, 10mol%) and the substrate-resin mixture were added to the fermentation broth. After 36 hours or complete conversion the resin was filtered off and was extracted with dichloromethane continuously overnight. The fermentation broth was centrifuged (10min, 4000rpm, 4°C), passed through a bed of Celite and was extracted with dichloromethane continuously overnight. Both extracts were combined, dried with sodium sulfate and the solvent was evaporated. Purification by column chromatography (LP/EtOAc = 2/1; 200g SiO_2) gave the desired lactone **16b** in 79% (4.42g, 31.6mmol) yield and 95% optical purity.

6.7 Formal Total Synthesis of Showdomycin

6.7.1 8-Oxabicyclo-[3.2.1]oct-6-en-3-one 16a



16a

Procedure: 1) Cu/Zn couple (20.6g; 0.31mol), furan (100ml, 1.39mol) and catalytic amounts of dibromoethane were suspended in dry acetonitrile (80mL). The reaction mixture was cooled to 10°C, set under N_2-atmosphere and sonificated (Bandelin Sonoplus Ultrasonic Homogenizer, HD 2200) for 30min under subsequent addition of tetrabromoacetone (38.2g; 1.02mol) dissolved in dry acetonitrile (20mL). The temperature has to be maintained below 25°C. The conversion was monitored by GC/MS. After complete conversion the reaction mixture was filtered through a bed of Celite. The crude reaction mixture was used without further work up or purification for the next reaction step.

2) Cu/Zn couple (47.3g; 0.72mol) and ammonium chloride (26.0g; 0.49mol) were suspended in dry ethanol and cooled to -78°C. Crude 1,5-dibromo-8-oxabicyclo-[3.2.1]oct-6-en-3-one (80% of the solution) in acetonitrile was added slowly and the temperature was maintained at -50°C. After 15min the remaining 20% of the solution were added and the reaction mixture was warmed to rt. Reaction monitoring was performed by GC/MS or TLC. After complete conversion, the Cu/Zn couple was filtered off, washed with dichloromethane and all volatiles were removed in *vacuo*. The crude product was cooled with an ice bath during neutralization with saturated bicarbonate. The resulting suspension was filtered again and washed extensively with dichloromethane. After separation, the combined organic layers were dried over sodium sulfate and evaporated (bath temp. below 30°C!).

The purity of the crude product was controlled by NMR-spectroscopy. After evaporating of all volatiles and drying in vacuum 72% of brown crystals of 8-oxabicyclo-[3.2.1]oct-6-en-3-one **16a** (9.11g; 0.74mmol) were isolated.

Yield: 9.11g (72%)

MW: 124.1, $C_7H_8O_2$

colorless crystals, m.p.: 36-38°C (Lit.[153] 38°C)

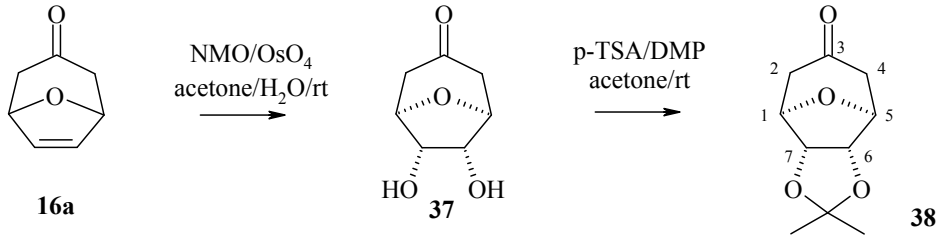
^{1}H-NMR ($CDCl_3$): δ 2.30 (d, J=16Hz, 2H, H-2eq/H-4eq), 2.80 (dd, J=16Hz, J=5Hz, 2H, H-2ax/H4-ax), 5.05 (d, J=5Hz, 2H, H-1/5,), 6.20 (s, 2H, H-6/7);

^{13}C-NMR: ($CDCl_3$): δ 46.6 (t, C-2/4), 77.1 (d, C-1/5), 133.3 (d, C-6/7), 205.2 (s, C-3);

m/z: 124 (48, M^+), 95 (8), 82 (81), 81 (100), 54 (33), 53 (33).

[153] Hoffmann, H.M.R.; Kim, H. *Eur. J. Org. Chem*. **2000**, 2195-2201.

6.7.2 (1R*, 5S*, 6S*, 7R*)-6,7-Isopropylidenedioxy-8-oxabicyclo[3.2.1]octan-3-one 38



16a **37** **38**

Procedure: Dihydroxylation of 8-oxabicyclo[3.2.1]oct-6-en-3-one **16a** was performed by OsO_4 in the presence of N-methylmorpholine oxide. Ketone (50mg, 0.40mmol) and NMO*H_2O (58.0mg, 0.43mmol, 1.2equiv.) were dissolved in acetone (4mL) and H_2O (1mL). Subsequently, catalytic amounts (5mg) of OsO_4 were added and the reaction was stirred at rt until complete conversion was observed (TLC control; LP/EtOAc = 1/1). The crude reaction mixture was diluted with saturated $NaHSO_3$ solution and stirred for 15 minutes. The aqueous phase was extracted with EtOAc and the combined organic layers were dried over sodium sulfate and concentrated *in vacuo*. The crude diol **37** was used without further work-up or purification in the following protection step.

^{1}H NMR (DMSO): δ 2.25 (d, J=16Hz, 1H), 2.61 (dd, J_1=6Hz, J_2=16Hz, 1H), 3.81-3.93 (m, 2H), 4.29 (d, J=6Hz, 2H), 4.94-5.06 (m, 2H);

^{13}C-NMR (DMSO): δ 46.0 (t), 74.0 (d), 81.6 (d), 205.7 (s, C=O);

The reaction mixture and catalytic amounts of p-TSA was suspended in a mixture of dry acetone (1mL) and DMP (4mL), and set under N_2-atmosphere and was stirred until complete conversion of the diol was observed (TLC control, LP/EtOAc = 1/1). Hydrolysis was performed with saturated bicarbonate solution and the aqueous phase was extracted 4 times with EtOAc. The combined organic layers were dried over sodium sulfate and the volatiles were evaporated.

After purification by column chromatography (basic silicagel, LP/EtOAc = 1/1) 60% (47mg, colorless crystals) of compound **36** were isolated.

Yield: 47.0mg (60%)

MW: 198.2, $C_{10}H_{14}O_4$

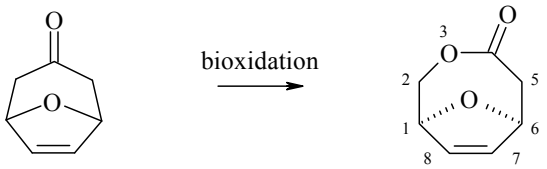
colorless crystals, m.p.: 117-120°C (Lit.[121] 117-119°C)

^{1}H NMR ($CDCl_3$): δ 1.28 (s, 3H, CH_3), 1.50 (s, 3H, CH_3), 2.29 (d, J=2Hz, 2H), 2.66 (dd, J_1=6Hz, J_2=16Hz, 2H), 4.15 (s, 2H), 4.53-4.61 (m, 2H);

^{1}H-NMR data are according to the literature.[121]

^{13}C-NMR ($CDCl_3$): δ 24.5 (q, CH_3), 25.9 (q, CH_3), 45.8 (t, C-2, C-4), 79.7 (d), 83.4 (d), 112.1 (s), 204.8 (s, C-3).

6.7.3 (1S,6S)-3,9-Dioxabicyclo[4.2.1]non-7-en-4-one 16b

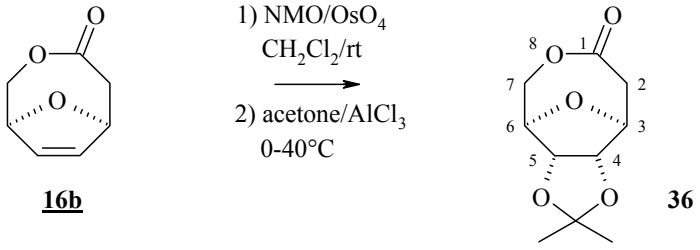


16a **16b**

Procedure: see section 6.6.5. procedure III

Yield: 3.90g (70%)
MW: 140.1, $C_7H_8O_3$

6.7.4 3,6-Anhydro-2-deoxy-4,5-O-(1-methylethylidene)-D-allo-heptonic Acid ζ-Lactone 36



16b **36**

Procedure: Dihydroxylation of (1S,6S)-3,9-dioxabicyclo[4.2.1]non-7-en-4-one **16b** was performed by OsO_4 in the presence of N-methylmorpholine oxide. Lactone (50mg, 0.36mmol) and NMO*H_2O (58.0mg, 0.43mmol, 1.2equiv.) were dissolved in dry dichloromethane (5.00mL). Subsequently, catalytic amounts (5mg) of OsO_4 were added and the reaction was stirred at rt until complete conversion was observed (TLC control; LP/EtOAc = 1/3). The crude reaction mixture was concentrated *in vacuo* (20°C bath temperature!), dissolved again with dichloromethane and concentrated at reduced pressure (4 times, 10mL dichloromethane). This procedure was performed to dry the crude diol and avoid problems in the next reaction step.

The crude diol was used without further work-up or purification in the following protection step. The reaction mixture was suspended in dry acetone (10mL), cooled to 0°C and set

under N_2-atmosphere. Freshly sublimed $AlCl_3$ (101mg, 0.76mmol, 2.1equiv.) was dissolved in dry, cooled diethylether (2.0mL) and was added slowly to the reaction mixture.
After complete addition of $AlCl_3$ the reaction mixture was heated to 40°C until complete conversion of the diol was observed (TLC control, LP/EtOAc = 1/1). Hydrolysis was performed with saturated and cooled bicarbonate solution and the aqueous phase was extracted 4 times with EtOAc. The combined organic layers were dried over sodium sulfate and the volatiles were evaporated.

After purification by column chromatography (basic silica gel, LP/EtOAc = 3/1) 48% (37mg, colorless crystals) of compound **36** were isolated.

Yield: 37.0mg (48%)
MW: 214.2, $C_{10}H_{14}O_5$

colorless crystals, m.p.: 163-167°C (Lit.[154] 167-168.5°C)

$[\alpha]_D^{20}$: +73.0 (c 0.66, $CHCl_3$) (Lit.[154] +82.6, c 0.6, $CHCl_3$)

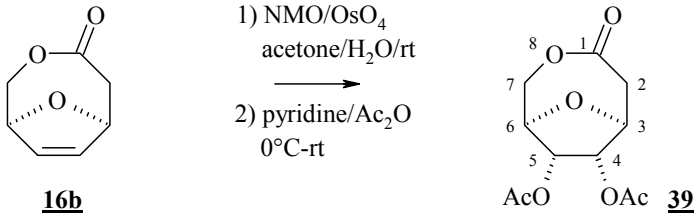
^{1}H NMR ($CDCl_3$): δ 1.32 (s, 3H, CH_3), 1.49 (s, 3H, CH_3), 2.96 (d, J=4Hz, 2H, H-2), 4.23-4.30 (m, 3H), 4.31-4.39 (m, 1H), 4.65 (d, J=5Hz, 2H), 4.95 (d, J=5Hz, 2H);
^{1}H-NMR data are according to the literature.[140]

^{13}C-NMR ($CDCl_3$): δ 24.3 (q, CH_3), 25.9 (q, CH_3), 42.5 (t, C-2), 71.5 (t, C-7), 78.3 (d), 81.5 (d), 82.4 (d), 83.5 (d), 112.4 (s), 172.2 (s, C-1);

m/z: 199 (100), 157 (19), 85 (23), 69 (21), 68 (17), 59 (26), 57 (15).

[154] Cruickshank, K.A.; Reese, C.B. *Synthesis* **1983**; *3*; 199-201.

6.7.5 3,6-Anhydro-2-deoxy-4,5-O-(bisacetyl)-D-allo-heptonic Acid ζ-Lactone 39



16b 39

Procedure: Dihydroxylation of (1S,6S)-3,9-dioxabicyclo[4.2.1]non-7-en-4-one **16b** was performed by OsO_4 in the presence of N-methylmorpholine oxide. Lactone (50mg, 0.36mmol) and NMO*H_2O (58.0mg, 0.43mmol, 1.2equiv.) were dissolved in dry dichloromethane (5.00mL). Subsequently, catalytic amounts (5mg) of OsO_4 were added and the reaction was stirred at rt until complete conversion was observed (TLC control; LP/EtOAc = 1/3). The crude reaction mixture was concentrated *in vacuo*. The crude diol **35** was used without further work-up or purification in the following protection step.

The reaction mixture was dissolved in dry pyridine (2mL) and cooled to 0°C. After addition of acetic anhydride (2mL) the reaction mixture was warmed to rt and stirring was continued until complete conversion of the diol was observed (TLC control, LP/EtOAc = 1/2). Hydrolysis was performed with saturated bicarbonate solution and the aqueous phase was extracted 4 times with EtOAc. The combined organic layers were dried over sodium sulfate and the volatiles were evaporated and crude **39** was isolated in 44% yield.

Yield: 40.9mg (44%)

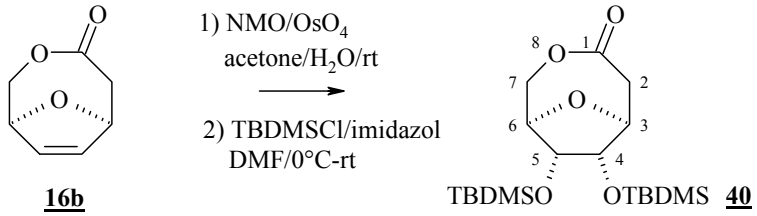
MW: 258.2, $C_{11}H_{14}O_7$

black oil

^{1}H NMR ($CDCl_3$): δ 2.03 (s, 3H, CH_3), 2.04 (s, 3H, CH_3), 2.96 (dd, J_1=2Hz, J_2=17Hz, 1H), 3.12 (dd, J_1=5Hz, J_2=17Hz, 1H), 4.22-4.46 (m, 4H), 5.10 (d, J=7Hz, 1H), 5.50 (d, J=7Hz, 1H);

^{13}C-NMR ($CDCl_3$): δ 20.0 (2xq, CH_3), 42.7 (t, C-2), 71.0 (d), 73.4 (d), 75.9 (d), 78.3 (d) 83.0 (d), 169.8 (s), 169.9 (s), 171.4 (s, C-1).

6.7.6 3,6-Anhydro-2-deoxy-4,5-O-[bis(*tert*-butyldimethylsilyl)]-D-allo-heptonic Acid ζ-Lactone 40



16b **40**

Procedure: Dihydroxylation of (1S,6S)-3,9-dioxabicyclo[4.2.1]non-7-en-4-one **16b** was performed by OsO_4 in the presence of N-methylmorpholine oxide. Lactone (50mg, 0.36mmol) and NMO*H_2O (58.0mg, 0.43mmol, 1.2equiv.) were dissolved in dry dichloromethane (5.00mL). Subsequently, catalytic amounts (5mg) of OsO_4 were added and the reaction was stirred at rt until complete conversion was observed (TLC control; LP/EtOAc = 1/3). The crude reaction mixture was concentrated *in vacuo*. The crude diol **35** was used without further work-up or purification in the following protection step.

The reaction mixture was dissolved in dry DMF (2mL) and cooled to 0°C. After addition of imidazol (122.6mg, 1.8mmol, 5eqiv.) the reaction mixture was stirred for 30 min and then TBDMSCl (130.2mg, 0.86mmol, 2.4 equiv.) was added. The reaction mixture was stirred until complete conversion of the diol was observed (TLC control, LP/EtOAc = 7/1). Hydrolysis was performed with saturated bicarbonate solution and the aqueous phase was extracted 4 times with EtOAc. The combined organic layers were washed with water (4 times) and dried over sodium sulfate and the volatiles were evaporated and **40** was isolated in 50% yield after column chromatographyas (silica gel, LP/EtOAc = 10/1) as colorless oil.

Yield: 72.5mg (50%)

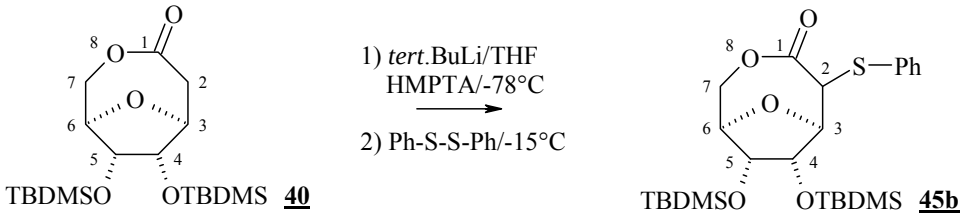
MW: 402.68, $C_{19}H_{38}O_5Si_2$

colorless oil

^{1}H NMR ($CDCl_3$): δ 0.00 (s, 12H, 4xCH_3-Si), 0.81 (s, 18H, 9xCH_3), 2.82-2.89 (m, 2H), 3.98-4.28 (m, 5H), 4.43 (d, J=6Hz, 1H),

^{13}C-NMR ($CDCl_3$): δ -4.4 (4xq, CH_3-Si), 18.2 (s), 18.4 (s), 26.0 (6xq, CH_3), 43.2 (t, C-2), 71.5 (d), 74.1 (d), 77.2 (d), 81.5 (d) 86.2 (d), 172.6 (s, C-1).

6.7.7 3,6-Anhydro-2-S-phenyl-4,5-O-(tert-butyldimethylsilyl)-D-allo-heptonic Acid ζ-Lactone 45b



Procedure: *tert.*-Butyllithium in pentane (0.97 M) was injected dropwise into a stirred, cold (-78 °C) solution of triphenylmethane (1 mg) and hexamethylphosphoric triamide (0.3 mL) in 10 mL of dry tetrahydrofuran under Ar until the solution was permanently red (0.01-0.02 mL)**.** Another 280 µL of *tert.*-butyllithium solution (0.28mmol, 1.1 equiv.) was added, and the mixture was stirred at -78 °C for 10 min. Lactone **40** (100mg, 0.25mmol) dissolved in dry THF was added dropwise and the reaction mixture was stirred for 15min, followed by addition of diphenyldisulfide (55.0mg, 0.25mmol, 1equiv.) dissolved in THF. After 20 min of stirring at at -15°C the described procedure was repeated twice. Finally, the reaction mixture was quenched with acetic acid and was stirred at 0°C for 5 minutes. All volatiles were removed and the crude reaction mixture was purified *via* coloumn chromatography. The desired product **45b** was obtained in traces only as a pale yellow oil (7%, silica gel, LP/EtOAc = 10/1).

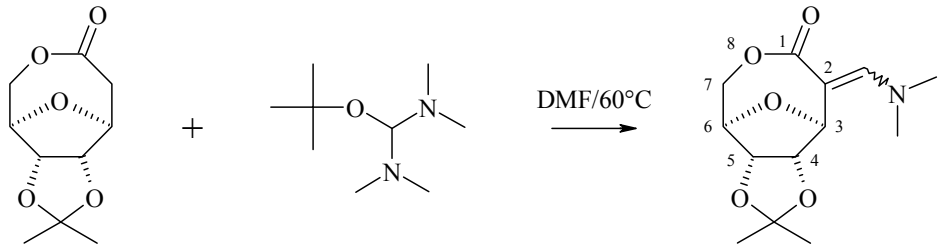
Yield: 8.90mg (7%)

MW: 510.85, $C_{25}H_{42}O_5SSi_2$

pale yellow oil

^{1}H NMR ($CDCl_3$): δ -0.05-0.06 (m, 12H, 4xCH_3-Si), 0.73-0.92 (bs, 18H, 9xCH_3), 3.99-4.21 (m, 3H), 4.25-4.34 (m, 2H), 4.43 (d, J=6Hz, 1H), 4.87 (d, J=13Hz, 1H), 7.20-7.29 (m, 3H, Ph), 7.37-7.48 (m, 2H, Ph).

6.7.8 (E,Z)-3,6-Anhydro-2-deoxy-2-[(dimethylamino) methylene]-4,5-O-(1-methylethylidene)-D-allo-heptonic Acid ζ-Lactone 46



36 **46**

Procedure: Acetonide **36** (100mg, 0.47mmol) was dissolved in dry DMF (1mL) and bis-(dimethylamino)-*tert.*-butoxymethane (1mL) was added dropwise. The reaction mixture was stirred at 60°C under N_2-atmosphere until complete conversion was observed (TLC control, 3-6hours). All volatiles were removed at reduced pressure and the crude reaction mixture was purified by flash chromatography (basic silica gel, LP/EtOAc = 1/1). The desired enaminone **48** was obtained in 89% yield (113mg, colorless oil) with a ratio of E/Z of 2/1.

Yield: 113mg (89%) (Lit.[121] 89%)
MW: 269.3, $C_{13}H_{19}NO_5$

colorless oil

E-isomer (analyzed in mixture with the Z-isomer)**:**
^{1}H NMR ($CDCl_3$): δ 1.32 (s, 3H, CH_3), 1.51 (s, 3H, CH_3), 3.14 (s, 6H, N-$(CH_3)_2$), 4.13-4.16 (m, 2H, H-7), 4.33 (s, 1H, H-6), 4.58 (d, J=6Hz, 1H, H-4), 4.90-4.93 (m, 1H, H-5), 5.05 (s, 1H, H-3), 7.34 (s, C=C<u>H</u>-N);

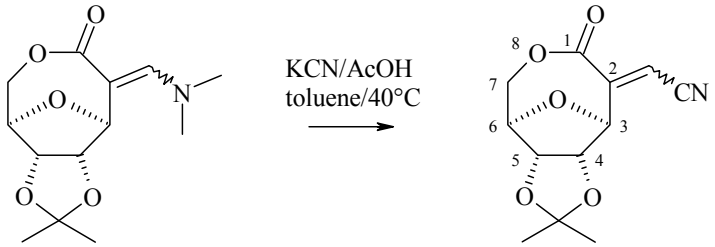
^{13}C-NMR ($CDCl_3$): δ 24.3 (q, CH_3), 26.0 (q, CH_3), 44.0 (q, N$(CH_3)_2$), 72.1 (t, C-7), 79.6 (d, C-3), 81.6 (d, C-6), 82.5 (d, C-5), 86.3 (d, C-4), 95.8 (s, C2), 111.7 (s, <u>C</u>$(CH_3)_2$), 153.5 (s, C=<u>C</u>-N$(CH_3)_2$), 174.4 (s, C-1);

Z-isomer:
^{1}H NMR ($CDCl_3$): δ 1.32 (s, 3H, CH_3), 1.50 (s, 3H, CH_3), 2.94 (s, 6H, N-$(CH_3)_2$), 4.18-4.23 (m, 2H, H-7), 4.28 (s, 1H, H-6), 4.37 (s, 1H, H-3), 4.66 (d, J=6Hz, 1H, H-4), 4.90-4.93 (m, 1H, H-5), 6.70 (s, C=C<u>H</u>-N);

^{13}C-NMR ($CDCl_3$): δ 24.3 (q, CH_3), 25.9 (q, CH_3), 44.2 (q, N$(CH_3)_2$), 71.9 (t, C-7), 81.1 (d, C-6), 82.3 (d, C-5), 85.7 (d, C-4), 87.9 (d, C-3), 95.0 (s, C-2), 111.6 (s, <u>C</u>$(CH_3)_2$), 153.8 (s, C=<u>C</u>-N$(CH_3)_2$), 168.7 (s, C-1);

m/z: 269 (36, M^+), 142 (100), 128 (58), 126 (51), 124 (23), 98 (56), 97 (33), 82 (59).

6.7.9 (E,Z)-3,6-anhydro-2-deoxy-2-[cyanomethylene]-4,5-O-(1-methylethylidene) -D-allo-heptonic Acid ζ-Lactone 47



46 **47**

Procedure: Enaminone **46** (160mg, 0.59mmol) was dissolved in dry toluene and glacial acid (1/1, 2mL). After addition of potassium cyanide (30mg) the reaction mixture was warmed to 40°C until complete conversion (after 3hours) was detected by TLC.

The reaction mixture was diluted with dichloromethane and cooled with an ice bath during neutralization with saturated bicarbonate. The organic layer was dried over sodium sulfate and all volatiles were removed at reduced pressure. The desired compound **47** was obtained in 77% yield (115mg, colorless oil) in an E to Z ratio of 3/1 without further purification (GC-MS purity >95%).

Yield: 115mg (77%)

MW: 251.2, $C_{12}H_{13}NO_5$

colorless oil

E-isomer (analyzed in mixture with the Z-isomer)**:**

^{1}H NMR ($CDCl_3$): δ 1.35 (s, 3H, CH_3), 1.54 (s, 3H, CH_3), 4.30-4.33 (m, 2H, H-7), 4.52-4.53 (m, 1H, H-6), 4.68-4.69 (m, 1H, H-4), 4.97-4.98 (m, 1H, H-5), 5.15 (s, 1H, H-3), 6.31 (s, C=C<u>H</u>);

^{13}C-NMR ($CDCl_3$): δ 24.4 (q, CH_3), 25.9 (q, CH_3), 72.7 (t, C-7), 81.1 (d, C-3), 81.7 (d, C-5), 82.6 (d, C-6), 83.4 (d, C-4), 112.0 (d, C=<u>C</u>-CN), 113.3 (s, <u>C</u>$(CH_3)_2$), 113.5 (s, C=C-<u>C</u>N), 151.3 (s, C-2), 166.1 (s, C-1);

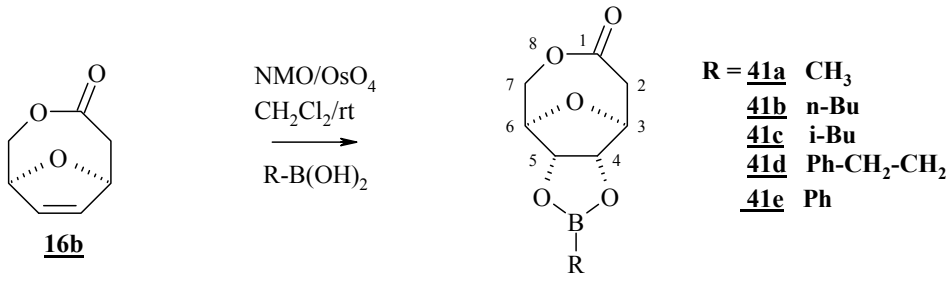
Z-isomer:

^{1}H NMR ($CDCl_3$): δ 1.33 (s, 3H, CH_3), 1.51 (s, 3H, CH_3), 4.26-4.30 (m, 2H, H-7), 4.51-4.52 (m, 1H, H-6), 4.63 (s, 1H, H-3), 4.71-4.72 (m, 1H, H-4), 4.92-4.93 (m, 1H, H-5), 5.99 (s, C=C<u>H</u>);

^{13}C-NMR ($CDCl_3$): δ 24.2 (q, CH_3), 25.8 (q, CH_3), 70.4 (t, C-7), 80.8 (d, C5), 83.2 (d, C-6), 83.4 (d, C-4), 83.8 (d, C-3), 110.6 (s, C=C-CN), 112.9 (s, $\underline{C}(CH_3)_2$), 150.7 (s, C-2), 164.3 (s, C-1);

m/z: 236 (100), 85 (28), 84 (17), 68 (48), 59 (58).

6.7.10 Synthesis of Boronic Esters

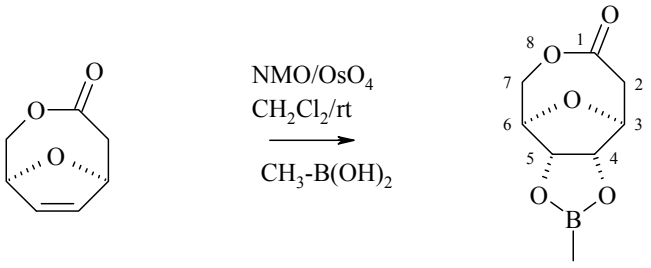


16b

6.7.10.1 General Procedure

Procedure: Dihydroxylation of 8-oxabicyclo[3.2.1]oct-6-en-3-one **16b** was performed by OsO_4 in the presence of N-methylmorpholine oxide. Lactone and NMO*H_2O (1.2equiv.) were dissolved in dry dichloromethane (10% solution). Subsequently, catalytic amounts (5mg) of OsO_4 and the corresponding boronic acid (1.2equiv.) were added and the reaction was stirred at rt until complete conversion (3-8hours) was observed (TLC control). The reaction mixture was quenched with 10% solution of $NaHS_2O_3$ at 0°C for 10 minutes. The two phases were separated and the aqueous layer was extracted three times with dichloromethane. The combined organic layers were dried over sodium sulfate and concentrated *in vacuo* (20°C!). No further purification of the boronic esters **41a-e** was necessary (>99% according to GC/MS). Purification of samples of the boronic esters was performed for combustion analysis.

6.7.10.2 Synthesis of 3,6-anhydro-2-deoxy-4,5-O-(methylborylene)-D-allo-heptonic Acid ζ-Lactone 41a



16b **41a**

Procedure: According to the general procedure above lactone **16b** (50.0mg, 0.36mmol) and methyl boronic acid (25.7mg, 0.43mmol, 1.2equiv.) were converted to the boronic ester. The desired compound **41a** was obtained in 96% yield (68.0mg, colorless crystals). The crude product was used in following synthetic steps without further purification (LP/EtOAc = 2/1, 5g SiO_2).

Yield: 68.0mg (96%)

MW: 198.0; $C_8H_{11}BO_5$

colorless crystals, m.p.: 147-150°C,

Calc.:	C 48.53%,	H 5.60%
Found:	C 48.38%	H 5.51%

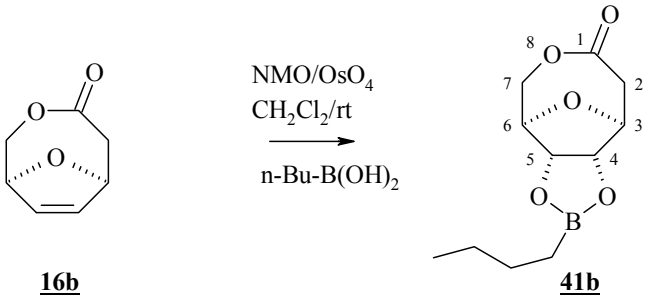
$[\alpha]_D^{20}$: +77.2 (c 1.10, $CHCl_3$)

^{1}H NMR ($CDCl_3$): δ 0.33 (s, 3H, CH_3-B), 2.98-3.04 (m, 2H, H-2), 4.30-4.39 (m, 3H), 4.40-4.47 (m, 1H), 4.78 (d, J=6Hz, 1H, CH-O-B), 5.08 (d, J=6Hz, 1H, CH-O-B);

^{13}C NMR ($CDCl_3$): δ 42.2 (t, C-2), 71.1 (t, C-7), 79.0 (d), 81.9 (d), 83.3 (d), 83.8 (d), 171.8 (s, C-1);

m/z: 198 (4, M^+), 168 (26), 140 (15), 111 (81), 97 (38), 84 (100), 83 (28).

6.7.10.3 Synthesis of 3,6-anhydro-2-deoxy-4,5-O-(butylborylene)-D-allo-heptonic Acid ζ-Lactone 41b



16b **41b**

Procedure: Lactone **16b** (50.0mg, 0.36mmol) and butylboronic acid (43.8mg, 0.43mmol, 1.2equiv.) were converted according to the general procedure. The desired compound **41b** was obtained in 72% yield (62.0mg, colorless oil). The crude product was used in following synthetic steps without further purification (LP/EtOAc = 4/1, 5g SiO_2).

Yield: 62.0mg (72%)

MW: 240.1; $C_{11}H_{17}BO_5$

colorless oil

Calc.:	C 55.04%,	H 7.14%
Found:	C 54.93%	H 7.08%

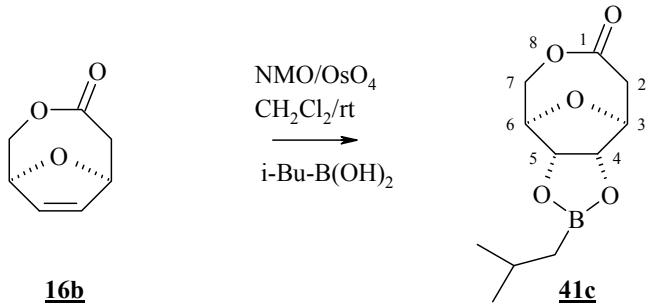
$[\alpha]_D^{20}$: +59.4 (c 1.62, $CHCl_3$)

^{1}H NMR ($CDCl_3$): δ 0.67-0.95 (m, 5H, CH_3, CH_2-B), 1.13-1.44 (m, 4H, CH_2-CH_2), 2.93 (d, J=4Hz, 2H, H-2,), 4.22-4.31 (m, 3H), 4.32-4.40 (m, 1H), 4.70 (d, J=6Hz, CH-O-B, 1H), 5.01 (d, J=6Hz, 1H, CH-O-B)

^{13}C NMR ($CDCl_3$): δ 13.8 (q, CH_3), 25.1 (t), 25.9 (t), 42.1 (t, C-2), 71.1 (t, C-7), 79.1 (d), 81.8 (d), 83.3 (d), 83.7 (d), 171.8 (s, C-1);

m/z: 240 (3, M^+), 210 (24), 182 (10), 153 (100), 139 (19), 126 (86), 96 (15), 69 (26).

6.7.10.4 Synthesis of 3,6-anhydro-2-deoxy-4,5-O-[(2-methyl propyl)borylene]-D-allo-heptonic Acid ζ-Lactone 41c



16b **41c**

Procedure: Lactone **16b** (50mg, 0.36mmol) and 2-methyl-propyl boronic acid (43.8mg, 0.43mmol, 1.2equiv.) were converted according to the general procedure. The desired compound **41c** was obtained in 83% yield (70.0mg, colorless crystals). The crude product was used in following synthetic steps without further purification (LP/EtOAc = 3/1, 5g SiO_2).

Yield: 70.0mg (83%)

MW: 240.1; $C_{11}H_{17}BO_5$

colorless crystals, m.p.: 74-77°C

Calc.:	C 55.04%,	H 7.14%
Found:	C 49.78%	H 6.99%

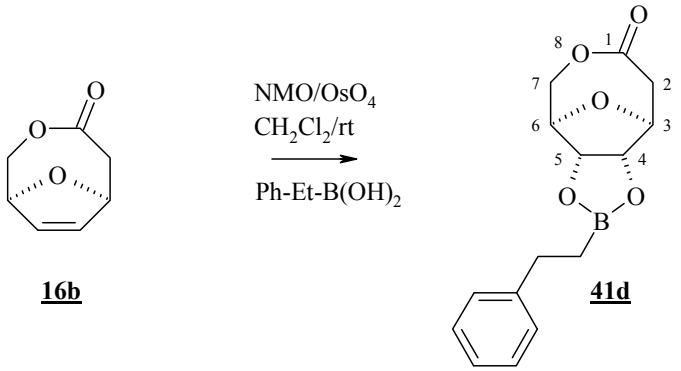
$[\alpha]_D^{20}$: +63.9 (c 1.02, $CHCl_3$)

^{1}H NMR ($CDCl_3$): δ 0.82 (d, 2H, J=7Hz, CH_2-B), 0.93 (d, 6H, J=7Hz, 2xCH_3), 1.85 (sep, J=7Hz, 1H, C<u>H</u>(CH_3)$_2$), 2.97-3.04 (m, 2H, H-2), 4.30-4.38 (m, 3H), 4.40-4.47 (m, 1H), 4.79 (d, J=6Hz, 1H, CH-O-B), 5.08 (d, J=6Hz, 1H, CH-O-B);

^{13}C NMR ($CDCl_3$): δ 24.6 (d, <u>C</u>H(CH_3)$_2$), 25.0 (q, 2xCH_3), 42.2 (t, C-2), 71.1 (t, C-7), 79.1 (d), 81.8 (d), 83.4 (d), 83.6 (d), 171.8 (s, C-1);

m/z: 225 (23), 210 (31), 183 (67), 153 (83), 126 (100), 97 (35), 83 (34), 69 (33), 56 (41).

6.7.10.5 Synthesis 3,6-anhydro-2-deoxy-4,5-O-[(2-phenyl ethyl)borylene]-D-allo-heptonic Acid ζ-Lactone 41d



16b **41d**

Procedure: Lactone **16b** (50mg, 0.36mmol) and phenylethyl boronic acid (64.5mg, 0.43mmol, 1.2equiv.) were converted according to the general procedure. The desired compound **41d** was obtained in 73% yield (75.0mg, colorless crystals). The crude product was used in following synthetic steps without further purification (LP/EtOAc = 4/1, 5g SiO_2).

Yield: 75.0mg (73%)

MW: 288.1; $C_{15}H_{17}BO_5$

colorless crystals, m.p.: 117-119°C

Calc.:	C 62.53%,	H 5.95%
Found:	C 62.47%	H 5.94%

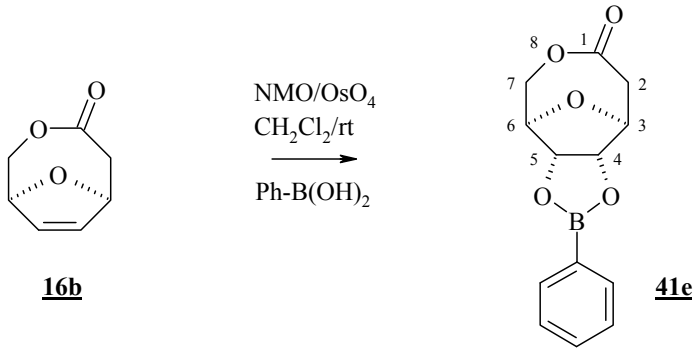
$[\alpha]_D^{20}$: +59.4 (c 1.82, $CHCl_3$)

^{1}H NMR ($CDCl_3$): δ 1.14 (t, J=8Hz, 2H, CH_2-B), 2.68 (t, J=8.1Hz, 2H, CH_2-Ph), 2.91 (d, J=3Hz, 2H, H-2), 4.20-4.28 (m, 3H), 4.30-4.37 (m, 1H), 4.70 (d, J=6Hz, 1H, CH-O-B), 5.00 (d, J=6Hz, 1H, CH-O-B), 7.02-7.32 (m, 5H, Ph)

^{13}C NMR ($CDCl_3$): δ 29.7(t, $\underline{C}H_2$-Ph), 42.1 (t, C-2), 71.1 (t, C-7), 79.0 (d), 82.0 (d), 83.3 (d), 83.9 (d), 125.7 (d, CH), 127.8 (d, 2xCH), 129.0 (d, 2xCH), 143.9 (s) 172 (s, C-1);

m/z: 288 (14, M^+), 105 (10), 104 (38), 91 (100).

6.7.10.6 Synthesis of 3,6-anhydro-2-deoxy-4,5-O-(phenylborylene)-D-allo-heptonic Acid ζ-lactone 41e



16b **41e**

Procedure: Lactone **16b** (50mg, 0.36mmol) and phenyl boronic acid (52.4mg, 0.43mmol, 1.2equiv.) were converted according to the general procedure. The desired compound **41e** was obtained in 80% yield (75.0mg, colorless crystals). The crude product was used in following synthetic steps without further purification (LP/EtOAc = 2/1, 15g SiO_2).

Yield: 150mg (80%)

MW: 260.1; $C_{13}H_{13}BO_5$

colorless crystals, m.p.: 140-143°C

Calc.:	C 60.04%,	H 5.04%
Found:	C 59.75%	H 5.08%

$[\alpha]_D^{20}$: +44.0 (c 1.26, $CHCl_3$)

^{1}H NMR ($CDCl_3$): δ 3.01-3.11 (m, 2H, H-2), 4.40-4.50 (m, 3H), 4.52-4.60 (m, 1H), 4.98 (d, J=6Hz, 1H, CH-O-B), 5.28 (d, J=6Hz, 1H, CH-O-B), 7.31-7.55 (m, 3H, Ph), 7.84 (m, 2H, Ph);

^{13}C NMR ($CDCl_3$): δ 42.2 (t, C-2), 71.2 (t, C-7), 79.1 (d), 82.6 (d), 83.3 (d), 84.4 (d), 127.9 (d, 2xCH), 131.7 (d, CH), 134.8 (d, 2xCH), 171.8 (s, C-1)

m/z: 260 (63, M^+), 230 (15), 173 (100), 172 (44), 159 (37), 146 (83), 105 (19) 59 (10).

Appendix

7 Christallographic Data

7.1 Crystal Data for (1S,6S)-3,9-Dioxabicyclo[4.2.1]non-7-en-4-one (16b)

Single-crystal X-ray diffraction data for compound **16b** were collected with a Bruker Smart APEX CCD 3-circle diffractometer operating with graphite monochromatized Mo-Kα radiation. Structure solution and refinement was performed with Bruker AXS SHELXTL software.

7.1.1 Crystal data and structure refinement for 16b

Identification code	km1225
Empirical formula	C7 H8 O3
Formula weight	140.13
Temperature	100(2) K
Wavelength	0.71073 A
Crystal system, space group	Monoclinic, P2(1)
Unit cell dimensions	a = 7.2667(7) A alpha = 90 deg. b = 5.5587(6) A beta = 90.4670(10) deg c = 7.7452(8) A gamma = 90 deg.
Volume	312.84(6) A^3
Z, Calculated density	2, 1.488 Mg/m^3
Absorption coefficient	0.117 mm^-1
F(000)	148
Crystal size	0.55 x 0.45 x 0.25 mm
Diffractometer	Bruker Smart APEX CCD 3-circle (sealed X-ray) tube, Mo Kα rad., graphite monochromator detector.distance 50 mm, 512x512 pixels)
Scan type / width / speed	ome-scan frames / dome=0.3deg 15sec per fram full sphere data collection,

	4x 606 frames
Theta range for data collection	2.80 to 29.97 deg.
Index ranges	-10<=h<=10, -7<=k<=7, -10<=l<=10
Reflections collected / unique	3412 / 986 [R(int) = 0.0139]
Completeness to theta = 29.97	99.3%
Absorption correction	Multi-scan (program SADABS; Sheldrick, 1996)
Max. and min. transmission	0.97 and 0.87
Structure solution	Direct methods (program SHELXS97)
Refinement method	Full-matrix least-squares on F2(prg SHELXL97)
Data / restraints / parameters	986 / 27 / 91
Goodness-of-fit on F2	1.067
Final R indices [I>2sigma(I)]	R1 = 0.0302, wR2 = 0.0810
R indices (all data)	R1 = 0.0304, wR2 = 0.0812
Absolute structure parameter	1.2(9)
Largest diff. peak and hole	0.309 and -0.221 eA-3

$R1 = \Sigma||F_o|-|F_c||/\Sigma|F_o|$, $wR2 = [\Sigma(w(F_o^2-F_c^2)^2)/\Sigma(w(F_o^2)^2)]^{½}$

Table 7.1. Crystal data and structure refinement for (1S,6S)-3,9-Dioxabicyclo[4.2.1]non-7-en-4-one **16b**

7.1.2 Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters (Å^2 x 10^3) for 16b

U_{eq} is defined as one third of the trace of the orthogonalized U_{ij} tensor.

	x	y	z	U_{eq}
O(1)	7438(1)	2042(2)	6340(1)	22(1)
O(2)	5743(1)	1425(2)	4041(1)	17(1)
O(3)	7231(1)	4623(2)	998(1)	16(1)
C(1)	7087(2)	2645(3)	4870(2)	15(1)
C(2)	8137(2)	4678(3)	4022(1)	17(1)
C(3)	8768(2)	4280(2)	2151(2)	15(1)
C(4)	9332(2)	1704(3)	1802(2)	17(1)
C(5)	7919(2)	565(2)	1078(2)	17(1)
C(6)	6343(2)	2317(2)	912(1)	14(1)
C(7)	4960(2)	2088(3)	2374(2)	16(1)

Table 7.2. Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters (Å^2 x 10^3) for (1S,6S)-3,9-Dioxabicyclo[4.2.1]non-7-en-4-one **16b**.

7.1.3 Hydrogen coordinates (x 10^4) and isotropic displacement parameters (Å^2 x 10^3) for 16b

Hydrogen atoms inserted in idealized positions and refined riding with the atoms to which they were bonded. All H atoms had $U_{iso} = U_{eq}$ x 1.2 (x 1.5 for CH_3) of their carrier atoms.

	x	y	z	U_{eq}
H(2A)	9240	5022	4738	21
H(2B)	7352	6133	4043	21
H(3A)	9789	5409	1858	18
H(4A)	10501	1020	2059	21
H(5A)	7903	-1072	726	21
H(6A)	5710	2113	-230	17
H(7A)	4312	3644	2500	20
H(7B)	4031	866	2043	20

Table 7.3. Hydrogen coordinates (x 10^4) and isotropic displacement parameters (Å^2 x 10^3) for (1S,6S)-3,9-Dioxabicyclo[4.2.1]non-7-en-4-one **16b**

7.1.4 Anisotropic displacement parameters (Å2 x 103) for 16b

The anisotropic displacement factor exponent takes the form:

$$-2\pi^2 [h^2 a^{*2} U_{11} + ... + 2 h k a^* b^* U_{12}]$$

	U_{11}	U_{22}	U_{33}	U_{23}	U_{13}	U_{12}
O(1)	22(1)	26(1)	17(1)	4(1)	-1(1)	-1(1)
O(2)	17(1)	19(1)	15(1)	1(1)	1(1)	-3(1)
O(3)	17(1)	13(1)	17(1)	2(1)	-3(1)	-2(1)
C(1)	14(1)	17(1)	15(1)	-1(1)	3(1)	0(1)
C(2)	19(1)	18(1)	14(1)	-2(1)	2(1)	-6(1)
C(3)	14(1)	18(1)	13(1)	1(1)	0(1)	-3(1)
C(4)	15(1)	22(1)	15(1)	2(1)	4(1)	4(1)
C(5)	21(1)	15(1)	16(1)	1(1)	4(1)	3(1)
C(6)	16(1)	12(1)	15(1)	0(1)	-1(1)	-1(1)
C(7)	14(1)	18(1)	17(1)	0(1)	-1(1)	-2(1)

Table 7.4. Anisotropic displacement parameters ($Å^2$ x 10^3) for (1S,6S)-3,9-Dioxabicyclo[4.2.1]non-7-en-4-one **16b**.

7.1.5 Bond lengths [Å] and angles [deg] for 16b

Table 7.5. Bond lengths [Å] and angles [deg] for (1S,6S)-3,9-Dioxabicyclo[4.2.1]non-7-en-4-one **16b**

Bond distances	
O(1)-C(1)	1.2120(15)
O(2)-C(1)	1.3469(15)
O(2)-C(7)	1.4541(15)
O(3)-C(6)	1.4360(15)
O(3)-C(3)	1.4368(14)
C(1)-C(2)	1.5165(18)
C(2)-C(3)	1.5394(16)
C(2)-H(2A)	0.9900
C(2)-H(2B)	0.9900
C(3)-C(4)	1.514(2)
C(3)-H(3A)	1.0000
C(4)-C(5)	1.3272(18)
C(4)-H(4A)	0.9500
C(5)-C(6)	1.5080(18)
C(5)-H(5A)	0.9500
C(6)-C(7)	1.5259(16)
C(6)-H(6A)	1.0000
C(7)-H(7A)	0.9900
C(7)-H(7B)	0.9900

Bond angles	
C(1)-O(2)-C(7)	124.91(10)
C(6)-O(3)-C(3)	104.88(9)
O(1)-C(1)-O(2)	117.04(12)
O(1)-C(1)-C(2)	120.67(11)
O(2)-C(1)-C(2)	122.28(10)
C(1)-C(2)-C(3)	117.09(11)
C(1)-C(2)-H(2A)	108.0
C(3)-C(2)-H(2A)	108.0
C(1)-C(2)-H(2B)	108.0
C(3)-C(2)-H(2B)	108.0
H(2A)-C(2)-H(2B)	107.3
O(3)-C(3)-C(4)	103.02(10)
O(3)-C(3)-C(2)	109.28(9)
C(4)-C(3)-C(2)	112.76(11)
O(3)-C(3)-H(3A)	110.5
C(4)-C(3)-H(3A)	110.5
C(2)-C(3)-H(3A)	110.5
C(5)-C(4)-C(3)	108.46(10)
C(5)-C(4)-H(4A)	125.8
C(3)-C(4)-H(4A)	125.8
C(4)-C(5)-C(6)	108.19(11)
C(4)-C(5)-H(5A)	125.9
C(6)-C(5)-H(5A)	125.9
O(3)-C(6)-C(5)	103.43(10)
O(3)-C(6)-C(7)	109.77(10)
C(5)-C(6)-C(7)	112.75(10)
O(3)-C(6)-H(6A)	110.2
C(5)-C(6)-H(6A)	110.2
C(7)-C(6)-H(6A)	110.2
O(2)-C(7)-C(6)	115.15(9)
O(2)-C(7)-H(7A)	108.5
C(6)-C(7)-H(7A)	108.5
O(2)-C(7)-H(7B)	108.5
C(6)-C(7)-H(7B)	108.5
H(7A)-C(7)-H(7B)	107.5

Torsion angles	
C7-O2-C1-O1	171.19(12)
C7-O2-C1-C2	-9.15(18)
O1-C1-C2-C3	135.94(13)
O2-C1-C2-C3	-43.70(17)
C6-O3-C3-C4	33.22(10)
C6-O3-C3-C2	-86.88(12)
C1-C2-C3-O3	78.08(14)
C1-C2-C3-C4	-35.84(14)
O3-C3-C4-C5	-20.53(12)
C2-C3-C4-C5	97.14(12)
C3-C4-C5-C6	-0.13(13)
C3-O3-C6-C5	-33.45(11)
C3-O3-C6-C7	87.09(10)
C4-C5-C6-O3	20.80(12)
C4-C5-C6-C7	-97.71(13)
C1-O2-C7-C6	56.42(16)
O3-C6-C7-O2	-81.14(13)
C5-C6-C7-O2	33.58(15)

7.2 Crystal Data for 3,6-anhydro-2-deoxy-4,5-O-(phenylborylene)-D-allo-heptonic Acid ζ-Lactone (41e)

Single-crystal X-ray diffraction data for compound **41e** were collected with a Bruker Smart APEX CCD 3-circle diffractometer operating with graphite monochromatized Mo-Kα radiation. Structure solution and refinement was performed with Bruker AXS SHELXTL software.

7.2.1 Crystal data and structure refinement for compound 41e

Identification code	1224m
Empirical formula	C13 H13 B O5
Formula weight	260.04
Temperature	100(2) K
Wavelength	0.71073 A
Crystal system, space group	Orthorhombic, P2(1)2(1)2(1)
Unit cell dimensions	a = 6.1889(2) A alpha = 90 deg b = 8.2869(3) A beta = 90 deg c = 23.2345(9) A gamma = 90 deg
Volume	1191.62(7) A^3
Z, Calculated density	4, 1.449 Mg/m^3
Absorption coefficient	0.110 mm^-1
F(000)	544
Crystal size	0.55 x 0.45 x 0.25 mm
Diffractometer	Bruker Smart APEX CCD 3-circle sealed X-ray tube, Mo Kalfa rad., graphite monochromator detector.distance 50 mm, 512x512 pixels)
Scan type / width / speed	ome-scan frames / dome=0.3deg /15sec per fram full sphere data collection, 4 x 600 frames
Theta range for data collection	2.61 to 29.99 deg.
Index ranges	-6<=h<=8, -9<=k<=11, -32<=l<=30
Reflections collected / unique	9219 / 2017 [R(int) = 0.0177]

Completeness to theta = 29.99	99.7%
Absorption correction	Multi-scan (program SADABS; Sheldrick, 1996)
Max. and min. transmission	1.00 and 0.88
Structure solution	Direct methods (program SHELXS97)
Refinement method	Full-matrix least-squares on F2 (prg SHELXL97)
Data / restraints / parameters	2017 / 54 / 172
Goodness-of-fit on F2	1.039
Final R indices [I>2sigma(I)]	R1 = 0.0347, wR2 = 0.0917
R indices (all data)	R1 = 0.0353, wR2 = 0.0926
Absolute structure parameter	1.8(8)
Largest diff. peak and hole	0.321 and -0.220 eA-3

$R1 = \Sigma||F_o|-|F_c||/\Sigma|F_o|$, $wR2 = [\Sigma(w(F_o^2-F_c^2)^2)/\Sigma(w(F_o^2)^2)]^{½}$

Table 7.6 Crystal data and structure refinement for ζ-lactone-3,6-anhydro-2-deoxy-4,5-O-(phenylborylene)-D-allo-Heptonic acid **41e**.

7.2.2 Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters (Å^2 x 10^3) for compound 41e

U_{eq} is defined as one third of the trace of the orthogonalized U_{ij} tensor.

	x	y	z	U_{eq}
O(1)	8454(2)	9848(1)	2649(1)	24(1)
O(2)	5259(2)	9303(1)	3002(1)	20(1)
O(3)	5156(2)	5523(1)	3345(1)	19(1)
O(4)	8682(2)	5849(1)	4238(1)	19(1)
O(5)	5442(2)	6820(1)	4572(1)	22(1)
B	7156(2)	5808(2)	4666(1)	18(1)
C(1)	7159(2)	8823(2)	2774(1)	17(1)
C(2)	7566(2)	7038(1)	2701(1)	18(1)
C(3)	7346(2)	6013(1)	3248(1)	16(1)
C(4)	7943(2)	6937(2)	3798(1)	16(1)
C(5)	5728(2)	7576(2)	4022(1)	18(1)
C(6)	4113(2)	6916(2)	3583(1)	19(1)
C(7)	3544(2)	8152(2)	3122(1)	22(1)
C(8)	7264(2)	4681(2)	5198(1)	19(1)
C(9)	9065(2)	3719(2)	5315(1)	21(1)
C(10)	9070(3)	2674(2)	5787(1)	25(1)
C(11)	7269(3)	2572(2)	6142(1)	27(1)
C(12)	5451(3)	3516(2)	6033(1)	26(1)
C(13)	5457(2)	4564(2)	5562(1)	22(1)

Table 7.6 Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters (Å^2 x 10^3) for compound **41e**.

7.2.3 Hydrogen coordinates (x 10^4) and isotropic displacement parameters (Å^2 x 10^3) for compound 41e

Hydrogen atoms inserted in idealized positions and refined riding with the atoms to which they were bonded. All H atoms had $U_{iso} = U_{eq}$ x 1.2 (x 1.5 for CH_3) of their carrier atoms.

	x	y	z	U_{eq}
H(2A)	9043	6892	2546	21
H(2B)	6543	6618	2410	21
H(3A)	8283	5034	3214	20
H(4A)	8996	7828	3723	19
H(5A)	5688	8780	4047	21
H(6A)	2765	6575	3787	23
H(7A)	3181	7574	2762	27
H(7B)	2240	8751	3245	27
H(9A)	10297	3776	5072	25
H(10A)	10307	2032	5865	30
H(11A)	7277	1854	6461	32
H(12A)	4221	3448	6276	32
H(13A)	4220	5208	5487	26

Table 7.7 Hydrogen coordinates (x 10^4) and isotropic displacement parameters (Å^2 x 10^3) for compound **41e**

7.2.4 Anisotropic displacement parameters ($Å^2$ x 10^3) for compound 41e

The anisotropic displacement factor exponent takes the form:

$$-2\pi^2 [h^2 a^{*2} U_{11} + ... + 2 h k a^* b^* U_{12}]$$

	U_{11}	U_{22}	U_{33}	U_{23}	U_{13}	U_{12}
O(1)	22(1)	22(1)	30(1)	5(1)	-2(1)	-5(1)
O(2)	20(1)	17(1)	24(1)	4(1)	2(1)	2(1)
O(3)	19(1)	16(1)	22(1)	2(1)	-2(1)	-4(1)
O(4)	17(1)	23(1)	17(1)	3(1)	-2(1)	5(1)
O(5)	24(1)	24(1)	17(1)	4(1)	4(1)	7(1)
B	21(1)	17(1)	16(1)	-2(1)	-2(1)	2(1)
C(1)	17(1)	18(1)	17(1)	2(1)	-1(1)	0(1)
C(2)	20(1)	16(1)	17(1)	0(1)	1(1)	1(1)
C(3)	18(1)	15(1)	17(1)	-1(1)	0(1)	1(1)
C(4)	15(1)	17(1)	17(1)	1(1)	-1(1)	2(1)
C(5)	19(1)	17(1)	17(1)	1(1)	2(1)	4(1)
C(6)	15(1)	22(1)	21(1)	6(1)	1(1)	1(1)
C(7)	14(1)	25(1)	27(1)	9(1)	-1(1)	1(1)
C(8)	23(1)	17(1)	16(1)	-2(1)	-2(1)	2(1)
C(9)	25(1)	20(1)	19(1)	-2(1)	-3(1)	3(1)
C(10)	35(1)	20(1)	21(1)	-2(1)	-6(1)	7(1)
C(11)	44(1)	21(1)	16(1)	0(1)	-2(1)	3(1)
C(12)	36(1)	26(1)	17(1)	-2(1)	3(1)	1(1)
C(13)	26(1)	21(1)	18(1)	-2(1)	0(1)	3(1)

Table 7.8 Anisotropic displacement parameters ($Å^2$ x 10^3) for compound **41e**.

7.2.5 Bond lengths [Å] and angles [deg] for compound 41e

Table 7.9 Bond lengths [Å] and angles [deg] for compound **41e**.

Bond distances		O(5)-B-O(4)	113.67(11)
O(1)-C(1)	1.2029(16)	O(5)-B-C(8)	121.99(12)
O(2)-C(1)	1.3498(16)	O(4)-B-C(8)	124.28(12)
O(2)-C(7)	1.4541(17)	O(1)-C(1)-O(2)	117.84(12)
O(3)-C(3)	1.4327(15)	O(1)-C(1)-C(2)	123.59(12)
O(3)-C(6)	1.4338(16)	O(2)-C(1)-C(2)	118.56(11)
O(4)-B	1.3725(17)	C(1)-C(2)-C(3)	115.75(10)
O(4)-C(4)	1.4373(14)	C(1)-C(2)-H(2A)	108.3
O(5)-B	1.3698(17)	C(3)-C(2)-H(2A)	108.3
O(5)-C(5)	1.4349(14)	C(1)-C(2)-H(2B)	108.3
B-C(8)	1.5500(18)	C(3)-C(2)-H(2B)	108.3
C(1)-C(2)	1.5102(17)	H(2A)-C(2)-H(2B)	107.4
C(2)-C(3)	1.5361(16)	O(3)-C(3)-C(4)	103.84(9)
C(2)-H(2A)	0.9900	O(3)-C(3)-C(2)	111.70(10)
C(2)-H(2B)	0.9900	C(4)-C(3)-C(2)	113.07(10)
C(3)-C(4)	1.5340(16)	O(3)-C(3)-H(3A)	109.4
C(3)-H(3A)	1.0000	C(4)-C(3)-H(3A)	109.4
C(4)-C(5)	1.5592(17)	C(2)-C(3)-H(3A)	109.4
C(4)-H(4A)	1.0000	O(4)-C(4)-C(3)	110.82(10)
C(5)-C(6)	1.5284(18)	O(4)-C(4)-C(5)	104.80(9)
C(5)-H(5A)	1.0000	C(3)-C(4)-C(5)	103.61(9)
C(6)-C(7)	1.5240(18)	O(4)-C(4)-H(4A)	112.3
C(6)-H(6A)	1.0000	C(3)-C(4)-H(4A)	112.3
C(7)-H(7A)	0.9900	C(5)-C(4)-H(4A)	112.3
C(7)-H(7B)	0.9900	O(5)-C(5)-C(6)	110.94(10)
C(8)-C(9)	1.3971(18)	O(5)-C(5)-C(4)	104.90(9)
C(8)-C(13)	1.4061(19)	C(6)-C(5)-C(4)	103.37(9)
C(9)-C(10)	1.3964(18)	O(5)-C(5)-H(5A)	112.3
C(9)-H(9A)	0.9500	C(6)-C(5)-H(5A)	112.3
C(10)-C(11)	1.390(2)	C(4)-C(5)-H(5A)	112.3
C(10)-H(10A)	0.9500	O(3)-C(6)-C(7)	111.92(10)
C(11)-C(12)	1.394(2)	O(3)-C(6)-C(5)	104.55(10)
C(11)-H(11A)	0.9500	C(7)-C(6)-C(5)	112.30(11)
C(12)-C(13)	1.3962(18)	O(3)-C(6)-H(6A)	109.3
C(12)-H(12A)	0.9500	C(7)-C(6)-H(6A)	109.3
C(13)-H(13A)	0.9500	C(5)-C(6)-H(6A)	109.3
		O(2)-C(7)-C(6)	114.01(10)
Bond angles		O(2)-C(7)-H(7A)	108.8
C(1)-O(2)-C(7)	121.18(10)	C(6)-C(7)-H(7A)	108.8
C(3)-O(3)-C(6)	104.98(9)	O(2)-C(7)-H(7B)	108.8
B-O(4)-C(4)	108.22(10)	C(6)-C(7)-H(7B)	108.8
B-O(5)-C(5)	108.33(10)	H(7A)-C(7)-H(7B)	107.6

C(9)-C(8)-C(13)	118.51(12)	C4-C5-C6-O3	24.58(11)
C(9)-C(8)-B	122.27(12)	O5-C5-C6-C7	151.07(10)
C(13)-C(8)-B	119.16(12)	C4-C5-C6-C7	-96.97(11)
C(10)-C(9)-C(8)	120.58(13)	C1-O2-C7-C6	64.76(15)
C(10)-C(9)-H(9A)	119.7	O3-C6-C7-O2	-88.22(14)
C(8)-C(9)-H(9A)	119.7	C5-C6-C7-O2	29.02(16)
C(11)-C(10)-C(9)	120.14(13)	O5-B-C8-C9	-175.96(12)
C(11)-C(10)-H(10A)	119.9	O4-B-C8-C9	6.81(19)
C(9)-C(10)-H(10A)	119.9	O5-B-C8-C13	6.88(19)
C(10)-C(11)-C(12)	120.35(13)	O4-B-C8-C13	-170.34(13)
C(10)-C(11)-H(11A)	119.8	C13-C8-C9-C10	-0.44(19)
C(12)-C(11)-H(11A)	119.8	B-C8-C9-C10	-177.61(12)
C(11)-C(12)-C(13)	119.29(14)	C8-C9-C10-C11	0.6(2)
C(11)-C(12)-H(12A)	120.4	C9-C10-C11-C12	-0.4(2)
C(13)-C(12)-H(12A)	120.4	C10-C11-C12-C13	0.1(2)
C(12)-C(13)-C(8)	121.13(13)	C11-C12-C13-C8	0.0(2)
C(12)-C(13)-H(13A)	119.4	C9-C8-C13-C12	0.2(2)
C(8)-C(13)-H(13A)	119.4	B-C8-C13-C12	177.43(12)

Torsion angles

C5-O5-B-O4	3.01(15)
C5-O5-B-C8	-174.48(11)
C4-O4-B-O5	-2.15(15)
C4-O4-B-C8	175.28(11)
C7-O2-C1-O1	175.11(11)
C7-O2-C1-C2	-6.29(17)
O1-C1-C2-C3	122.95(13)
O2-C1-C2-C3	-55.57(16)
C6-O3-C3-C4	43.81(11)
C6-O3-C3-C2	-78.36(11)
C1-C2-C3-O3	85.98(13)
C1-C2-C3-C4	-30.72(15)
B-O4-C4-C3	-110.72(11)
B-O4-C4-C5	0.44(12)
O3-C3-C4-O4	85.40(11)
C2-C3-C4-O4	-153.34(10)
O3-C3-C4-C5	-26.52(11)
C2-C3-C4-C5	94.73(11)
B-O5-C5-C6	108.49(11)
B-O5-C5-C4	-2.48(13)
O4-C4-C5-O5	1.24(12)
C3-C4-C5-O5	117.49(10)
O4-C4-C5-C6	-115.07(10)
C3-C4-C5-C6	1.18(11)
C3-O3-C6-C7	78.54(12)
C3-O3-C6-C5	-43.26(11)
O5-C5-C6-O3	-87.37(11)

7.3 Homology Approximation of $CHMO_{Acineto}$

Homology approximation of CHMO, based on a sequence alignment (40.3% sequence identity to 1W4X of PAMO) was estimated by Deep View/Swiss-PdbViewer 3.7 (SP5)). Incorporation of NADPH and the Arg 327 were based on previous published data for 1W4X of PAMO.

7.3.1 Sequence Alignmnet of $CHMO_{Acineto}$ and $PAMO_{Thermo}$

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CHMO_acineto  1    MSQKMDFDAI VIGGGFGGLY AVKKLRDELE LKVQAFDKAT DVAGTWYWNR
1W4X          10   RRQPPEEVDV LVVGAGFSGL YALYRLRELG RSVHVIETAG DVGGVWYWNR
                     *  .   . .. *.  .           **    *. .. *  **.* *****

CHMO_acineto  51   YPGALTDTET HLYCYSWDKE LLQSLEIKKK YVQGPDVRKY LQQVAEKHDL
1W4X          60   YPGARCDIES IEYCYSFSEE VLQEWNWTER YASQPEILRY INFVADKFDL
                   ****  * *.   ****   * .**  .   . *   *.. .* .  **.* **

CHMO_acineto  101  KKSYQFNTAV QSAHYNEADA LWEVTTEYGD KYTARFLITA LGLLSAPNLP
1W4X          110  RSGITFHTTV TAAAFDEATN TWTVDTNHGD RIRARYLIMA SGQLSVPQLP
                   . .  *.*.*  .* ..**   * * *. **  .  **.** *  * ** * **

CHMO_acineto  151  NIKGINQFKG ELHHTSRWPD D-VSFEGKRV GVIGTGSTGV QVITAVAPLA
1W4X          160  NFPGLKDFAG NLYHTGNWPH EPVDFSGQRV GVIGTGSSGI QVSPQIAKQA
                   *. *...* * .* **. **  . * * * ** *******.*. **   .*  *

CHMO_acineto  200  KHLTVFQRSA QYSVPIGNDP LSEEDVKKIK DNYDKIWDGV WNSALAFGLN
1W4X          210  AELFVFQRTP HFAVPARNAP LDPEFLADLK KRYAEFREES RNTPGGTHRY
                     * ****.. ...**  * * *  * .  .*   *  ...   .*.. .

CHMO_acineto  250  ESTVPAMSVS AEERKAVFEK AWQTGGGFRF MFETFGDIAT NMEANIEAQN
1W4X          260  QGPKSALEVS DEELVETLER YWQEGGPDIL AAYRDILRDR DANERVAEFI
                   ..  .*. **  **    .*.  ** **   .             . .  .

CHMO_acineto  300  FIKGKIAEIV KDPAIAQKLM PQDLYAKRPL CDSGYYNTFN RDNVRLEDVK
1W4X          310  RNKIRNTVRD PEVAERLVPK GYPFGTKRLI LEIDYYEMFN RDNVHLVDTL
                     *  . .    . *     .    . .** .  .  **. ** ****.* *

CHMO_acineto  350  ANPIVEITEN GVKLENGDFV ELDMLICATG FDAVDGNYVR MDIQGKNGLA
1W4X          360  SAPIETITPR GVRTSEREY- ELDSLVLATG FDALTGALFK IDIRGVGNVA
                   . **  **   **.  . ..  *** *. *** ***. *   . .**.*   .*

CHMO_acineto  400  MKDYWKEGPS SYMGVTVNNY PNMFMVLGPN GPF--TNLPP SIESQVEWIS
1W4X          409  LKEKWAAGPR TYLGLSTAGF PNLFFIAGPG SPSALSNMLV SIEQHVEWVT
                   .*. *  **  .*.*..  . **.* . **  .*   .*.   *** .***..

CHMO_acineto  448  DTIQYTVENN VESIEATKEA EEQWTQTCAN IAEMTLFPKA QSWIFGANIP
1W4X          459  DHIAYMFKNG LTRSEAVLEK EDEWVEHVNE IADETLYPMT ASWYTGANVP
                   * * *   *  .   **  *  *..* .   . **. **.*..  **  ***.*

CHMO_acineto  498  GKKNTVYFYL GGLKEYRSAL ANCKNHAYEG FDIQLQRSDI KQPANA
1W4X          509  GKPRVFMLYV GGFHRYRQIC DEVAAKGYEG FVLT
                   **     .*. **.  **     .    .*** * .
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Curriculum Vitae

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Education

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Summer	2001	Industrial placement (3 month) - Syngenta Basel (Herbicide Chemistry)
	1997 – 2003	Study of Organic Chemistry – Institute of Applied Synthetic Chemistry, Vienna University of Technology (www.ias.tuwien.ac.at)
March	2003	Completion of Diploma Thesis in Organic Chemistry: *Enantiodivergent Baeyer-Villiger Oxidation of Functionalized Prochiral Cyclohexanone Derivatives Utilizing Recombinant Cells* (Supervisors: Prof. Marko D. Mihovilovic and Prof. Peter Stanetty)
March	2003	Diploma degree in Organic Chemistry
January	2003-2004	Industrial placement (part time) – GSK Vienna (Registration)

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October	2003	Start of study in Molecular Biology – University of Vienna
September	2004	NMR-Summer-School Niederöblan, Austria
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Languages

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Publication activity

- 12 publications
- 7 posters
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Professional membership

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IT skills

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Other relevant skills

- driving licence (kategory B and A/500cm^3)
- NMR-, GC-GC/MS- and MPLC-operator in the research group of Prof. Marko D. Mihovilovic and Prof. Peter Stanetty (Institute of Applied Synthetic Chemistry, Vienna University of Technology).

Oral presentations:

- Rudroff F. **Application of Baeyer-Villiger Biooxidation Products in the Synthesis of Natural Products and Bioactive Compounds** *GRCBiocatalysis* **2006**, Smith Field, Rhode Island, USA.

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