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Carboligation using the aldol reaction

A comparison of stereoselectivity and methods

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Abstract

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The research summarized in this thesis focuses on synthesizing aldehyde and aldol compounds as substrates and products for the enzyme D-fructose-6-aldolase (FSA). Aldolases are important enzymes for the formation of carbon-carbon bonds in nature. In biological systems, aldol reactions, both cleavage and formation play central roles in sugar metabolism. Aldolases exhibit high degrees of stereoselectivity and can steer the product configurations to a given enantiomeric and diastereomeric form. To become truly useful synthetic tools, the substrate scope of these enzymes needs to become broadened.

In the first project, phenylacetaldehyde derivatives were synthesized for the use as test substrates for *E. coli* FSA. Different methods were discussed to prepare phenylacetaldehyde derivatives, the addition of a one carbon unit to benzaldehyde derivatives using a homologation reaction was successful and was proven efficient and non-sensitive to the moisture. The analogues were prepared through two steps with 75-80 % yields for both *meta*- and *para*-substituted compounds.

The second project focuses on synthesizing aldol compound using FSA enzymes, both wild type and mutated variants selected from library screening, the assay has been successfully used to identify a hit with 10-fold improvement in an R134V/S166G variant. This enzyme produces one out of four possible stereoisomers.

The third project focuses on the synthesis of a range of aldol compounds using two different approaches reductive cross-coupling of aldehydes by SmI_2 or by organocatalysts using cinchonine. Phenylacetaldehydes were reacted with hydroxy-, dihydroxyacetone and hydroxyacetophenone in presence of cinchonine, the reaction was successful with hydroxyacetophenone in moderate yields and 60-99 % *de* ratio. On the other hand, the aldehydes reacting with methyl- and phenylglyoxal in the presence of SmI_2 resulted in moderate yields and without stereoselectivity.

Keywords: aldol reaction, cinchonine, FSA enzyme, homologation reactions, phenylacetaldehyde derivatives, samarium diiodide.

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To my parents
To Hebbo
To Summer and Sarah

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Al-Smadi D, Enugala T.R, Norberg T, Kihlberg J, Widersten M. (2018) Synthesis of Substrates for Aldolase-Catalysed Reactions: A Comparison of Methods for the Synthesis of Substituted Phenylacetaldehydes. *Synlett,* **29**, 1187-1190.

II Ma H, Engel S, Enugala T.R, Al-Smadi D, Gautier C, Widersten M. (2018) New Stereoselective Biocatalysts for Carboligation and Retro-Aldol Cleavage Reactions Derived from D-Fructose 6-Phosphate Aldolase. *Biochemistry,* **57***,* 5877-5885.

III Al-Smadi D, Enugala T.R, Norberg T, Kessler V, Kihlberg J, Widersten M. A Comparison of Synthetic Approaches to Derivatives of 1,4-Substituted 2,3-Dihydroxybutanones. *Manuscript*.

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Contribution Report

The author wishes to clarify his contribution to the papers included in the thesis:

I Contributed in synthesis planning and redesign of the methods, performed all synthesis, evaluation experiments and characterization of the products, and contributed to writing of the manuscript.

II Performed the synthesis of aldehydes and non-enzymatic synthesis of the aldol compounds.

III Contributed in synthesis planning and redesigning the methods, performed all the synthesis and characterization of products, and contributed to writing of the manuscript.

Contents

Abbreviations

BINOL	1,1'-bi-2-naphthol
SmI_2	samarium diiodide
DHA	dihydroxyacetone
DHAP	dihydroxyacetone phosphate
FSA	Fructose 6-phosphate aldolase
PEP	phosphoenolpyruvate
G3P	glyceraldehyde 3-phosphate
E. coli	*Escherichia coli*
DERA	deoxyribose 5-phosphate aldolase
MnO_2	Manganese dioxide
IBX	2-iodoxybenzoic acid
PCC	pyridiniumchlorochromate
DMSO	dimethylsulfoxide
DCM	dichloromethane
DMF	dimethylformamide
DCE	1,2-dichloroethane
F6P	fructose-6-phosphate
TalB	transaldolase B
DHPP	3,4-dihydroxy-5-phenylpentane-2-one
^{1}H-NMR	proton nuclear magnetic resonance
NMR	nuclear magnetic resonance
HPLC	high performance liquid chromatography
$CHCl_3$	chloroform
THF	tetrahydrofuran

1. General Introduction

The aldol reaction is one of the most powerful transformations in organic chemistry since it involves formation of new carbon-carbon bonds. The products of these reactions are known as *aldols* and can be found in several synthetic or naturally occurring molecules, such as carbohydrates, as stereogenic alcohol units.

Aldol compounds can be produced either synthetically or in nature by enzymes that are called *aldolases*. However, various challenges have been associated with aldol reactions that include issues of chemo-, regio-, diastereo-, and enantioselectivity, and thus, many powerful stoichiometric processes have been developed to address these issues.[1,2]

In recent years, stereochemistry, dealing with the three-dimensional behavior of chiral molecules, has become a significant area of research in modern organic chemistry. The concept of stereochemistry can, however, be traced as far back as the nineteenth century. In 1801, the French mineralogist Hauéy noticed that quartz crystals exhibited hemihedral phenomena, which implied that certain facets of the crystals were exposed as nonsuperimposable species showing a typical relationship between an object and its mirror image. [3] In 1809, the French physicist Malus, who also studied quartz crystals, observed that they could induce the polarization of light.[3]

1.1 Selectivity and specificity

Selectivity is one of the main challenges to the synthetic chemists when producing desired products. Each product formed is characterized in terms of structure, stereo-configuration and yield. For instance, if the product of a certain reaction contains only one structural isomer, the reaction is described as *selective* with respect to formation of that particular structural isomer. If only one of two or more possible diastereomers are formed, the reaction is described as selective with respect to formation of that diastereomer. If only one member of a pair of enantiomers is formed, the reaction is described as selective with respect to formation of that enantiomer. Moreover, if the products are a mixture of isomers one could describe the degree of selectivity of the reaction with respect to the formation of a specific structure, diastereomer or enantiomer.

Furthermore, when performing and comparing the product or mixture of products from a reaction of two (or more) isomeric starting materials, the yields of the products of various structures and configurations may or may not differ depending on the starting materials. If the products of two reactions with structurally isomeric starting materials are different, the reaction is considered specific toward structural isomers. If the products of two reactions with diastereomeric starting materials are different, the reaction is considered specific toward diastereomers. If the products of the two reactions with enantiomeric starting materials are different, the reaction is said to be specific toward enantiomers.

1.2 Asymmetric aldol reactions

Asymmetric aldol reactions are powerful for the construction of carbon–carbon bonds in an enantioselective fashion, first discovered in 1872 by Wurtz.[4] These reactions present many challenges regarding the selectivity to the synthetic chemist, and in biological systems, aldol and retro-aldol reactions play critical roles in sugar metabolism emphasizing the importance of control of the stereoconfiguration in the products.

Aldol reactions have been extensively studied from different angles, including the development of new stereoselective reactions. The developed catalysts are transition metal coordination complexes with chiral ligands, small chiral organic molecules and biocatalytic approaches employing aldolase enzymes.[5-10]

Various metal-catalyzed processes have been reported for the direct catalytic asymmetric aldol reaction. The proposed mechanisms of these synthetic catalysts share resemblance with the mechanism of type II aldolases,[11-13] which exploits a zinc ion to acidify an α-proton of the donor component to form and stabilize a reactive enolate.[14]

Silver and gold have been used with ferrocenyl ligands to catalyze the direct aldol reaction with high enantioselectivity maintained.[15-19] In 1999, Motherwell and coworkers described how rhodium-catalyzed isomerization of allylic lithiumalkoxides allowed regioselective generation of the metal enolates of ketones or aldehydes and that these enolates added to aldehydes and ketones in an aldol fashion (Figure 1).[20]

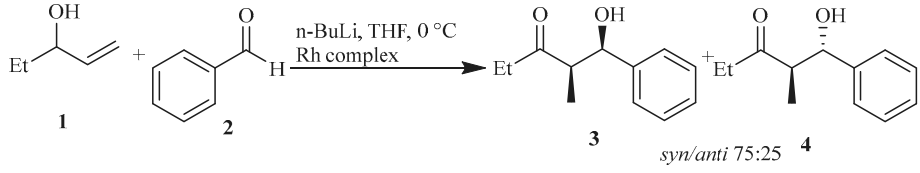


Figure 1. Formation of aldols from ketone enolates catalyzed by rhodium complex.[20]

Rhodium was also used to catalyze direct aldol reactions, and 1 % mol of a ferrocenyl rhodium complex reacted with α-cyanocarboxylates to produce adducts up to 91 % *ee* ratio (Figure 2).[21]

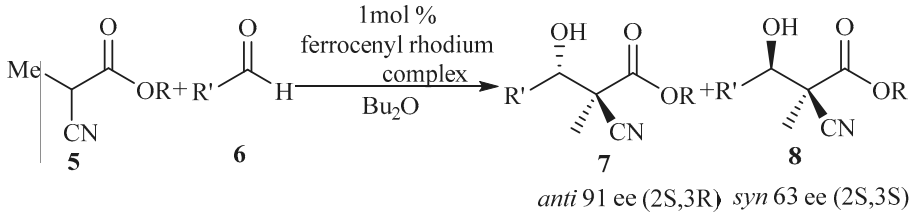


Figure 2. α-cyanocarboxylates reacting with aldehyde catalyzed by a ferrocenyl rhodium complex.[21]

A wide range of asymmetric reactions have been studied that are catalyzed by simple amino acids, primarily proline. Hajas-Parrish-Eder-Sauer-Wiechert cyclization was the first to report a direct asymmetric aldol reaction catalyzed by L-proline in 1971,[2,22,23] the reaction proceeded with 3 mol % catalyst to form a cyclic aldol, followed by dehydration to give the product in excellent yield (Figure 3).

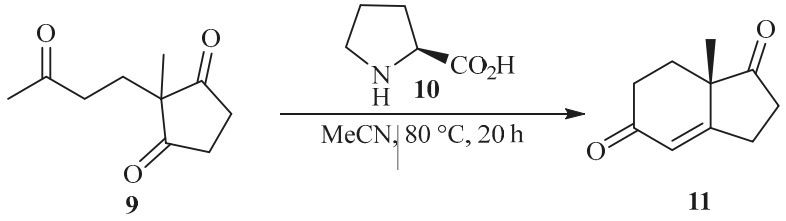


Figure 3. Intramolecular L-proline catalyzed cyclization reaction[2].

Later, List and co-workers, in 2000, described the intermolecular proline-catalyzed direct aldol reaction using 20-30 mol % L-proline to produce aldol compound **14** (Figure 4).[24]

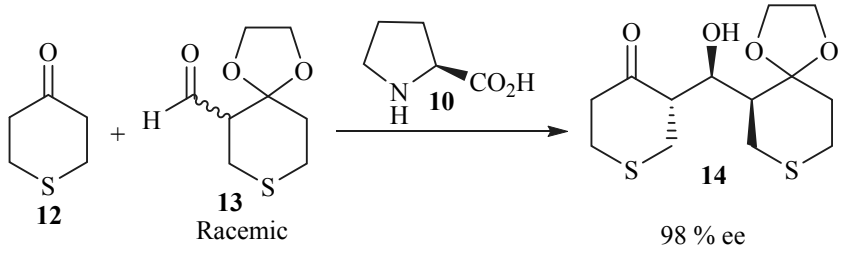


Figure 4. Intermolecular L-proline catalyzed aldol reaction.[24]

Furthermore, carbohydrate synthesis was also achieved by trimerization of an aldehyde in the presence of only 10 % L-proline to generate a cyclic hexose in 41 % yield and with excellent *ee* ratio and 51 % yield open chain sugar in 4:1 *dr* ratio (Figure 5). [25]

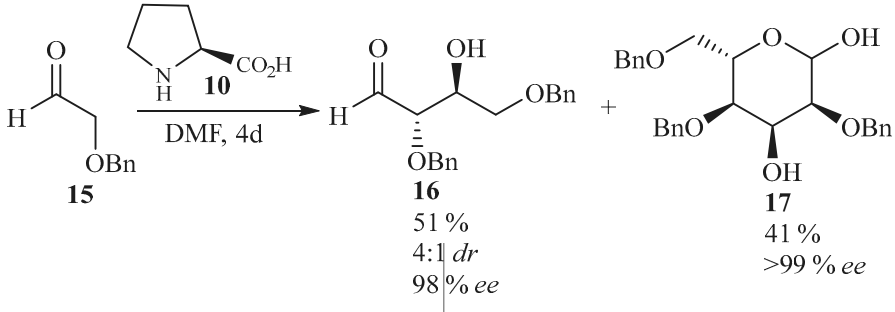


Figure 5. Proline-catalyzed sugar synthesis.

The, 1,1'-bi-2-naphthol (BINOL)-based catalysis is an important development of the asymmetric aldol reactions.[26] Several catalysts have been developed based on BINOL.[20,27] Shibasaki *et al.*, reported a novel barium complex to catalyze reaction of an aldehyde and an unmodified ketone, and also found that the most effective barium catalyst gave 77-99 % yield and 70 % *ee* (Figure 6).[28]

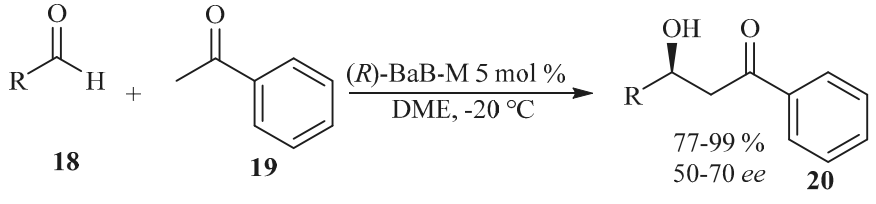


Figure 6. Direct catalytic asymmetric aldol reactions by a BINOL barium complex.[28]

1.3 Synthetic reactions using samarium diiodide (SmI_2)

Samarium diiodide is another reagent that can be used for production of aldol compounds.[29,30] There are two major classes of reactions mediated by samarium diiodide, (i) reductive couplings resulting in formation of C-C bonds and (ii) reductive manipulations of functional groups.

In the last 40 years, samarium diiodide promoted couplings to form C-C bonds have been shown to be powerful synthetic tools.[31-42] For instance, SmI_2-mediated aldehyde-aldehyde coupling gives access to 1,2 diols, however, without stereoselectivity.[43]

Cross-coupling reactions can be promoted by SmI_2 with either one or two-electron mechanisms, radical intermediates forming carbon-carbon bond. It

should be noted that, although the proposed mechanism for SmI_2 mediated cross-couplings of carbonyl derivatives with olefins includes the generation of ketyl-type radical intermediates ("carbonyl-first"),[44] the mechanistic studies propose that in many cases these reactions may also proceed via an alternative reaction pathway involving reduction of the olefin ("olefin-first").[45]

Moreover, specific examples may involve an anionic C−C bond-forming process. However, the current mechanistic evidence does not allow the two pathways to be distinguished. In the section below, the reactions of carbonyl compounds for which distinct mechanisms have been proposed are highlighted and additionally discussed due to the fact that when designing Sm(II)-mediated cross-coupling reactions,[43] changes in the electronic properties of coupling partners can be exploited to increase the efficiency of a given synthetic process. In 1983, the first SmI_2-mediated pinacol coupling of an aldehyde with ketones was reported by Kagan.[29,31-33,46] This early procedure resulted in mixture of diastereoisomers. However, the achieved high yields for coupling of aliphatic and aromatic ketones and aldehydes predicted a potential of SmI_2 for synthesis of vicinal diols. Since then, both inter- and intramolecular SmI_2-mediated pinacol-type couplings have been reported in the literature.[47,48] Few years back, this method served as a valuable alternative to the synthesis of 1,2-diols by dihydroxylation of alkenes and has been widely utilized in target-oriented synthesis.[46] Later, highly *cis*-diastereoselective intramolecular aldehyde-aldehyde pinacol coupling was reported in 2006 by d'Alarcao for the synthesis of galactosaminyl D-chiro inositols using SmI_2 and *t*-BuOH at -78 °C (Figure 7).[49]

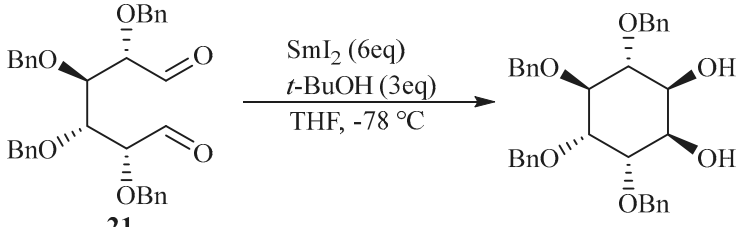


Figure 7. Intramolecular pinacol coupling as a part of a total synthesis.[49]

Ionic reactions mediated by samarium diiodide can be divided into three classes, Refomatsky, Grignard/Barbier[50-52] and aldol reactions. This thesis will focus on the aldol reactions which are directly related to the thesis project. Several alternative approaches to SmI_2-mediated aldol reactions have been reported, and most of these reactions are limited to specific substrates and products. SmI_2-mediated aldol reactions of acylepoxide and 2-acylaziridine have

been used as part of total synthesis project and they showed high *syn*-selectivity in products of either intra- or intermolecular reactions (Figure 8).[53,54]

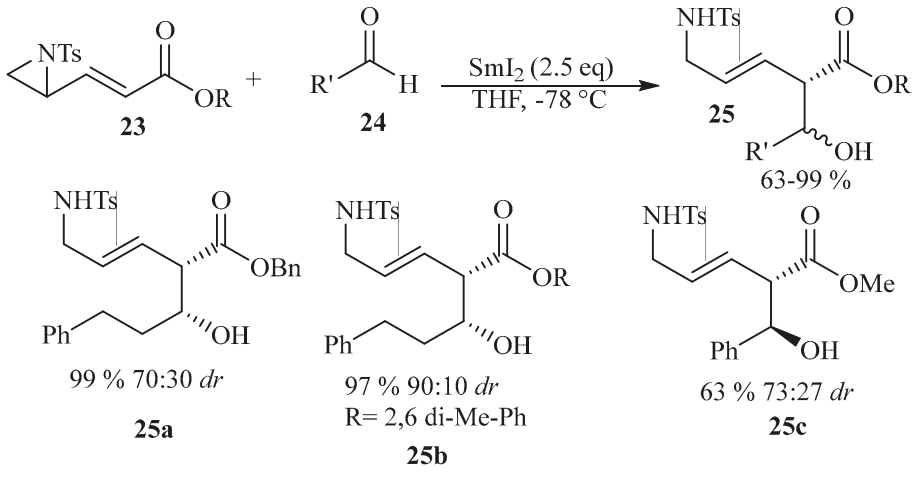


Figure 8. SmI_2-enolates of aldol reactions.[53,54]

In 2008, Procter reported the synthesis of *cis*-hydrindanes via 5-*exo*-trig ketyl radical-olefin coupling with excellent diastereocontrol at the three stereocenters generated during the reaction.[55,56] Furthermore, in 2009, Procter reported the 5-*exo*-trig ketyl-olefin cyclization/intramolecular aldol cascade for the synthesis of complex spirocyclic lactones, forming two new rings and affording four contiguous stereocenters in a single step reaction with excellent yields and high *syn*-selectivity (Figure 9).[57,58]

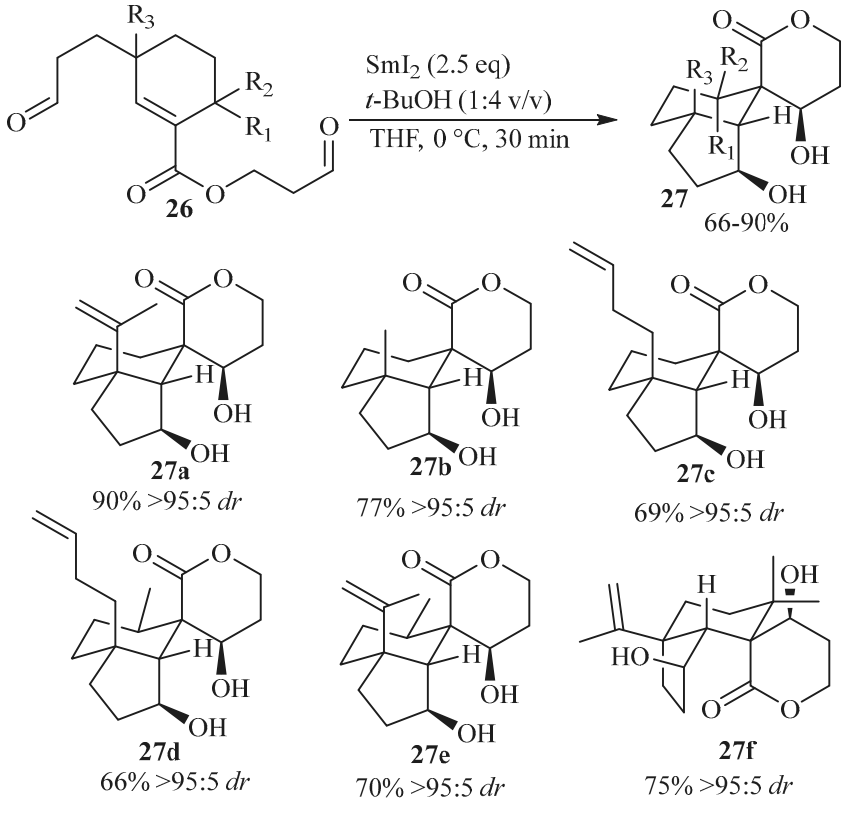


Figure 9. Dialdehyde aldol spirocyclization cascade reaction.[57,58]

The proposed mechanism involves the following steps: (i) *anti*-selective ketyl olefin cyclization; (ii) reduction to the Sm(III) enolate; and (iii) chelation controlled aldol cyclization through a six-membered transition state. Interestingly, the cyclization cascade of a substrate containing an α-*gem*-dimethyl group gave the product with the opposite configuration (>95:5 *dr*) at the quaternary stereocenter formed during the aldol cyclization. The authors proposed that this selectivity comes from a different conformation of the enolate intermediate.[54,55]

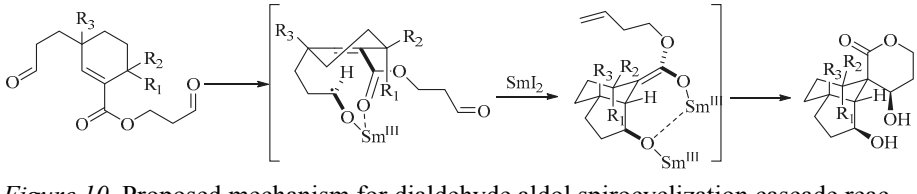


Figure 10. Proposed mechanism for dialdehyde aldol spirocyclization cascade reaction.

In 2013 Reisman and co-workers reported sequential 6-endo-Trig/cyclization of an aldehyde-ester as part of total synthesis of (–)-Longikaurin E in 57% yield and more than 95:5 diastereomeric ratio (Figure 11).[59,60]

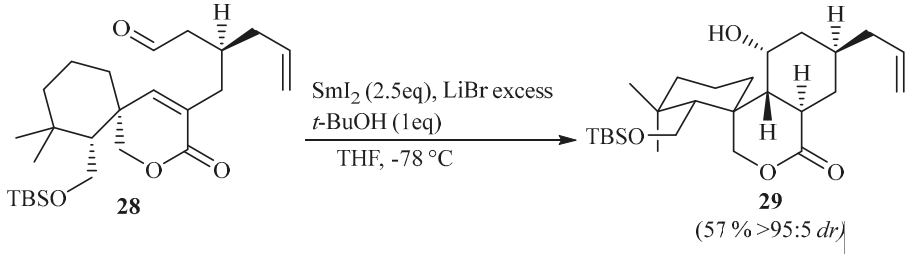


Figure 11. 6-endo-Trig/pinacol-type cyclization in the total synthesis of (−)-longikaurin E.[59,60]

Although, the progress in this field has been massive, there are still challenges that need to be solved.[43]

1.4 Aldolases.

Aldolases are lyase enzymes that catalyze a symmetric addition reaction between a ketone or aldehyde as a donor and with an aldehyde as acceptor. Today, more than 30 aldolases present in several organisms have been identified.[11] In the cell, aldol and retro-aldol reactions are essential steps in cellular metabolism especially for carbohydrates and keto acids.

Aldolases are key enzymes for many metabolic reactions, glycolysis, fructose metabolism, pentose phosphate pathways and other reactions. According to their catalytic mechanisms, aldolases can be classified into two major classes;[12] Class I aldolases that activate the donor molecule via a catalytic Lys residue and form a covalent Schiff base intermediate, and class II aldolases that require a divalent metal ion (Zn^{2+}, Fe^{2+} in most cases, or Co^{2+} in few cases) to stabilize reaction intermediates by polarizing the substrate carbonyl groups, thus stabilizing the reactive enolates.[11,61,62]

1.4.1 Class I aldolases.

Due to their biocatalytic potential in organic synthesis, class I aldolases have attracted great attention. These enzymes are co-factor free and generally the stereochemistry at the newly formed stereocenter is controlled by the enzyme which facilitates predictions of the product structure.[11] Since class I aldolases are tolerant regarding the structure of the acceptor molecules and more restricted about the donor molecule structure, these aldolases have been further classified into five subgroups based on their donor performance.[63] These subgroups include: dihydroxyacetone phosphate (DHAP) dependent aldolases,

dihydroxyacetone (DHA) dependent aldolases, phosphoenolpyruvate (PEP) and pyruvate dependent aldolases, glycine dependent aldolases and acetaldehyde dependent aldolases.

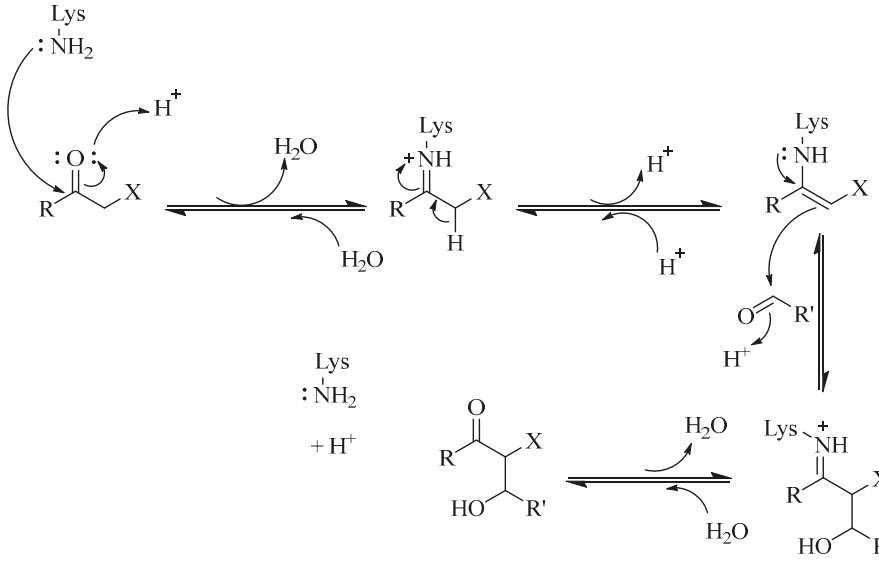
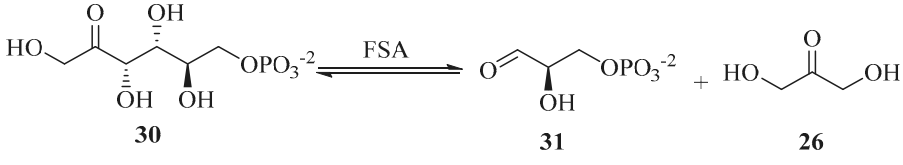


Figure 12. Outline of the mechanism of aldol addition catalyzed by class I aldolases.

1.4.2 Fructose-6-phosphate aldolase (FSA)

FSA is the first reported enzyme that catalyzes the cleavage of fructose 6-phosphate (F6P) to generate dihydroxyacetone and glyceraldehyde 3-phosphate (G3P) (Figure 13). FSA exists as two isoenzymes, and the gene for both isoenzymes are present but not transcribed under normal conditions in *E. coli*. However, the physiological function of FSA is still unclear.[64]



30 **31** **26**

Figure 13. The cleavage of fructose 6-phosphate catalyzed by FSA. With fructose, fructose 1-phosphate, glucose 6-phosphate, and fructose 1,6-bisphosphate there is no observed cleavage by FSA. In the aldol addition reaction, dihydroxyacetone is considered to be a standard donor. Erythrose and glycolaldehyde are weak acceptors. In addition dihydroxyacetone phosphate does not serve as a donor compound.[64]

The crystal structure of FSA was reported by G. Schneider and coworkers at 1.93 Å resolution (Figure 14).[65] The overall structure of FSA is a 250 kDa decamer of ten identical subunits. Each subunit of FSA folds into an α/β TIM

barrel fold where the catalytic Lys85 is located on the β4 strand. Five identical subunits of 220 amino acid residues first assemble into a pentamer, and two ring-like pentamers pack like a doughnut to form the decamer. An important interaction in the pentamer is through the C-terminal helix from one monomer that runs across the active site of the neighboring subunit. An inner channel of 30 Å in diameter passes through the middle of the decamer. In addition, the interaction between a number of helices and N-terminal loops in the interface of adjacent subunits also help in maintaining the pentameric structure of FSA. The major interactions between two pentameric subunits are the interactions between the residues which are close to the border of the inner channel of the decamer. Overall, the decameric structure of FSA is strongly packed and thus contributing greatly to FSA's high thermostability.[65]

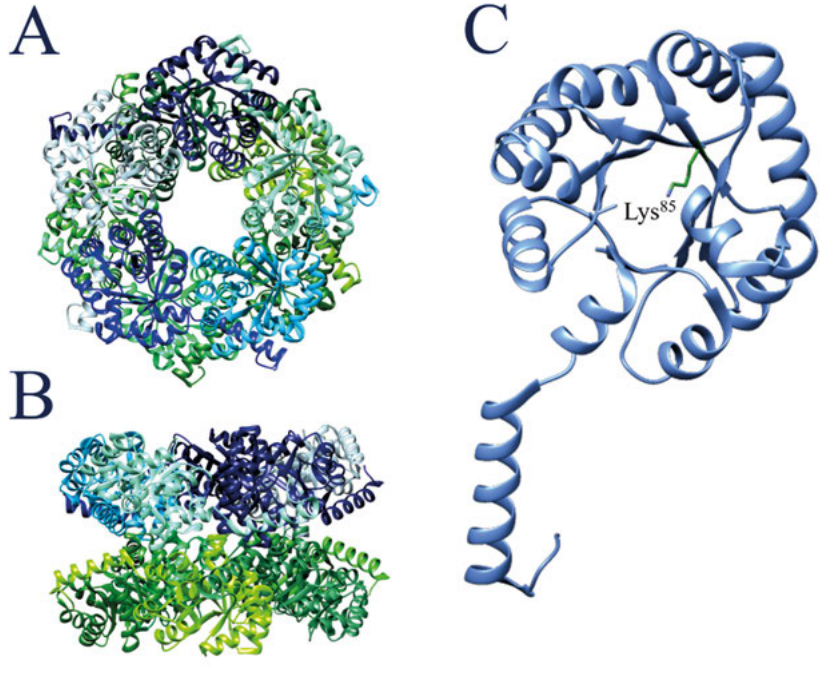


Figure 14. The crystal structure of FSA, a 250 kDa homodecamer. (A) Structure of one subunit, catalytic Lys85 is shown in stick. (B) Overall structure, top view. (C) Overall structure, side view. PDB entry: 1L6W.[65]

1.4.3 Application of FSA in organic synthesis.

After the identification of FSA, its applicability to organic syntheses have been studied. One of the most attractive properties of FSA is its unusual independence on phosphorylated donor compounds.[66,67] Also, wild type FSA shows cross-coupling activity with two short-chain aldehydes. Protein engineering by rational mutagenesis of FSA has also been performed to improve on the substrate scope.[66,68-71]

Roldan *et al.* reported production of aldol compounds from simple aliphatic ketones and L-glyceraldehyde-3-phosphate using FSA variants (Figure 15).[72]

Recently, Junker et.al reported a switch of reaction selectivity by directed evolution *in vitro* to synthesize other related aliphatic products.[73]

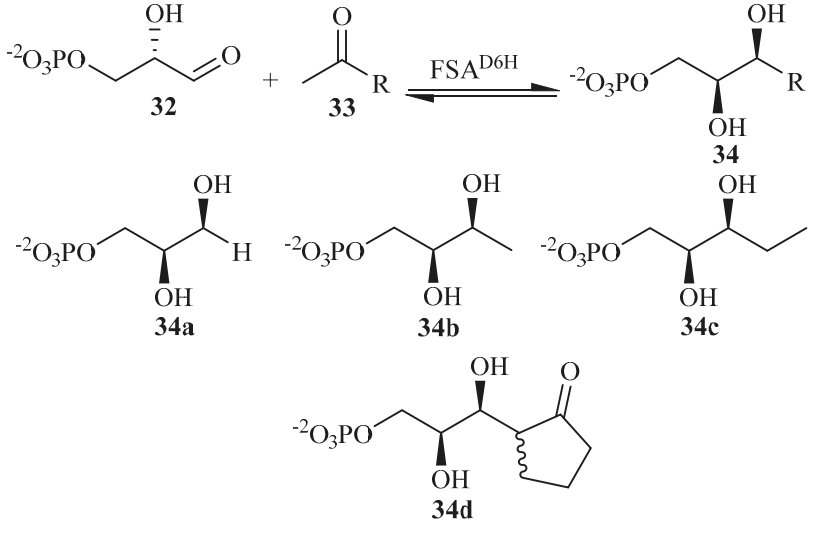


Figure 15. Synthetic experiment conducted with L-G3P as the acceptor and the various ketones as donors.

2. Aim of the research

Aldolases are highly promising enzymes for biocatalysis of stereoselective aldol and retro-aldol reactions, the main goal of this project was to develop new methods based on established approaches to produce new chemical compounds which can be used for isolation and characterization of new aldolase enzymes, and in studies of their catalytic properties and substrate selectivities, especially stereoselectivities.

Progress toward the aims:

Synthesis of phenylacetaldehyde derivatives (Paper I)

In the first paper, the aim was to compare currently available methods for the preparation of new phenylacetaldehyde derivatives in good yields, with the purpose to use these compounds as new aldolase substrates.

Stereoselective biocatalysts for carboligation reactions (Paper II).

The goal here was to study both the FSA catalyzed aldol coupling of several phenylacetaldehyde derivatives with hydroxyacetone and dihydroxyacetone and the enzyme catalyzed retro-aldol reactions of corresponding aldols.

1,4-substituted 2,3-dihydroxybutanone derivatives synthesis (Paper III).

The aim here was to synthesize new derivatives of 1,4-substituted 2,3-dihydroxybutanones that can be used as reference compounds and substrates in aldolase catalyzed reactions. Furthermore, we wanted to study the stereoselective control using two distinct methods: samarium diiodide coupling and aldol reaction with an organocatalyst reagent (cinchonine).

3. Synthesis of phenylacetaldehyde derivatives (Paper I)

3.1 Introduction

There are three major methods to prepare aldehydes, reduction of carboxylic acids or esters, oxidation of primary alcohols or carbon chain-extension of a lower-homolog aldehyde. The Rosenmund reaction is an important reductive method that can be applied for synthesis of various types of aldehydes,[74-76] this method, however, was not investigated in this work. Several reagents were tested for oxidation of alcohols to produce the corresponding aldehydes such as Swern oxidation,[77,78] manganese dioxide,[79] 2-iodoxybenzoic acid (IBX) and pyridinium chlorochromate (PCC).[79] Carbon-carbon extension is another extensively used method and was also tested in this work.

3.2 Results and discussion

The different reaction approaches were tested with *p*-methoxyphenylacetaldehyde as a model target compound. Firstly, Swern oxidation, i.e. activated dimethyl sulfoxide (DMSO) with oxalyl chloride in presence of triethylamine and dichloromethane (DCM), was tested for alcohol to aldehyde oxidation.[77,78] It was not a successful approach since the reaction was moisture sensitive and resulted in formation of side products and gave only low (~20 %) yields after column chromatography purification (Figure 16).

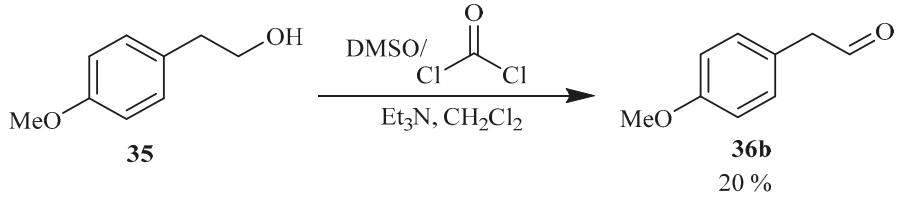


Figure 16. Reaction of *p*-methoxyphenyl-2-ethyl alcohol with activated DMSO with oxalyl chloride in presence of triethyl amine and DCM (Swern oxidation).

The Dess-Martin periodinane reagent has been shown to be useful for oxidation of alcohols to aldehyde and ketones.[80] We tested another derivative from

Dess-Marten, 2-iodoxybenzoic acid (IBX), on *p*-methoxyphenylethanol and it resulted in reasonable yields (~75 %) after column chromatography purification. However, problems with product purity due to IBX leftovers made us abandon also this approach for aldehyde synthesis (Figure 17).[79]

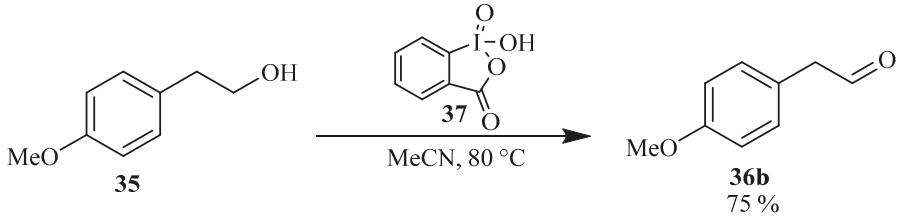


Figure 17. Reaction of *p*-methoxyphenyl-2-ethyl alcohol with 2-iodoxybenzoic acid (IBX) in acetonitrile at 80 °C.

Use of MnO_2 in dimethylformamide (DMF) likewise resulted in good yields (~75 %) after chromatography. The product was, however, the lower homolog (*p*-methoxybenzaldehyde) in which the benzylic carbon atom had been cleaved off under the reaction conditions (Figure 18).[79] Similar results have obtained with the PCC oxidation reagent. For this reason, also these methods were abandoned for the current purpose.

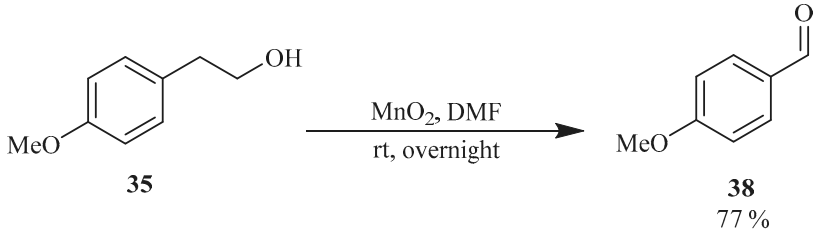
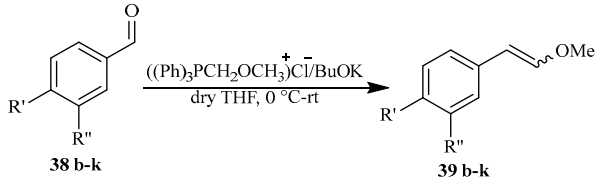


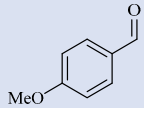
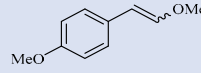
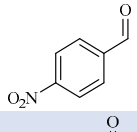
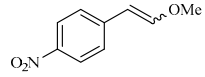
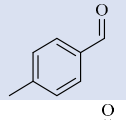
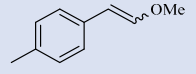
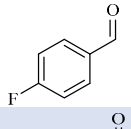
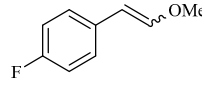
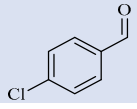
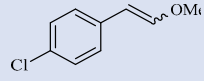
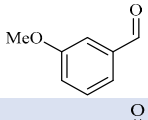
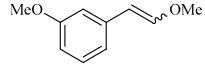
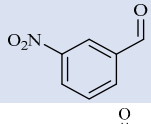
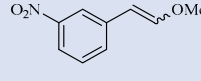
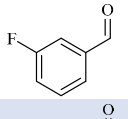
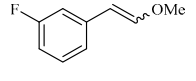
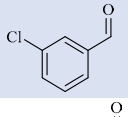
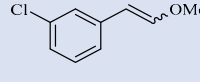
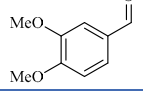
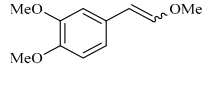
Figure 18. Reaction of *p*-methoxyphenyl-2-ethyl alcohol with manganese dioxide in DMF at room temperature.

Since several benzaldehyde derivatives are commercially available, one-carbon chain extension was the next tested approach for the preparation of the desired phenylacetaldehyde derivatives.[81-83] We applied a modified original Wittig-type reaction of the benzaldehyde with methoxymethylenetriphenylphosphonium chloride. The reagent was initially reacted with *para*-methoxybenzaldehyde as our test compound, which resulted in the corresponding enol ether intermediate with 1:1 ratio of *E/Z* isomers. The yield was excellent (~95 %) after chromatography. The procedure was repeated on nine more derivatives in all cases with excellent yields (Table 1).

Table 1. Reaction of substituted benzaldehydes **38b-k** with methoxymethylene-triphenylphosphine

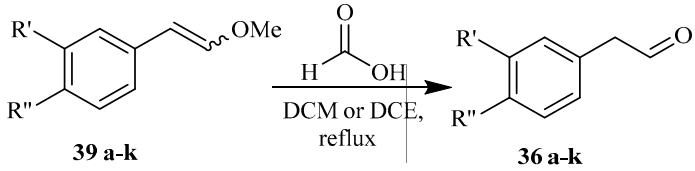


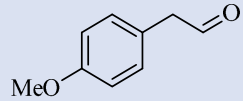
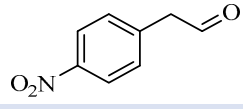
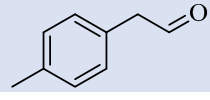
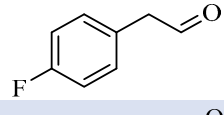
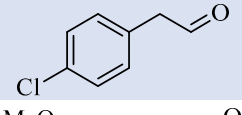
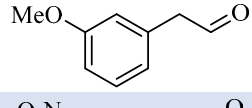
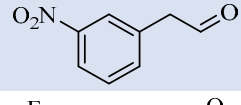
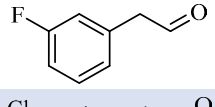
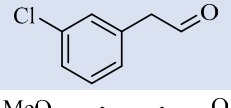
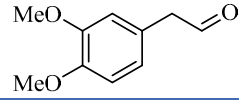
38 b-k **39 b-k** 91-97 %

Entry	Starting aldehyde (38)	Reaction time	Product (39)	Yield (%)	E/Z ratio
b		16		95	1/1
c		16		97	1/1
d		16		95	1/1
e		16		91	1/1
f		16		93	1/1
g		16		97	1/1
h		24		95	1/1
i		24		98	1/1.5
j		24		98	1/1.5
k		16		97	1/1.3

The enol ether **39b** was subsequently hydrolyzed by 10 % formic acid in DCM or 1,2-dichloroethane (DCE) which resulted in *p*-methoxy phenylacetaldehyde in 81 % yield. The yields with other derivatives were between 65-88 % (Table 2). The reaction times were different between the derivatives, for instance *m*-chloro, *m*-flouro and di-methoxy derivatives resulted in lower yields if the reaction mixtures were kept for longer times while for other derivatives, overnight reflux was required for adequate yields.

Table 2. Hydrolysis of the resulting enol ethers **39b-k** to produce phenylacetaldehydes **36b-k.**



Entry (39)	Hydrolysis time	Product (36)	Yield (%)
b	16		85
c	16		88
d	16		80
e	16		70
f	16		86
g	24		79
h	36		68
i	3		65
j	4		73
k	3		81

The best hydrolysis results in our study were obtained with 10 % formic acid in DCM and DCE. Interestingly, the addition of small amounts of water during hydrolysis resulted in more complex product mixtures, indicating initial formation of an intermediate formic acid adduct. In some cases, the crude aldehyde products were sufficiently pure for further use without chromatographic purification.

3.3 Conclusion

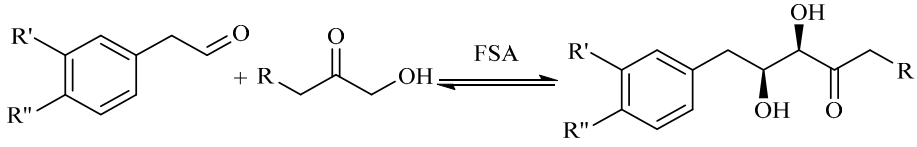
In this study, several methods to synthesize substituted phenylacetaldehydes have been tested and evaluated. The Wittig-type carbon-chain extension protocol based on treatment of aldehydes with methoxymethylenetriphenylphosphine followed by hydrolysis was found to be the most efficient method and gave consistently high yields. The used approach also allowed to a greater extent the use of commercially available reagents and starting materials as compared to the methods based on alcohol oxidation. This method was used to prepare several substituted phenylacetaldehydes that can be used as test substrates in aldolase catalyzed reactions and as reagents for aldol synthesis.

4. Stereoselective biocatalytic aldol and retro-aldol reactions using fructose 6-phosphate aldolase (FSA) (Paper II)

4.1 Introduction

Aldolases are enzymes that play an important role in biological systems. D-fructose-6-phosphate aldolase (FSA) catalyzes the asymmetric aldol cross-coupling of aldehyde and short-chain aliphatic ketones. Thus, this enzyme provides an environmentally friendly tool for efficient catalysis of asymmetric carboligation reactions. in this work, we analyzed the activities of wild-type FSA and three variant enzymes for their ability to catalyze the formation of diols and triols from aldehydes synthesized in Paper I and hydroxy- or dihydroxyacetone.

Table 3. Ketone and aldehyde substrates tested for FSA catalyzed aldol addition.



36 a-k **40 a-b** **41 a-v**

	substituent		
compound	R	R”	R’
40a	H		
40b	OH		
36a		H	H
36b		OCH_3	H
36c		NO_2	H
36d		CH_3	H
36e		F	H
36f		Cl	H
36g		H	OCH_3
36h		H	NO_2
36i		H	F
36j		H	Cl
36k		OCH_3	OCH_3

4.2 Results and discussion

In this study, positions 134 and 166 (Figure 19), which are proposed to interact with the phosphate group in F6P or G3P, were randomly mutated to allow for widening of the acceptor substrate scope. After selection of the mutant-library for enzymes that displayed improved retro-aldol cleavage activity with 3,4-dihydroxy-5-phenyl-pentane-2-one (**41a**),[84] three variant FSA enzymes were identified as putative hits. These selected FSA variants contain a hydrophobic residue at position 134 (Met, Ile or Val) combined with a small residue at position 166 (Ala or Gly) (Table 4). The R134V/S166G mutant was the most active variant in retro-aldol cleavage of derivatives of 1,4-substituted 2,3-di-hydroxybutanones **41** (Table 5).

Table 4. Amino acid sequences in wild type FSA and three mutants selected from library of mutated enzymes.

	134	166	184
WT	Arg	Ser	Ile
IG	Ile	Gly	Thr
VG	Val	Gly	Ile
MA	Met	Ala	Ile

4.2.1 Catalytic mechanism and activity

In addition to the Schiff base forming Lys85, the proposed catalytic mechanism of FSA depends on a Tyr residue at position 131 acting as a general acid/base via a catalytic water molecule, and hydrogen bonding with a Gln59 and Thr109. [71,85-87] However, a previous study showed that mutagenesis of Tyr31 into Phe has no negative effects on the aldol addition reaction between cinnamaldehydes and hydroxyacetone.[71]

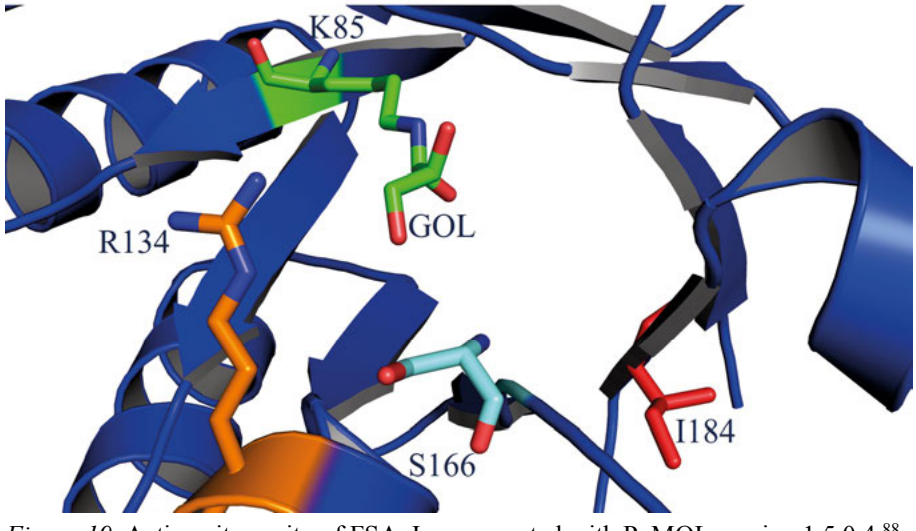


Figure 19. Active-site cavity of FSA. Image created with PyMOL version 1.5.0.4.[88]

4.2.2 Retro-aldol cleavage activities

The retro-aldol activities tested with **41a**, and the activity shown by wild type FSA is similar to what has been reported for the cleavage of F6P under modified conditions.[89] Both the R134V/S166G and R134M/S166A variants showed important increase in their activities, with the former variant displaying an 11-fold higher activity as compared to the wild type enzyme. Table 6

shows the steady state activities in the catalyzed retro-aldol reactions with aldols **41a**, **41e** and **41m** (Table 3).

Table 5. Steady state kinetic parameters for retro-aldol cleavage of **41a**, **41e** and **41m**

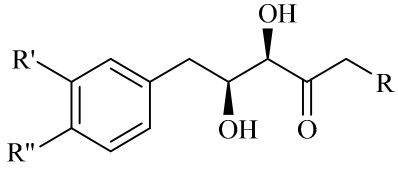
enzyme	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($s^{-1} \times M^{-1}$)
wild type / 41a	1.6±0.1	3.6±0.5	440±40
VG / 41a	18±4	28±7	640±30
IGT / 41a	1.7±0.06	5.0±0.3	340±9
MA / 41a	3.5±0.1	7.5±0.5	470±10
wild type / 41e	0.93±0.1	12±2	77±5
VG / 41e	9.1±2	15±4	610±60
IGT / 41e	8.0±3	32±10	250±20
MA / 41e	7.4±1	13±3	570±40
wild type / 41m	0.59±0.03	9.5±0.8	62±2
VG / 41m	7.9±2	9.0±3	870±100

The aldol compounds **41e** and **41m** were prepared from **36b** and **36g** in presence of **42a** using SmI_2, reagent (see Paper III for details).

4.2.3 Aldol addition activities

The aim of the initial mutagenesis and screening process was to identify variants of FSA that had acquired improved aldol addition activities as compared to non-enzymatic synthesis approaches.

Table 6. FSA catalyzed aldol reaction between phenylacetaldehyde (**36a**), and hydroxyacetone (**40a**). The formed product is (3*R*,4*S*)- dihydroxy-5-phenylpentan-2-one (**41a**).



Aldol	R	R"	R'	Aldol	R	R"	R'
41a	H	H	H	**41m**	H	H	OCH_3
41b	OH	H	H	**41n**	OH	H	OCH_3
41c	H	CH_3	H	**41o**	H	H	F
41d	OH	CH_3	H	**41p**	OH	H	F
41e	H	OCH_3	H	**41q**	H	H	Cl
41f	OH	OCH_3	H	**41r**	OH	H	Cl
41g	H	F	H	**41s**	H	H	NO_2
41h	OH	F	H	**41t**	OH	H	NO_2
41i	H	Cl	H	**41u**	H	OCH_3	OCH_3
41j	OH	Cl	H	**41v**	OH	OCH_3	OCH_3
41k	H	NO_2	H				
41l	OH	NO_2	H				

The three described variants and the wild type were tested for catalysis of aldol addition reactions with *meta*- and *para*-phenylacetaldehyde derivatives and hydroxy- and dihydroxyacetone. The results represented a range of activities compared to the wild type FSA. The most important results showed that the reaction catalyzed by the R134V/S166G variant gave the highest product formation with *p*-methoxyphenylacetaldehyde in combination with dihydroxyacetone. In addition, the R134M/S166A variant displayed the highest activity with both *p*-methylphenylacetaldehyde and di-methoxyphenylacetaldehyde combined with dihydroxyacetone. Finally, wild type FSA showed lower activities with *meta*- and *para*-substituted phenylacetaldehyde with dihydroxyacetone as compared with hydroxyacetone (Figure 20).

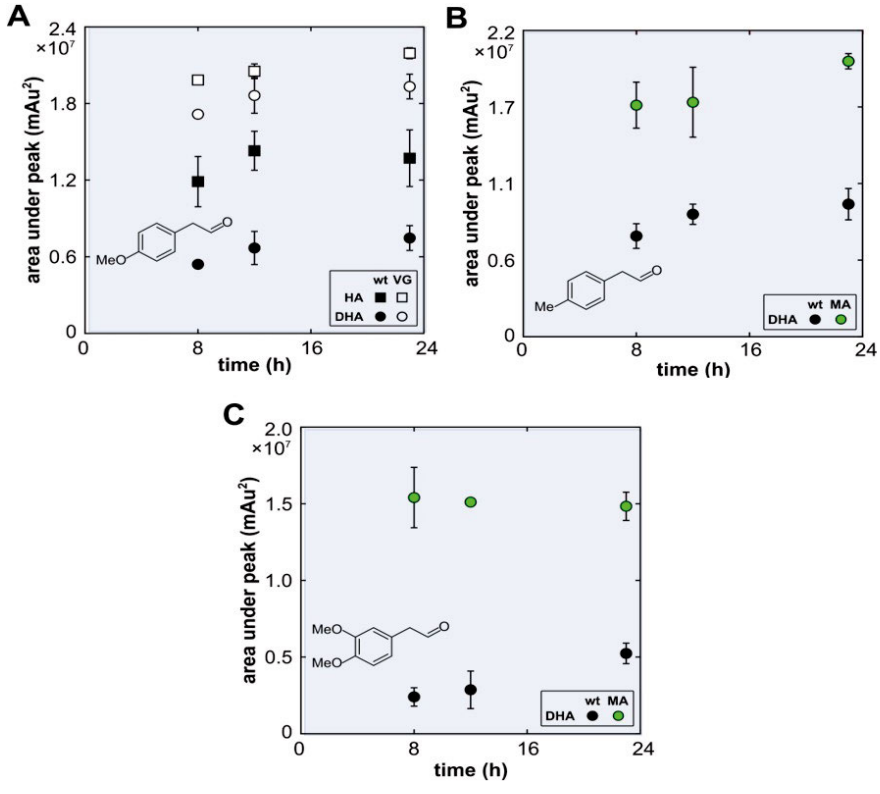


Figure 20. Formation of aldol addition product as a function of time. (**A**) Difference in catalyzed aldol product formation between wild type (filled symbols) and the R134V/S166G variant ("VG", unfilled symbols) with aldehyde **36b** and either ketone **40a** (squares) or **40b** (circles). (**B**) Difference in catalyzed aldol product formation between wild type (black circles) and the R134M/S166A variant ("MA", green circles) with aldehyde **36d** and ketone **40b** and (**C**), with aldehyde **36k** and ketone **40b**.

In addition, *p*-flourophenylacetaldehyde (**36f**) in combination of either ketone is a relatively poor substrate for all the tested enzymes as judged by the presence of substantial amount of unreacted aldehyde in the reaction mixtures even after extensive reaction time. A similar result was observed with the dimethoxy substituted **36k** when reacted with **40b**. Although the R134M/S166A variant shows the highest visible activity with this substrate combination, large amounts of aldehyde were left in the (product) mixture even after 23h incubation.

Later, the steady state kinetic parameters were determined for a chosen set of variants and substrate combinations (Table 7). In general, the wild type shows a ~10-fold decrease in turnover number with *p*-methoxy substituted **36b** with **40a** and similarly with the *p*-methyl substituted **36d** together with ketone **40b**.

Table 7. Steady state parameter values determined in the presence of 50 mM ketone (**40a** or **40b**) and varied concentrations of aldehyde at pH 8 and 30 °C.

enzyme	product	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($s^{-1}\times M^{-1}$)
wild type / 40a + 36a	**41a**	2.2±0.2	6.8±3	320±40
VG / 40a + 36a	**41a**	1.7±0.2	2.7±1	620±100
wild type / 40b + 36a	**41b**	0.99±0.3	11±4	93±10
VG / 40b + 36a	**41b**	0.49±0.1	4.5±2	110±20
wild type / 40b + 36d	**41d**	0.11±0.06	9.1±7	12±3
MA / 40b + 36d	**41d**	0.78±0.7	23±20	33±5
wild type / 40a + 36b	**41e**	0.18±0.06	5.5±4	33±9
VG / 40a + 36b	**41e**	0.35±0.04	0.91±0.4	390±100
wild type / 40b + 36b	**41f**	>0.01[b]	>2.5[b]	2.5±0.6
VG / 40b + 36b	**41f**	0.035±0.005	2.4±0.8	14±3

In the formation of **41a**, **41b**, **41d**, **41e**, and **41f** effects on both K_M and k_{cat} are observed, in all tested cases leading to increases in k_{cat}/K_M. For instance, the R134M/S166A variant shows a 7-fold higher turnover number as compared to the wild type enzyme for producing **41d**. The reaction efficiencies dropped significantly when the ketone donor changed from hydroxyacetone to dihydroxyacetone in all tested cases.

4.2.4 Stereo configuration of aldol products

Our study shows that all aldols produced enzymatically had only *syn* configuration of the asymmetric centers as suggested by the similarity of the NMR spectra with the corresponding spectra of close analogs with known structures (see chapter on synthesis of 2,3-diols). More specifically, the ^{1}H-NMR coupling constant of the protons bound to the chiral carbons and the coupling constants of the enzymatically synthesized **41e** is identical to that of the *syn*-diastereomer synthesized using SmI_2[48] and different from those of the *anti*-diastereomer.

In addition, **41d**, **41e**, **41f** and **41n** that were produced enzymatically, all elute as single peaks under chiral HPLC conditions that separate the two possible *syn*-enantiomers, and thus strongly suggests enantiopure products. **41e**, prepared by a chemical method and used as reference compound (see next section)[48] helped in the clarification of their stereoconfigurations. The enzyme produced **41e** coelutes with the first eluted peak in chiral chromatography,

which agrees with the elution profile of the FSA catalyzed product between cinnamaldehyde and **40a** that has been determined to be (3*R*,4*S*).[71]

4.3 Conclusion

Aldolase catalyzed production of asymmetric hydroxylated compounds is a powerful addition to the synthetic tool-box. In this study, the formation of aldol compounds was described in two methods although the focus was on the enzyme reactions and modification of the enzyme active site to obtain the optimum results. The most important advantage of the enzyme catalyzed reactions is the possibility to achieve enantiopure products without any further purification. The same selectivity, however, prevent production of other stereoisomers suggesting that alternative complementary methods for aldol synthesis are also needed.

5. Synthesis of 1,4-substituted 2,3-diol butanone derivatives (Paper III)

5.1 Introduction

The asymmetric aldol reaction is an important approach to form carbon-carbon bonds.[90] The use of enzymes for aldol formation has proven to be very powerful in the production of a range of aldol compounds. However, the biocatalyst substrate scope can be restricted, and the products may be of limited stereoisomeric range. On the other hand, there are synthetic reagents that display wider scopes in substrate structures but instead are not stereoselective leading to low degrees of enrichments of individual stereoisomers.

5.2 Results and Discussion

In this study, two different methods were applied to form 1,4-substituted 2,3-dihydroxybutanones from *meta*- and *para*-substituted phenylacetaldehydes and with either hydroxyacetone, dihydroxyacetone, 2-hydroxyacetophenone or methyl- and phenylglyoxal. The main goal was to assess efficient routes to the disubstituted diol products (Figure 21), and: (i) to identify facile access to any of the four possible stereoisomers of these aldols, and (ii), to produce reference compounds that can be used for benchmarking of biocatalytic (aldolase) synthetic routes.

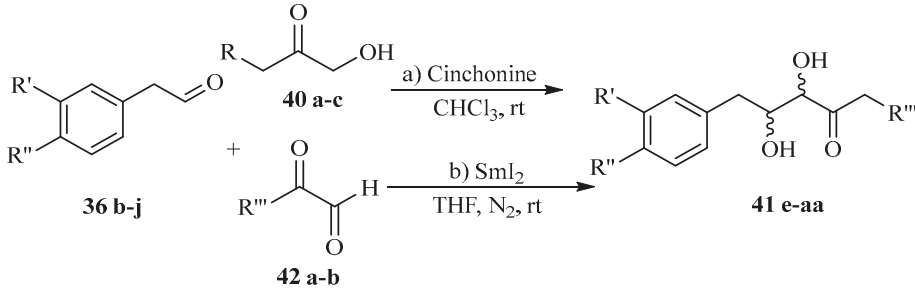
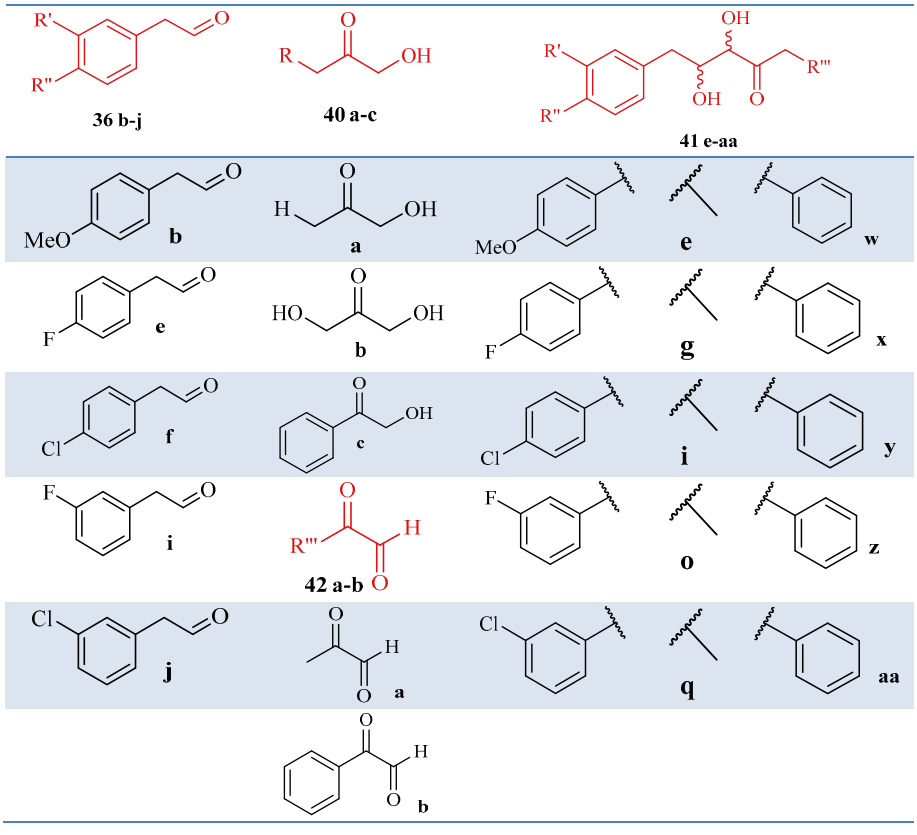


Figure 21. Synthesis of 1,4-substituted 2,3-dihydroxybutanones using either direct aldol coupling with cinchonine (**a**) or reductive cross-coupling with SmI_2 (**b**).

Hydroxyacetone, dihydroxyacetone, and hydroxyacetophenone were tested in a cinchonine-catalyzed direct aldol reaction with a series of phenylacetaldehydes containing both electron donating (methoxy) and electron withdrawing (Cl, F) substituents. The same phenylacetaldehyde derivatives were also tested in SmI_2 mediated reductive couplings with methyl- and phenylglyoxal for production of the corresponding products using reductive cross-coupling.

Table 8. Substrates and diol products.

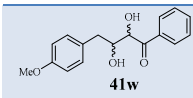
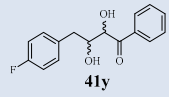
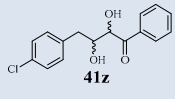
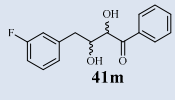
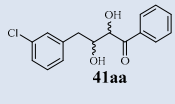


5.2.1 Cinchonine catalyzed aldol reaction

Cinchonina derivatives have been reported as useful catalysts in asymmetric aldol reactions. [91] Here, cinchonine was used to test direct aldol addition of unprotected hydroxyketones (**40a-c**) to phenylacetaldehyde derivatives (**36 b-j**), the results are shown in Table 9.

Two equivalents of ketones (**40a-c**) were used due to their commercially availability and low cost since the aldehydes are not commercially available and have to be synthesized in two steps.[92]

Table 9. Synthesized diols using SmI_2 with phenylglyoxal and cinchonine with hydroxyacetophenone, total and isomeric yields.

compound	synthesis approach	fraction *anti* (a) (%)	*anti*-enantiomer ratio (%)	fraction *syn* (b) (%)	*syn* enantiomer ratio (%)	yield (%)
 **41w**	SmI_2	42	50:50	58	50:50	60
	cinchonine	< 1%	-	> 99%	78:22	46
 **41y**	SmI_2	44	50:50	56	50:50	43
	cinchonine	20	58:42	80	75:25	41
 **41z**	SmI_2	48	50:50	52	50:50	54
	cinchonine	< 1%	-	> 99%	75:25	47
 **41m**	SmI_2	46	50:50	54	48:52	49
	cinchonine	2	62:38	98	73:27	40
 **41aa**	SmI_2	47	49:51	53	48:52	56
	cinchonine	5	57:43	95	75:25	55

This method was only useful with hydroxyacetophenone **40c** and the aldehydes; 20-30 mol-% of the catalyst in chloroform ($CHCl_3$) over 60 hours at room temperature resulted in 40-44 % yield with moderate to excellent diastereomeric excess of the *syn* isomers (*de*= 60-99 %). Our results showed that the most stereoselective reactions were with *para*-methoxy- and *para*-chlorophenylacetaldehyde (**36b** and **36f**). Hydroxy- and dihydroxyacetone were also tested under the same conditions, but without success even when reacted for longer periods.

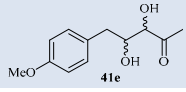
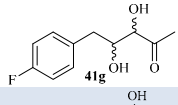
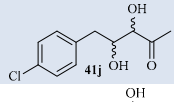
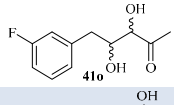
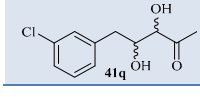
5.2.2 Samarium diiodide promoted reductive cross coupling

Previous reports on the synthesis of unsubstituted diols[48] using samarium diiodide describing couplings of glyoxals (**42a**, **b**) to phenylacetaldehyde challenged us to compare this approach with the organocatalytic route described in the previous section. Two equivalents of either methyl- or phenylglyoxal

over the aldehydes (**36b-j**) were used in the presence of 3.8 eq SmI_2 (in tetrahydrofuran (THF)) at room temperature. The reactions containing phenylglyoxal **42b** were faster and gave higher degrees of conversions than those including methylglyoxal **42a**.

The SmI_2 promoter does not favor production of particular stereoisomers, resulting in *de* and *ee* values close to zero. However, the separation of *anti* and *syn* diastereomer fractions can be easily achieved reversed phase HPLC. Hence, the use of SmI_2 extend the product scope to also provide 1-methylsubstituted aldols **41e-aa** (Tables 8,10).

Table 10. Synthesized diols using methylglyoxal and SmI_2, total and isomeric yields.

Compound	synthesis approach	fraction *anti* (a) (%) [b]	*anti*-enantiomer ratio (%)	fraction *syn* (b) (%)	*syn* enantiomer ratio (%)	yield (%)
 **41e**	SmI_2	41	50:50	59	50:50	35
 **41g**	SmI_2	48	~50:50	52	50:50	21
 **41j**	SmI_2	51	45:55	49	50:50	23
 **41o**	SmI_2	48	49:51	52	51:49	25
 **41q**	SmI_2	54	~50:50	46	51:49	22

5.3 Conclusion

In this study, two methods for the synthesis of the same product molecules were compared. The products were often mixtures of stereoisomers and thus required further purification by chromatography. Several advantages and disadvantages were observed during the synthetic work with both of these methods.

Cinchonine is a tertiary amine catalyst and has been reported as a *syn*-selective catalyst in the direct asymmetric aldol reaction of aromatic hydroxy ketones. The cinchonine catalyzed reactions, in this work, however, gave less than moderate yields despite long reaction times and did not work well with either hydroxyacetone or dihydroxyacetone.

On the other hand, samarium diiodide mediated reactions were efficient with both methyl- and phenylglyoxal in low to moderate yields, although there

was no stereoselectivity and all four possible product stereoisomers were produced. In addition, this reagent is quite expensive and very sensitive to air oxidation leading to lower yields if not performed with fresh reagent.

6. Concluding remarks and future work

In this thesis, aldehydes and 1,4-substituted 2,3-diol butanone derivatives were prepared to use as substrates and products for an aldolase enzyme. In Paper I, several methods have been tested to prepare phenylacetaldehyde derivatives, the Wittig-type carbon-chain extension was found to be the most efficient method and used to prepare several substituted phenylacetaldehydes.

In paper II, FSA enzyme was used to catalyze asymmetric aldol reactions with hydroxylated compounds as an added synthetic tool. A next step could be to further improve the catalytic activity between aldehyde derivatives and arylated ketone donors such as hydroxyacetophenone. Future generations of FSA-libraries are being constructed and will be screened in the near future.

In paper III, two methods were tested to prepare 1,4-substituted 2,3-diol butanone derivatives. The samarium diiodide mediated reaction resulted in low to moderate yields without stereoselectivity, on the other hand, the cinchonine catalyst could be used to prepare same compounds but is inactive with hydroxy- and dihydroxyacetone. In the future, other organocatalysts can be used to prepare the derivatives that cinchonine were unable to produce in more stereoselective and better yield.

7. Sammanfattning på svenska

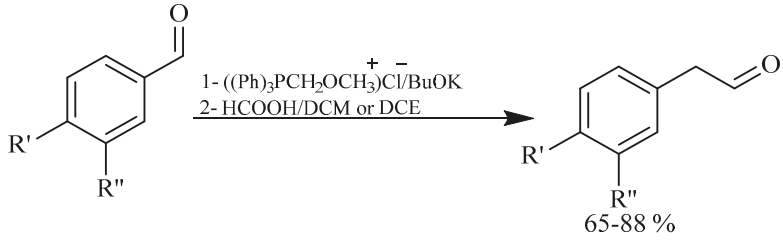
Organisk kemi definieras som kolföreningarnas kemi. Begreppet fick sin moderna betydelse då Friedrich Wöhler år 1828 visade att man ur enkla föreningar från mineralriket kan framställa urinämne, som produceras i alla däggdjurs urin och alltså tillhör de molekyler som finns i levande organismer. Innan dess trodde man att de "organiska" ämnena i levande organismer var väsensskilda från andra ämnen. Wöhlers arbete visade att det inte fanns någon sådan avgörande skillnad, och termen "organisk kemi" omdefinierades därför till "kolföreningarnas kemi", eftersom man redan visste att de allra flesta ämnen som finns i levande organismer innehåller grundämnet kol. Organisk syntes, som är ett av huvudtemana i denna avhandling, innebär att man från enkla kolföreningar i laboratoriet framställer mer komplicerade sådana, ofta identiska med eller liknande de som förekommer i levande organismer. Wöhlers framställning av urinämne kan ses som den första organiska syntesen. Sedan dess har en imponerande utveckling av den organiska kemin ägt rum, och man kan idag med hjälp av organisk syntes framställa alla de enklare typerna av organiska molekyler och även de mer komplicerade som man finner i levande organismer såsom kolhydrater, lipider, peptider, proteiner och nukleinsyror. Gränserna för vad som kan åstadkommas i laboratoriet flyttas ständigt framåt, och skiljelinjen mellan den organiska kemin och biokemin, som studerar ämnen och processer levande organismer, blir alltmer diffus. Denna avhandling illusterar detta, eftersom dess andra huvudtema är studiet av enzymer, en klassisk gren av biokemin.

Enzymer är stora organiska molekyler, oftast proteiner, som kan påskynda (katalysera) kemiska reaktioner mellan andra molekyler. Enzymerna har en speciell kavitet (det aktiva sätet) där de molekyler som ska reagera kan fastna och därvid komma varandra såpass nära att en kemisk reaktion mellan dem kan ske. Molekylerna som enzymerna på detta sätt kan få att reagera kallas substrat och de molekyler som produceras kallas produkter. Så gott som alla kemiska reaktioner i levande celler är enzymkatalyserade, endast på detta sätt kan de fås att gå med tillräcklig precision och hastighet för att upprätthålla livsprocesserna.

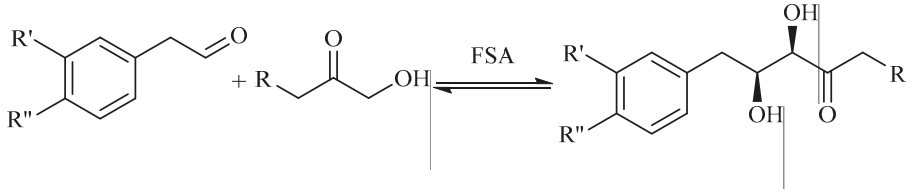
Målet för detta avhandlingsarbete har varit att med klassisk organisk syntes framställa molekyler i laboratoriet för att senare kunna användas som substrat i enzymreaktioner. De framställda molekylerna liknar i vissa delar enzymernas naturliga substrat.

Organiska molekyler klassificeras efter struktur och egenskaper i olika grupper, t.ex. alkoholer, aldehyder och ketoner. Aldehyder och ketoner innehåller en kolatom som är dubbelbunden till en syreatom (C=O-grupp), detta ger molekylen elektrofila (elektronattraherande) egenskaper. Alkoholer innehåller en eller flera hydroxylgrupper (OH-grupper). En aldol är en molekyl som ofta innehåller minst en OH-grupp och även en C=O-grupp, dvs molekylen är både en aldehyd och en keton.

Den första delen av avhandlingen beskriver försök att framställa olika aldehyder med hjälp av flera metoder, där syftet var att undersöka vilken metod som gav bäst utbyten (dvs mängd produkt i jämförelse med mängd använt substrat). Metoden som innebar att man förlängde (homologerade) bensaldehydmolekyler med en kolatom-enhet fungerade bäst, och en hel serie aldehyder framställdes i två steg och med 65–88 % utbyte.

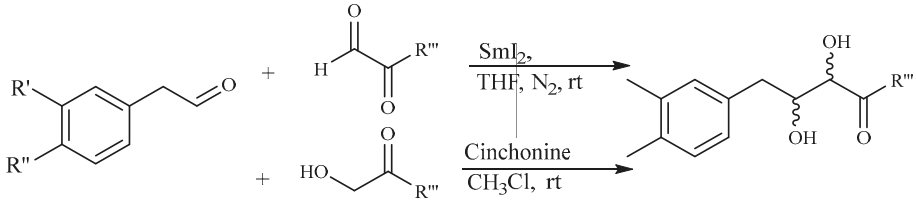


Den andra delen av avhandlingen beskriver syntes av aldolmolekyler med hjälp av FSA-enzymer (både det naturliga enzymet och varianter som framtagits genom riktad selektion). En ny framgångsrik analysmetod möjliggjorde identifikation av en enzymvariant med tio gånger högre synteseffektivitet. Samtliga enzymreaktioner var både diastereoselektiva och enantioselektiva, dvs producerade endast en av fyra möjliga aldolprodukter.



Den tredje delen av avhandlingen beskriver syntes av aldolmolekyler med hjälp av två olika organiska syntesmetoder, kallade SmI_2-metoden och chinconin-metoden. De aldehyder (fenylacetaldehyder) vars framställning beskrivits i den första delen av avhandlingen reagerades med hydroxyaceton, dihydroxyaceton och hydroxyacetofenon i närvaro av cinchonin. Endast hydroxyacetofenon gav aldolprodukter i rimliga utbyten. Reaktionerna var i hög grad diastereoselektiva, och även i en viss grad enantioselektiva. Samma aldehyder reagerades med metylglyoxal och fenylglyoxal i närvaro av SmI_2, här

erhölls aldolprodukter med båda glyoxalerna. Reaktionerna var i detta fall varken diastereoselektiva eller enantioselektiva.



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ابي امي اخوتي اختي وعائلتي الكريمة اهديكم ثمرة تعبي خلال هذه السنوات واشكر لكم دعمكم
المتواصل وحبكم حتى وصلت الى هذه المرحلة ارجو من الله ان يحفظكم ويرعاكم
زوجتي العزيزة لا اجد كلمات تعبر لكي عن حبي وشكري لدعمك المتواصل ومساندتي خلال
هذه الرحلة الشاقة التي لولا الله ولولاكي لم اكن لاستطيع انجازها
بناتي مهجة قلبي لقد اضفتن طعما للحياة بوجودكن معنا حفظكن الله وانبتكن نباتا حسنا

والحمد لله الذي تتم بنعمته الصالحات

9. References

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