Enzymatic cascade reactions involving phosphorylated intermediates: immobilization and process optimization
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Chapter 1

Introduction

1. Formation and hydrolysis of phosphate esters
2. C-C bond formation
3. Principles of enzyme immobilization
4. Process development
5. Scope of this thesis
1. Formation and hydrolysis of phosphate esters

Phosphate esters play a very important role in all organisms and in all cell processes.\textsuperscript{[1]} DNA and RNA contain phosphate in their backbone and cellular membranes have phospholipids as components. Adenosine triphosphate (ATP) forms the energy container of metabolism inside cells for the metabolism and it is also referred as "molecular unit of currency" of intracellular energy transfer.\textsuperscript{[2,5]} Phosphate has also a regulatory function in trans-membrane signalling, immune response, host-pathogen interactions, and cellular control by protein phosphorylation and dephosphorylation.\textsuperscript{[6]} Phosphate has a key role in many other biochemical reactions, such as photosynthesis, sugar and lipid metabolism, nitrogen cycle, and activation of metabolites. Enzyme cofactors are also often phosphorylated, like nicotinamide adenine dinucleotide phosphate (NAD\textsuperscript{+} and NADPH).\textsuperscript{[7]} In cells, phosphate groups are introduced by phosphorylating enzymes, such as phosphorylases or phosphotransferases (kinases), which use ATP as energy rich phosphate donor. However, many other enzymes handle phosphorylated compounds, such as aldolases, mutases, restriction endonucleases, (deoxy)ribonucleases, DNA/RNA ligases, DNA/RNA polymerases, reverse transcriptases. Further there are hydrolytic enzymes that hydrolyse phosphorylated compounds but, as described in this thesis, some of these are also able to carry out transphosphorylation reactions.

Phosphate esters play also a pharmaceutical role in prodrugs to improve drug delivery due to the high solubility and easy removal of the phosphate group, thus increasing the bioavailability of the active molecule.\textsuperscript{[8,9]} Phosphate ester containing compounds have also found application as seasoning or taste enhancers and as active ingredients in cosmetics.\textsuperscript{[10]}

Phosphate esters are also employed as valuable intermediates in synthetic chemistry as a source of organolithium compounds,\textsuperscript{[11]} as precursors to alkenes,\textsuperscript{[12]} as substrates for stereoselective displacement with Grignard reagents,\textsuperscript{[13,14]} or to activate building blocks in the synthesis of many carbohydrates.\textsuperscript{[15-17]} Given the \textit{in-vivo} and \textit{in-vitro} importance of the phosphate group, many chemical and biochemical phosphorylation methods have been developed.\textsuperscript{[18-20]} Chemical procedures are in general very reactive, give side reactions, and often require protection and deprotection steps. In contrast, the more environmentally friendly enzymatic phosphorylations allow eliminating many of these steps and often are diastereo-,\textsuperscript{[21]} enantio-, and regioselective.\textsuperscript{[22,23]}

\textit{Chemical preparation of phosphomonoesters}

Phosphomonoesters can be prepared by two kinds of phosphorus chemistry,
referred as P(V) and P(III). P(V) is a tetra-coordinated phosphorus with oxidation state +5, typical of phosphates, whereas P(III) with an oxidation state of +3 exists in two forms: one tri-coordinated like in phosphites and one tetra-coordinated like in H-phosphonates.

A tetra-coordinated P(V) has a very stable phosphoryl group (P=O) where the phosphorus atom is a strong electrophile which reacts with strong nucleophiles. P(V) compounds are very stable during storage and easy to handle, but reacts slowly compared to the trivalent P(III) derivatives. The most widely used P(V) phosphorylating agents are chlorophosphates; in particular for the phosphorylation of nucleosides phosphor yl chloride (POCl₃) is mainly used.[²⁴, ²⁵]

A good alternative to POCl₃ are N-phosphoryl oxazolidinones in combination with lithium and magnesium alkoxides which are more stable and easy to prepare and manipulate.[²⁶, ²⁷] Other P(V) compounds are phosphorodichloridates [(RO)POCl₂] and phosphorochloridates [(RO₂)POCl], which react via the formation of a phosphate triester intermediate. They have been used in many procedures, but the application is still limited.[²⁸-²⁷] Other P(V) compounds are phosphoric acid, employed in the direct equimolar condensation with alcohols,[²⁸-²⁰] pyrophosphates,[¹¹, ¹²] and phosphoric acid monoesters in combination with activating agents.[¹³-¹⁵]

The trivalent P(III) compounds, like phosphites, possess a trigonal pyramidal geometry with a lone electron pair on the phosphorus atom. This confers a basic and soft nucleophilic character to the phosphorus, which therefore reacts with soft electrophiles. However, in case of protonation or presence of electron-withdrawing substituents, the phosphorus can also react with nucleophiles. This makes P(III) derivatives very reactive and thus attractive phosphorylating agents although difficult to handle. They easily hydrolyse and spontaneously oxidise upon storage. Phosphoramidites are perhaps the most widely used in the phosphorylation of saccharides, nucleotides, phospholipids and other compounds.[⁶] They are easy to prepare, but require care in handling and have limited shelf lives. Other P(III) phosphorylating agents are phosphorochloridates, which are used with acid scavengers to yield first a phosphite triester intermediate. This is oxidized to the phosphate triester, followed by deprotection to yield the monoester.[¹⁷]

The tetra-coordinated P(III) has a strong resemblance to the P(V) with a tetrahedral structure and oxidation state +3, but does not possess the lone electron pair on the phosphorus center. However, it possesses a P-H bond not present in the other already described phosphorus geometry and therefore they are also referred as H-phosphonates.[⁴⁸] In this class of compounds, the phosphorus center is electrophilic, but less prone to oxidation than trivalent P(III) derivatives. Tetravalent P(III) compounds can be easily converted into
various P(V) derivatives using different oxidizing reagents (e.g. iodine/water, elemental sulfur, elemental selenium etc.). Some H-phosphonate derivatives need to be activated in order to react with electrophiles and form the phosphomonoester.\footnote{\textsuperscript{49-51}}

Once phosphorylated, biomolecules often need to be separated for further studies. Therefore, to simplify the product isolation it is convenient to form neutral phosphate esters by using blocking groups, easy to remove once the compound is isolated. Moreover, large biomolecules containing many phosphorylating sites require different protection and deprotection steps in order to achieve the desired protection/deprotection of phosphorylated sites have been developed. Some protecting groups are removed via a less specific P-O bond fission, whereas others via a C-O cleavage that reduces side reactions and thus are more widely used.\footnote{\textsuperscript{52}} The most common protecting groups are aryl groups, easily removed with platinum or palladium catalysts,\footnote{\textsuperscript{53,54}} basic hydrolysis,\footnote{\textsuperscript{42,55-57}} oxidative treatment,\footnote{\textsuperscript{45,58}} or addition of ZnCl$_2$ or CuCl$_2$.\footnote{\textsuperscript{59,60}} Also alkyl groups are used as protecting agents\footnote{\textsuperscript{61-66}} as well as allyl groups.\footnote{\textsuperscript{67-71}}

\section*{Enzymatic preparation of phosphomonoesters}

Enzymatic phosphorylation may replace chemical methods avoiding the need of protecting groups and harsh reaction conditions. In fact, enzymes are naturally diastereo-, regio-, and stereospecific and operate in mild aqueous conditions. Enzymes belonging to the classes of transferases and hydrolases can perform phosphorylations usually in a reversible fashion with formation and removal of the phosphate ester. The right choice of the phosphate donor is a very important factor to consider when enzymes are used. The suitability of a phosphate donor depends on thermodynamics, and in particular on the free energy of hydrolysis ($\Delta G^\circ$ hydro) of the phosphate donor and the acceptor. Using this value, it is possible to assign a phosphorylating potential to each donor and determine the efficiency of the phosphate transfer.\footnote{\textsuperscript{18,19,72}} Donors with high phosphorylating potentials are thermodynamically favoured, like phosphoenolpyruvate, adenosine 5’-triphosphate (ATP), and pyrophosphate (PP). PP$_1$ and other polyphosphates are better donors than ATP and are stable, cheap, and easy to prepare. Agents having a low phosphorylating potential are for example glucose-1-phosphate and glucose-6-phosphate. They can be successfully used if the phosphorylation is coupled to a more thermodynamically favoured reaction.\footnote{\textsuperscript{73}}

The most common phosphorylating enzymes are phosphorylases, which transfer glycosyl groups from and to phosphate, kinases, which use ATP as phosphate donor and require ATP regeneration, and phosphatases, which hydrolyse phosphate esters but can also transfer the phosphate to an acceptor.
1. Phosphorylases
Phosphorylases belong to the class of hexosyltransferases and are able to transfer phosphate to and from glycosyl molecules. For example polysaccharide phosphorylase in presence of phosphate removes a glucose unit from starch or glycogen forming glucose-1-phosphate.\cite{74,76} At the non-reducing end of glycogen, phosphorylases hydrolyse the bond between two glucose residues with the aid of a proton donated by the inorganic phosphate and bound to the active site. Such proton is transferred to the C1 position of glucose, generating glucose-1-phosphate. Phosphorylases have been used in industry for the large scale production of glucose-6-phosphate from glucose-1-phosphate by interconversion by phosphoglucomutase (EC 5.4.2.2).\cite{77,78} Glucose-1-phosphate was also used as activated donor together with glucose for the synthesis of trehalose by trehalose phosphorylase.\cite{79} Transglucosylation using glycerol-1-phosphate was also possible using analogues of glucose as glycosyl acceptor.\cite{80} A phosphorylase was also used to synthesize $\alpha,\alpha$-trehalose from sucrose in a coupled enzyme system (sucrose phosphorylase, glucose isomerase, and trehalose phosphorylase).\cite{81} Maltose phosphorylase has been used to convert maltose into D-glucose and $\beta$-D-glucose-1-phosphate, which was then coupled to an appropriate carbohydrate to yield novel disaccharides thanks to the synthetic activity of the phosphorylase.\cite{82} Trisaccharides were also synthesized from $\beta$-D-glucose-1-phosphate as donor and disaccharide as acceptor.\cite{83} Several oligosaccharides were synthesized by glycosyl transfer from $\beta$-D-glucose-1-phosphate to isokestose or nystose using kojibiose phosphorylase.\cite{84} Sucrose phosphorylase, which converts sucrose into D-fructose and $\alpha$-D-glucose-1-phosphate, can also perform a transglycosylation of benzoic acid using sucrose as donor.\cite{85} Nucleoside phosphorylase was used in the enzymatic production of viral inhibitor Ribavirin.\cite{86,87}

2. Kinases
In cells kinases usually perform transfer of mono-, di-, or tri-phosphate groups from ATP to alcohols.\cite{4,20} In some cases the kinases also use other nucleotides.\cite{88,89} Kinases are able to transfer a phosphate to different acceptors - alcohols, carboxylic acids, amines, phosphates - while converting ATP to adenosine 5'-diphosphate (ADP). Interestingly some kinases from gram-positive bacteria were found to accept also polyphosphate instead of ATP as phosphate donor.\cite{90-94} However, only kinases which transfer phosphate from ATP to alcohols have been successfully used in biocatalysis.

Hexokinase is the most widely employed kinase and phosphorylates D-glucose to D-glucose-6-phosphate (G6P). G6P can then be used in a regeneration system of nicotinamide cofactors.\cite{73,95,96} Hexokinase can also phosphorylate
position 6 of other hexoses,\textsuperscript{[97]} as well as thio- or aza- analogues.\textsuperscript{[98,99]} The pentose D-ribose-5-phosphate can be prepared with D-ribose and ribokinase.\textsuperscript{[21]} Also glycerol kinase accepts a broad range of substrates from glycerol\textsuperscript{[100]} to dihydroxyacetone and other prochiral or racemic primary alcohols.\textsuperscript{[23,101]} With these substrates yields and enantiomeric excess are usually good.\textsuperscript{[22,102]} Adenylyl kinase can phosphorylate nucleotides and their analogues,\textsuperscript{[103-105]} NAD$^+$ kinase has been used to produce the expensive phosphorylated enzymatic cofactor NADP$^+$ from NAD$.\textsuperscript{[106,107]}

The major disadvantage of using kinase for industrial purposes is that they require stoichiometric amounts of the expensive ATP. Moreover the accumulation of ADP affects the thermodynamic equilibrium by limiting the final yield. However, when a regeneration systems of ATP is present only catalytic amounts of ATP are needed.\textsuperscript{[101,108,109]} The regeneration system usually consists of an auxiliary “regenerating” kinase, which uses a cheap donor phosphate in stoichiometric amounts to regenerate ATP from ADP (Fig. 1).

![Diagram](image)

**Fig. 1:** Scheme of the phosphorylation by kinases and the ATP regeneration system

The most widely used regeneration systems are acetyl phosphate/acetyl kinase, which produces acetate as byproduct,\textsuperscript{[110-112]} and phosphoenolpyruvate/pyruvate kinase, which produces pyruvate.\textsuperscript{[113]} Other less efficient regeneration systems are propionyl phosphate/acetyl kinase,\textsuperscript{[114]} carbamoyl phosphate or methoxycarbonyl phosphate/carbamate kinase,\textsuperscript{[114,115]} and phosphocreatinine/creatinine kinase.\textsuperscript{[116,117]}

Although these regeneration systems can be optimized, most phosphate donors are not stable in solution and the by-product of the regenerating kinase can inhibit the regenerating kinase, thus requiring the dilution of the reaction mixture.

3. **Phosphatases**

Phosphatases (EC 3.1.3) are hydrolytic enzymes that remove the phosphate
group from phosphorylated substrates. In organisms they play a relevant role in microbial virulence,\cite{118} signal transduction,\cite{119} energy conversion, and metabolism.\cite{120,121} Many phosphatases are found in nature, but in particular non-specific alkaline phosphatases and acid phosphatases have been studied and details of their enzymatic mechanism are available.\cite{122} Alkaline phosphatases are non-specific phosphomonoesterases with a pH optimum between 9 and 10. They contain Zn$^{2+}$ and Mg$^{2+}$.\cite{123} In addition to their use in molecular biology for the dephosphorylation of the 5\textsuperscript{'} end of DNA,\cite{124} alkaline phosphatases have been employed in the dephosphorylation of many other compounds, such as polypropenol phosphates,\cite{125} sphingoid base 1-phosphate,\cite{126} nucleotides,\cite{127} and aromatic phosphate esters.\cite{128,129} Alkaline phosphatase can also perform transphosphorylation reactions in which a primary alcohol is phosphorylated by transfer of a phosphate molecule from the phosphate donor pyrophosphate (PP).\cite{120,131} In particular, glycerol-1-phosphate was synthesized in a regioselective but not stereoselective way to a mixture of DL-glycerol-1-phosphate. Details on the phosphorylation by alkaline phosphatase, its immobilization, and its use to produce glycerol-1-phosphate and DHAP as substrates in cascade reactions are described in Chapter 6 of this thesis.

Non-specific acid phosphatases, which are extensively studied in our group, are non-metal soluble periplasmic proteins or membrane-bound lipoproteins, which operate at acidic to neutral pH values.\cite{132} They are divided in class A, B, and C.\cite{133-135} PhoN-Sf from \textit{Shigella flexneri}, which is extensively used in the studies described in this thesis, belongs to class A1.\cite{136} Class A1 acid phosphatases have a broad substrate specificity, are able to dephosphorylate 5\textsuperscript{'}-nucleotides monophosphate (5\textsuperscript{'-NMPs}), hexoses-, pentoses-, and aryl-phosphate like pNPP and phenolphthalein phosphate.\cite{133-135} Class A2 acid phosphatases, such as PhoN-Se from \textit{Salmonella enterica ser. typhimurium} possess an even wider substrate specificity, including secondary alcohols and both 5\textsuperscript{'}- and 3\textsuperscript{'-NMPs.}\cite{137,138} Acid phosphatases are also able to regioselectively dephosphorylate 2\textsuperscript{'-carboxy-D-arabinitol-1,5-diphosphate into the 1-phosphate derivative, whereas alkaline phosphatase gives a 4 : 1 mixture of 1- and 5- phosphate derivatives.\cite{139} Polyprenyl pyrophosphates are also hydrolyzed by acid phosphatases in good yields and without side reactions.\cite{140,141} Rac-threonine can be resolved via hydrolysis of the phosphate ester with acid phosphatase, representing the only example of kinetic resolution of chiral alcohols via hydrolysis of phosphate esters.\cite{142,143} Also D-allo-threonine and D-threonine were resolved with this method.\cite{144} Acid phosphatases have also been employed in the synthesis of optically pure polyols: they dephosphorylate the sugar-like product of the aldol
reaction catalyzed by DHAP-dependent aldolases under mild conditions and without isolating this unstable intermediate.\cite{145-147}

In addition to the hydrolytic activity towards phosphate esters, acid and alkaline phosphatases are able to carry out transphosphorylation reactions in which a phosphate unit is transferred from a donor (phosphomonoesters or pyrophosphate PP$_i$) to an acceptor alcohol in two-step reaction (Fig. 2).\cite{148,149}

![Fig. 2: Mechanism of transphosphorylation, hydrolysis, and dephosphorylation of acid and alkaline phosphatases.](image-url)

The essential intermediate is an enzyme-phosphate species (E·P$_i$), which is formed after binding of the PP$_i$ in the active site and hydrolysis of one phosphate unit. E·P$_i$ usually undergoes hydrolysis in presence of water but, if a competing alcohol acceptor is present, the phosphate group can be transferred to the alcohol leading to the phosphorylated product. For this reason, the process is usually referred as transphosphorylation. The $K_m$ value for the alcohol, therefore, is a very important factor that determines whether an effective phosphorylation occurs. The enzyme can also bind the phosphorylated alcohol and release the free alcohol, forming again the E·P$_i$ intermediate. The phosphate is then released by the hydrolytic activity of the enzyme using a water molecule. Thus phosphatases are able to hydrolyze PP$_i$, transfer a phosphate to the acceptor alcohol, or hydrolyze phosphate esters. The transphosphorylation reaction is essentially reversible and the equilibrium position depends on the conditions and the amounts of reagents and products present in the reaction mixture. Acid phosphatase was successfully employed in the regioselective phosphorylation of nucleotides using PP$_i$. In particular inosine and guanosine were phosphorylated to inosine-5‘-monophosphate (5‘-IMP) and guanosine-5‘-monophosphate (5‘-GMP), widely used in food industry as taste enhancers (umami).\cite{148,150-152} Since 2003, 5‘-IMP and 5‘-GMP have been produced at multiple thousands of tons per year by Ajinomoto Co. Inc., Japan using an acid phosphatase.\cite{153} PhoN-Sf could phosphorylate only on the 5‘ position of inosine, whereas PhoN-Se yielded a mixture of 3‘- and 5‘-IMP, showing that
regiospecificity is present both in the phosphorylating and hydrolytic mode.\cite{149} Regiospecific phosphorylation of glucose yielding glucose-6-phosphate was also observed with PhoN-Sf and PhoN-Se.\cite{149,154} These enzymes have a very broad substrate specificity as demonstrated by the phosphorylation of other alcohols and sugars.\cite{154}

4. Synthetic methods towards DHAP

Dihydroxyacetone (DHA) is also phosphorylated to dihydroxyacetone phosphate (DHAP) by both PhoN-Sf and PhoN-Se.\cite{17,154,155} DHAP is a highly valuable compound used in aldol reaction with DHAP-dependent aldolases to produce chiral sugars.\cite{4,156-158} DHAP is thus a very important intermediate that can be synthesized both chemically and enzymatically. In both cases, the bottleneck is the instability of DHAP, which undergoes interconversion to glyceraldehyde-3-phosphate or degradation to dimethylglyoxal under alkaline conditions.\cite{16} In its chemical synthesis complicated multistep procedures involving protection/deprotection are required and a stable precursor is produced and converted to DHAP right before its use.\cite{159-169} On the other hand, enzymatic routes to DHAP generally involve the in situ synthesis of DHAP and integration of this step in a more complicated pathway. DHAP can be synthesized from DHA using phosphorylating enzymes, e.g. glycerol kinase,\cite{22,23} DHA kinase,\cite{170,171} or acid phosphatase.\cite{17,154,155} Also glyceraldehyde can be converted to DHAP after phosphorylation to glyceraldehyde-1-phosphate and oxidation using L-glyceraldehyde-1-phosphate oxidase.\cite{172} The phosphorylation of glyceraldehyde can be carried out with kinases,\cite{23} phytase,\cite{16} alkaline phosphatase,\cite{130,131} acid phosphatase,\cite{154,173} or chemo-enzymatically with lipase.\cite{165} \textit{Rac}-glycidol can also serve as substrate.\cite{174} DHAP can also be produced starting from larger sugars by aldol cleavage. Fructose-1,6-bisphosphate can be cleaved by fructose-1,6-bisphosphate aldolase into DHAP and glyceraldehyde-3-phosphate, which is isomerized to DHAP by triose phosphate isomerase.\cite{175} Other sugars, like sucrose, fructose, and glucose can be converted enzymatically to DHAP.\cite{176}

In this thesis, we have studied the enzymatic synthesis of DHAP by converting glyceraldehyde into DHAP by PhoN-Sf and L-glycerol-1-phosphate oxidase (Chapter 2), the synthesis of DHAP by a continuous flow method using immobilized PhoN-Sf (Chapter 3), and the phosphorylation of DHA by alkaline phosphatase (Chapter 6). Similarly, we have synthesized glyceraldehyde-1-phosphate using both the alkaline phosphatase and as well as PhoN-Sf.
2. **C-C bond formation**

Stereoselective C-C bond formation is one of the most important reactions in organic synthesis. Traditional chemical C-C coupling is tedious and complicated by iterative protection and deprotection, especially in the synthesis of carbohydrates where many hydroxyl groups are present. Moreover, stereoccontrol is also difficult to maintain in chemical procedures. Enzymatic methods can be very helpful since they are chemo-, regio-, diastereo-, and enantio-selectively controlled, as enzymes naturally are.\(^{[177-180]}\)

Enzymatic C-C coupling is typically carried out by a group of enzymes belonging to the lyases group: the aldolases. They catalyze the stereoselective addition of a nucleophilic ketone donor to an electrophilic aldehyde acceptor. In nature, aldolases are involved in the metabolism and catabolism of carbohydrates catalyzing the reversible breakdown of sugars but in vitro the equilibrium can be driven towards the synthesis of large molecules.\(^{[168,181-184]}\)

Aldolases can be classified according to the catalytic mechanism. We distinguish type I and type II aldolases (Fig. 3).

![Mechanism of type I and type II aldolases](image)

**Fig. 3:** Mechanism of type I (A) and type II aldolases (B) with the ketone donor DHAP.

Type I aldolases, mainly present in animals and higher plants, possess an enamine mechanism with the formation of a Schiff base intermediate between a lysine in the active site and the carbonyl of the donor.\(^{[185]}\) The tautomerization of this imine to enamine allows the stereoselective addition to the acceptor aldehydes.

Type II aldolases are present in fungi and bacteria and contain a Zn\(^{2+}\) cofactor in the active site. Zn\(^{2+}\) acts as a Lewis acid that polarises the carbonyl of the donor forming the nucleophilic enediolate. This then adds to the acceptor aldehyde.
with the aid of a glutamate residue that removes and donates protons.\cite{186,187} The mechanism of type I aldolases was mimicked in protein engineering to design particular catalytic antibodies with aldolase catalytic activity.\cite{188} Some chemical catalysts used in organic chemistry, such as an asymmetric Et₂Zn/linked-BINOL complex are designed according to the mechanism of type II aldolases.\cite{189} Aldolases can also be classified according to the ketone donor they depend on. The donor can be acetaldehyde, phosphoenolpyruvate, pyruvate, DHAP, glycine, or hydroxypyruvate (Fig. 4).\cite{168}

![Figure 4: Aldolases grouped according to the donor molecule they use.](image)

**Acetaldehyde-dependent aldolases**

2-Deoxyribose-5-phosphate aldolase (DERA) is the only aldolase that performs a cross-aldol reaction of two aldehydes. It catalyzes the synthesis of 2-deoxyribose-5-phosphate from acetaldehyde and D-glyceraldehyde-3-phosphate.\cite{190,191} This class of aldolases have a broad tolerance towards the acceptor substrate with a preference for the D-2-hydroxyaldehydes, and azido-, thio-, and α-methyl- substituted aldehydes. Aldehydes up to four carbons are accepted. The tolerance towards the donor is also relatively large and in addition to acetaldehyde also propanal, acetone, and fluoroacetone can be used.\cite{190,192} Since the product of the DERA reaction is an aldehyde, this can be used as acceptor substrate for a second aldol condensation.\cite{193} DERA has been used together with DHAP-dependent aldolases to produce 5-deoxyketoses with three substituents on the axial position\cite{194} and deoxy sialic acid derivatives.\cite{195} DERA was also used to prepare some intermediates of novel compounds used in cancer therapy called epothilones.\cite{196}
Chapter 1

Pyruvate- and phosphoenolpyruvate-dependent aldolases

These aldolases are used for the preparation of α-ketoacids. In vivo the PEP-dependent aldolases are involved in the biosynthesis of ketoacids and in vitro they can perform synthetic reactions thanks to the irreversible release of inorganic phosphate, which modifies the equilibrium. Pyruvate-dependent aldolases are involved in the catabolism, but can be used for synthetic purpose with an excess of pyruvate to optimize the equilibrium.\textsuperscript{[197,198]}

The most well known pyruvate-dependent aldolase is the commercially available N-acetylneuraminic acid (NeuAc) aldolase, also known as sialic acid aldolase, whose natural substrates are N-acetyl-D-mannosamine and pyruvate. This enzyme couples pyruvate and also fluoropyruvate\textsuperscript{[199]} to many aldehydes, including hexoses, pentoses, and tetroses and their substitutes on C4, C5, and C6.\textsuperscript{[198,200-203]} On C2 only small substituents are allowed, whereas on C3 only an hydroxyl group is tolerated.\textsuperscript{[204-209]} Also disaccharides are accepted to produce disaccharides with a sialic acid end.\textsuperscript{[210]} The stereoselectivity in the C-C bond formation is not intrinsic in the enzyme but depends on the structure of the substrates. The preferred S configuration on C3 of the substrate leads to a product with S configuration, whereas R C3 gives R products.\textsuperscript{[186,204,211]} NeuAc aldolase has been used for the synthesis of D- and L- sugars, sialic acid, and their isomers.\textsuperscript{[198]} An azasugar like pyrrolidine was obtained from a N-Cbz-D-mannosamine after reductive amination and was further converted into 3-(hydroxy-methyl)-6-epicastanospermine.\textsuperscript{[212]} Other aldolases from this group like KDO (2-keto-3-deoxy-octonate) and KDPG (2-keto-3-deoxy-6-phosphogluconate) aldolases form products with S configuration but accept other substrates than their natural ones with very low rate.\textsuperscript{[213,214]}

Glycine-dependent aldolases

This class of pyridoxalphosphate (PLP)-dependent aldolases catalyze the reversible addition of glycine to an acceptor aldehyde forming β-hydroxy-α-aminoacids. The most used are hydroxymethyltransferases (SHMT) and threonine aldolases (ThrA), involved in the degradation of threonine.\textsuperscript{[215]} Although they have been used extensively for the resolution of racemic β-hydroxy-α-aminoacids, little is reported on the C-C formation. Because of its broad substrate acceptance (aldehydes up to ten carbon units) L-threonine aldolase is mainly used but it shows low stereospecificity in the C-C coupling.\textsuperscript{[144,216-218]} However α-substituted hydroxaldehydes were stereoselectively coupled to the donor. Unsaturated aminoacids were synthesized starting from thiophenol-substituted aldehydes, since α,β-unsaturated compounds were not accepted.\textsuperscript{[215,219,220]}
**DHAP-dependent aldolases**

The most studied aldolases are DHAP-dependent and they synthesize a ketose-1-phosphate from the nucleophilic donor DHAP and the electrophilic acceptor aldehyde.\cite{221,222} This class of enzymes is extremely selective for the ketone donor DHAP, but is less strict towards the acceptor aldehyde. The aldol coupling generates two new stereocenters at C3 and C4, thus in principle four stereoisomers can be formed. Since each aldolase generates a single isomer and four aldolases with different specificity are available, each isomer can be easily obtained by choosing the appropriate aldolase (Fig. 5). However, the stereospecificity is total at C3, whereas in some cases the specificity for C4 depends on the structure of the aldehyde.\cite{221,222}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig5.png}
\caption{Stereospecificity of the four DHAP-dependent aldolases.}
\end{figure}

The isomer with configuration $3S,4R$ is formed by the fructose-1,6-bisphosphate aldolase (FruA). In vivo, this enzyme is involved in the glycolytic pathway and performs the aldol addition of D-glyceraldehyde-3-phosphate to DHAP to give fructose-1,6-bisphosphate. FruA is present in both procaryotes and eucaryotes and the type I and II are available. The type II bacterial FruA is in general more stable in solution than the mammalian counterpart.\cite{223} The aldolase isolated from rabbit muscle (RAMA) is the most frequently employed aldolase of this kind.\cite{224,225} RAMA exhibits a stringent specificity for the donor since analogues of DHAP (thio- and aza-analogs, carbonated forms, phosphate bioisosteres and borates) are only poorly accepted, showing only 10% of the activity towards DHAP.\cite{227,228} On the other hand more than 100 aldehydes have been successfully used as acceptors (aliphatic, heteroatom-containing substituted aldehydes, monosaccharides), with the exception of sterically hindered, aromatic, and $\alpha,\beta$-substituted aldehydes.\cite{175} The $\alpha$-substituted aldehydes can
also be used in racemic mixtures since the enzyme is specific only for one isomer. For example FruA reacts with D-glyceraldehyde-3-phosphate 20 times faster than with the L isomer, whereas other aldolases accept the L isomer only.\cite{4,5} Because of its properties FruA is the most widely employed enzyme in organic chemistry for C-C bond formation, especially for the synthesis of sugars and their analogues, hetero-containing carbohydrates, deoxysugars, halogen-substituted, higher-carbon, and $^{13}$C-labelled sugars.\cite{6,7,8,9,10,11,12} L-fuculose-1-phosphate aldolase (FucA), L-rhamnulose-1-phosphate aldolases (RhuA), and tagatose-1,6-bisphosphate aldolase (TagA) give rise to the products with configuration $3R,4R$, $3R,4S$, and $3S,4S$, respectively (Fig. 5). In vivo FucA and RhuA perform the aldol coupling between DHAP and S-lactaldehyde and are present in microorganism only as type II aldolases. TagA couples D-glyceraldehyde-3-phosphate to DHAP and is present in nature either as type I or type II.\cite{13,14,15} Similarly to FruA, these aldolase are very tolerant towards the acceptor aldehydes, but are very specific for DHAP. However, they are not very stereospecific in the C-C coupling: the configuration at C3 is usually maintained whereas the specific configuration at C4 depends on the enzyme and on the structure of the acceptor aldehyde.\cite{16} Therefore diastereomeric mixtures are often obtained, limiting the large scale industrial application of these aldolases.\cite{17,18} However, the stereoselectivity can be improved by choosing aldehydes with specific structures and via protein engineering.\cite{19}

Only one special aldolase has been found able to accept other substrates than DHAP. The so called fructose-6-phosphate aldolase (FSA) is a type I aldolase from \textit{E. coli} which is able to accept DHA instead of DHAP yielding a product with configuration $3S,4R$.\cite{20,21} The use of DHA, which is much more stable and cheap than DHAP, circumvents the issues of the use of DHAP. Moreover, the final dephosphorylation is not needed after aldol coupling. Other DHA analogues, like monohydroxyacetone, glycolaldehyde, and 1-hydroxy-2-butanol, are also accepted.\cite{22,23} However, the drawback of FSA is the stereospecificity of the product configuration which is $3S,4R$, limiting the use of FSA to the synthesis of only one of the four possible stereoisomers. Bypassing the use of the unstable DHAP is possible also using arsenate and borate esters which can replace the phosphate in enzymatic reactions.\cite{24,25}

\textit{Transketolase}

Transketolase is an aldolase which catalyzes the transfer of a 2-C hydroxyacetyl group from a ketose phosphate (D-xylulose-5-phosphate) to an aldol phosphate (D-ribose-5-phosphate) to form D-sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate (Fig. 6A).\cite{26,27} It requires thiamine diphosphate (ThDP) and Mg$^{2+}$ as cofactors and is present in bacteria, yeast, plants,
mammalian,[272-277] and also as recombinant enzyme.[278,279] The successful use of transketolase in organic synthesis lies in its broad donor specificity. Xylulose-5-phosphate, sedoheptulose-7-phosphate, and fructose-6-phosphate can be used as donor of the two-carbon unit. Above all, hydroxypyruvate (HPA) is the preferred substrate in organic synthesis applications because in the two-carbon unit transfer CO₂ is released, which is easily removed making the reaction irreversible (Fig 6B).[280]

![Chemical structures](image)

**Fig. 6:** Transketolase natural reaction with the transfer of a two-carbon unit (A). Scheme of the *in-vitro* transfer of a two-carbon unit from hydroxypyruvate to an aldehyde (B).

Transketolase also shows a broad substrate tolerance[281] and complete stereospecific in the creation of the new stereocenter, which is always the S configuration.[271,282-285] The substrate aldehydes are in general α-hydroxyaldehydes, aldoses, and also α,β-unsaturations are tolerated.[280,233,271,285-302] In addition to the stereoselectivity in the C-C bond formation, transketolase is also selective for (R)-2-hydroxyaldehydes, thus forming an hydroxyketone with configuration (3S,4R) as in the case for FruA.[301,303-308] For this reason, FruA and transketolase are used as complementary tools to synthesize the same products starting from different substrates. Moreover, transketolase has the advantage of being more stable in solution than RAMA.[175]

Transketolase has been used for the synthesis of 6-deoxy-L-sorbose, a precursor or furaneol, a caramel-like flavour[309] and in the synthesis of the unnatural sugar 4-deoxy-D-fructose-6-phosphate.[310] A multi-enzymatic approach using the DHAP-dependent aldolase RAMA and transketolase was developed to synthesize D-xylulose-5-phosphate.[295,311] A chemo-enzymatic synthesis led to N-hydroxy-pyrroldine, a potential glycosidases inhibitor.[296]
3. Principles of enzyme immobilization

Although the efficiency of biocatalytic processes has been widely recognized for the mild reaction conditions and high selectivity and specificity,[179,312-314] large-scale application and commercialization of enzymes is still limited because of the lack of economic feasibility. Common issues are low long-term operational stability and difficult reusability.[315-317]

Among the efforts to improve operations based on enzymatic processes, enzyme immobilization offers many advantages. It improves the operational stability, recovery and reusability of enzymes, facilitates the separation from the products and prevents protein contamination in the product.[318-320]

Four different immobilization strategies are described in Fig. 7. The enzyme can be trapped (Fig. 7A) or encapsulated (Fig. 7B) on a carrier or a solid support. Self immobilization (Fig. 7C) allows to obtain a carrier-free immobilized enzyme.[321] Entrapment is a method to cage the enzymes in gels or fibers without modifying its structure (Fig. 7A).[322-325] The synthesis of the sol-gel matrix takes place in presence of the enzyme, is usually benign, and protects the enzyme from the contact with the environment, limiting the denaturing effect of gas bubbles, organic solvents, and mechanical stress.[320] Nevertheless, enzyme leaching and mass transfer limitation can occur. Typical materials are alginate, chitosane, collagen, cellulose, and carrageenan. Porous glass is the most used in biosensors applications. Hybrid carriers has also been created in order to prevent enzyme leakage and increase mechanical stability.[326] Nanostructure supports are also very convenient and used in fine chemistry, biomedicine, biosensors, and biofuels.[327-330]

![Fig. 7: Enzyme immobilization strategies: entrapment (A), encapsulation (B), self immobilization (C), solid support (D).](image)

Encapsulation also protects the enzyme from the external environment similarly to entrapment (Fig. 7B). To confine the biocatalyst, membrane encapsulation in liposomes and microcapsules is common.[331-334] However, not many biocatalytic methods are based on this strategy because it does not allow an efficient mass transfer.[335]
Self-immobilization by cross-linking allows to immobilize enzymes without any support by using bifunctional agents like glutardialdehyde (Fig. 7C). Cross-linked enzyme crystals (CLECs) are obtained by crystallization of the pure enzyme and following cross-linking. Cross-linked enzyme aggregates (CLEAs) are prepared by precipitation of enzymes from a water phase by the addition of organic solvent such as acetone, ammonium sulfate, ethanol, or 1,2-dimethoxyethane. Afterwards, a cross-linking agent like glutardialdehyde is added.

The immobilization on solid carriers is based on a specific interaction between the surface of enzyme and the support (Fig. 7D). The polar groups of the amino acid residues of the enzyme (amino groups of lysine and acidic groups of glutamic acid) but also sugars in glycosylated proteins are usually involved. Covalent, non-covalent, or ionic interactions can take place. Carriers should be inert, resistant, stable, and able to maximize enzyme activity/stability and minimize inhibition. The physical and chemical structure of the carrier can be varied and porous or non-porous, hydrophobic or hydrophilic materials like polysaccharides, synthetic polymers, or glass are used. As general rule, a large surface area is a key factor for the interaction of the carrier with the enzyme and its stabilization, thus small particles or porous materials are often used. Non-covalent adsorption takes place via hydrophobic or hydrophilic interactions, van der Waals forces, or hydrogen bonding. This immobilization method is mild and the structure and activity of enzymes is maintained, but leaching from the carrier in water media can also occur. Supports like EP-100 polypropylene, Accurel MP1004 polypropylene, octyl-silica and octyl-agarose bind enzymes via Van der Waals interactions. For binding to occur, hydrophobic regions on the surface of the enzyme need to be present and this method is used to immobilize lipases. Cellulose, lignin, Avicel, Celite, porous glass, clay, silica gel are common hydrophilic carriers which form hydrogen bond with hydrophilic amino acids present on the surface of the enzymes or sugars in glycosylated proteins. Ionic interactions can be used to immobilize enzymes via ionic and strong polar bonds with charged residues. Depending on the net charge of the enzyme, cation or anion exchanger may be used. However, the strength of the interaction depends strongly on pH and concentrations of salts not only during immobilization but also during operation. Therefore, leaching is very frequent under operational conditions though it can be avoided with a carrier of appropriate pore size. Covalent binding involves the formation of multiple bonds between the side chain of amino acids of the enzyme and the functionalities present on the carrier. Being the strongest interaction, this method prevents leaching and is preferred in water-based processes. The reactive groups are attached to the carrier through a spacer of
variable length. Longer spacers allow more conformation flexibility to the protein and may be favourable if conformational changes are required for the enzymatic activity. Shorter spacers limit structural flexibility and thermal vibration, preventing unfolding and denaturation. The nucleophilic amino group of lysine, arginine, or histidine attacks the functionality on the support, usually epoxide or aldehydes ([344],[349],[349-351]). Very popular epoxy-functionalized carriers are Eupergit C and Eupergit C 250 L.[352] In order to react with the enzymes, amino groups on the support need to be activated by multifunctional reagents like glutardialdehyde. Also sugar residues of glucosylated proteins can be used.[344,345] Other methacrylic carriers like Sepabeads can have either epoxy or amino groups. Other supports are agarose, glyoxyl agarose, and aminated glyoxyl agarose (MANA). Carbobdiimides can form amide bonds with carboxylate groups on the carrier and vice versa.[343]

1. Epoxy-functionalized

![Epoxy-functionalized](image)

2. Amino-functionalized activated with carbodiimide

![Amino-functionalized activated with carbodiimide](image)

3. Amino-functionalized activated with glutardialdehyde

![Amino-functionalized activated with glutardialdehyde](image)

**Fig. 8:** Scheme of the most common covalent binding of amino or acidic groups of enzyme on functionalized carriers.

The choice of the appropriate immobilization strategy is usually a trial and error approach whose outcome is a compromise between activity, stability, method simplicity, purpose of use, and cost effectiveness. First of all the enzyme should retain its activity during and after immobilization. The reactivity of the protein groups during binding is enhanced by alkaline pH values, high temperature, and
long reaction times,[353] but these extreme conditions may cause denaturation and activity loss. In particular multi-interactions of the enzyme with the support result in the distortion of the protein structure and deactivation.[318-319,344]

The activity of an immobilized catalysts may also be limited by the diffusional rate of substrates and products.[354-355] An inefficient mass transfer limits not only the reaction rate but can also cause product inhibition or an unwanted pH gradient. The most simple solution is to decrease the particle size or immobilize the catalysts on the outermost part of the support.[356-358] Good diffusion can be achieved with non-porous supports whereas big pore size is preferable in case of porous carriers because a large pore diameter compared to the substrate maintains the diffusion coefficient close to that found in water.[359] For example, proteases immobilized on macroporous supports were still able to hydrolyze macromolecules like proteins.[360]

Immobilization is key in the reusability of enzymes. It rigidifies the structure of enzymes, reduces the mobility, and improves the intrinsic stability. Good carriers should thus have a large internal surface and a high concentration of functional groups available for binding.[344] The most powerful strategy to obtain stable catalysts is the multipoint covalent attachment on a short spacer of solid supports. Moreover, porous supports protects most enzymes from protein aggregation, proteolysis, and from the interaction with hydrophobic surfaces - bubbles or drops of immiscible solvent - that cause enzyme inactivation.[344,361] Multimeric proteins can also be immobilized but usually subunits dissociation occurs.[362] Full maintenance of the quaternary structure of proteins is ensured by the cross-linking in CLEAs and CLEC preparations.[363] On the other hand immobilization on solid support depends on the complexity of the multimeric enzyme: dimeric enzymes are often stabilized by adsorption and covalent attachment,[364] but for trimeric and tetrameric proteins usually heterofunctional supports are preferred carrying epoxy, glyoxyl, and adsorbent groups.[365] In more extreme circumstances, stabilization is achieved by further chemical cross-linking with polymers and other post-immobilization techniques.[364,366]

Another important factor to consider is the mechanical resistance of the support and its physical properties, in particular when the immobilized enzyme has to be used in an industrial large-scale setup.[361] CLECs and CLEAs are not very mechanically resistant towards stirring. Depending on the reactor configuration, coupling to pre-existing solids is the best solution. Flexible materials such as agarose and cellulose are used in reactors with mechanical stirring, whereas stronger carriers like porous glass and silicates are used in fixed bed reactor. Polyacrylic supports stable at high pressure are commonly used in column reactors.

Simplicity of the immobilization procedure is also a factor for the large scale
application of immobilized enzymes.\[^{[361]}\]

Some procedures also allow the immobilization of impure or partially purified enzymes, whose use is preferred in industry because it limits the cost of purification.\[^{[367,368]}\] The simplest method to bind a specific target protein in an impure preparation is affinity immobilization, which couples protein purification with immobilization. In fact, both techniques exploit a specific feature of the target protein that makes it different from the other contaminants. The matrix can be either be precoupled to an affinity ligand for a target protein or the enzyme itself can be conjugated with an affinity ligand toward the matrix, like a His tag. Other options are the selective precipitation in CLEAs preparations\[^{[369]}\] and the physical adsorption on solids.\[^{[370]}\] However, to avoid desorption and enzyme leaching highly activated supports which adsorb most enzymes are necessary, thus reducing the purification efficiency.\[^{[371,372]}\] The introduction of tags in enzymes with high and specific affinity for the support is probably the best option to achieve simultaneous purification and immobilization. His-tagged enzyme can be immobilized on solid metal chelates,\[^{[373-377]}\] whereas poly Asp, Glu, Arg, or Lys tags are adsorbed on ionic exchange materials at moderately high ionic strength.\[^{[378-382]}\] Nanomagnetic supports are used to immobilize an enzyme directly in the reactor where the enzyme is expressed.\[^{[383]}\]

Co-immobilization of different catalysts on the same support is possible. This is preferred when several enzymes are used to get a product from a substrate in cascade reactions,\[^{[384]}\] because it enhances the substrate/product diffusion and availability especially on porous supports.\[^{[361]}\] However, the immobilization procedure has to be unique and all the catalysts should have a similar operational stability. This in practice is very difficult to achieve. In some cases, the independent immobilization of each component under their respective optimal conditions is favourable. Nevertheless, co-immobilization is necessary when the intermediates of the cascade reaction are unstable, giving side-reactions with other products\[^{[385]}\] or in redox reactions requiring cofactor regeneration.\[^{[386]}\] Some combi-CLEAs have been successfully prepared for this purpose.\[^{[387,388]}\]

4. Process development

Biocatalytic processes are used in industry mainly for the synthesis of chiral compounds in the pharmaceutical sector,\[^{[312,313,389-390]}\] but in the last years also applications in the production of cosmetic ingredients, polymers, and biofuels have been developed. Soluble enzymes are usually employed in the early stage of
the process development because just by changing the concentration of catalysts and the reaction conditions the reaction can be easily optimized. However, in order to render the process economically competitive, the expensive catalysts have to be recycled. In industry, recycling of soluble catalysts is carried out with ultrafiltration membranes which usually require a high energy consumption. Therefore, the easy reuse of immobilized enzymes has found much larger application also in the industrial multi-ton scale production. For example high fructose corn syrup is produced by glucose isomerase immobilized on an inorganic carrier, penicillin G acylase covalently attached to polyacrylate is used for the production of penicillins, and low-lactose milk is produced by lactase immobilized on an ion-exchange resin. Fat modification is carried out with T lipase on silica, whereas lipase B from C. antarctica is used for resolutions of pharmaceutical intermediates. It has been estimated that an effective industrial process should possess a productivity of around 10 tons of product per kg of immobilized enzyme used. This means that the immobilized biocatalysts should be extremely stable and be reused for at least 200 cycles. This is exemplified by glucose isomerase that produces the high fructose corn syrup: it possesses an operating half-life of one year. The economical feasibility of a technology is not only based on the intrinsic efficiency of the catalysts, but also the development of an efficient process is very important. The design of a biocatalytic process is based on a mathematical model derived from engineering principles to save manpower and time typical of experimental investigation. Bioprocess engineering thus has to complement enzyme technology with the development of more stable and reusable biocatalysts to be used in very efficient processes. Unlike chemical reaction engineering, whose design paradigm has already been established, the design of biocatalytic processes has been developed only recently. In 1996, Lily and Woodley developed a systematic approach to identify the most suitable options for efficient biocatalytic process and integrated in this system all processes, from the production of the biocatalyst to the reactor selection and product recovery. Such rational design helps to identify a number of process constrains to allow the elimination of some alternatives and consider only some others in an iterative way until a model is developed. Single-enzyme processes usually possess a low number of constrains which facilitate the optimization of the process. In contrast, in multi-enzymatic reactions the complexity increases and the process optimization is much more difficult. Multi-enzymatic processes have been validated at laboratory scale for the in situ cofactor regeneration, deracemization and cascade catalysis for the production of pharmaceuticals and fine chemicals. A few examples at pilot and industrial scale have been also reported.
Chapter 1

In multi-enzyme processes two or more enzymes catalyse reactions in a defined sequence of steps, mimicking the metabolic activity of living microorganisms. However, in vitro cascade reactions developed for synthetic purposes are based on the simultaneous use of catalysts, which may come from different hosts. Therefore, it is likely that the enzymes operate at different conditions and have different reaction rates.\[^{399,403,405,406}\] If the enzymes do not operate in the same way under a common set of conditions, the reactions have to be carried out separately and several reactors are required. One example is the five-enzyme synthesis of 12-ketoursodeoxycholic acid using dehydrogenases to catalyze two oxidative steps followed by reduction.\[^{407}\] The membrane reactor was designed in order to separate the oxidative from the reductive reactions and eliminate the formation of by-products.\[^{407}\]

To facilitate the process design, it is essential to analyze the optimal conditions and compatibility of enzymes to make them work under the same operational conditions. Usually maximum productivity can be achieved only by means of a balanced compromise. In this way one-pot cascade processes have been developed with two or more enzymes active and stable under the same set of conditions (media, temperature, pH,\(_i\)). If this is accomplished, multiple steps can be carried out within a single reactor. One example is a four-step enzymatic cascade for the synthesis of non-natural carbohydrates from glycerol.\[^{16}\]

Although the reaction was carried out in one pot, a controlled switch of pH was required in order to alter selectively the activities of the different enzymes. A similar cascade is reported in Chapter 2 of this thesis: the use of another enzyme allows to perform the cascade in one pot without the need of the pH switch.\[^{173}\]

In one-pot strategies the separation and the purification of the intermediates is completely avoided, reducing the downstream processes and operating costs. Moreover the intermediates are generated at low concentrations and their accumulation and possible inhibitory effects are limited.\[^{395}\] This is particular advantageous from a synthetic point of view since the product of one reaction is the substrate for the consecutive reaction, and this allows the optimization of the thermodynamic equilibrium towards the formation of the desired product.

The critical step in the development of a biocatalytic process on large scale but also for a lab-scale experiment is the choice of an appropriate reactor. This usually depends on cost, space, mass transfer, kinetics, and reusability of the catalyst.\[^{398,408}\] Two major classes of reactors can be identified: the first includes stirred tank reactor (STRs) where the concentration of every compound is homogeneous and can be described with ordinary differential equations. The second class are characterized by the presence of a gradient of concentration through the reactor, which require partial differential equations for their description. Reactors with these characteristics are plug-flow reactors PFR,
packed-bed reactors PBR, and fluidized-bed reactors FBR. Membrane reactors share the characteristic of these two classes.

Batch stirred-tank reactors (BSTRs) were first developed as natural scaling-up of traditional synthetic setup on lab-scale (Fig. 9). They require a very simple setup, equipment, and ease operation, but the volumetric productivity is low. The most used batch reactors are the continuous stirred tank reactors (CSTR), which are operated in continuous mode limiting also the possibility of substrate and product inhibition (Fig. 9). However, the mechanical stress given by the stirring cause a very rapid inactivation of the immobilized biocatalysts which have to be soon replaced.\[393\]

![Diagram](image)

**Fig. 9:** Scheme of a batch stirred tank reactos (BSTR) on the left and a continuous stirred tank reactor (CSTR) on the right.

The mechanical stress is less invasive in bubble column or air-lift reactors where the reaction medium is kept mixed and aerated by the introduction of air from the bottom of the reactor (Fig. 10). Similarly, in a continuous fluidized-bed reactor (CFBR) the reaction mixture is mixed via the continuous feed from the base of the reactor (Fig. 10). These two types of reactors are used with highly viscous reaction media or when contact/reaction of a liquid and a gas is required.\[409\] However, not many processes using this technology is reported, but the advantages in multi-synthetic processes are clear.

Continuous packed-bed reactors (CPBRs) with immobilized enzymes are often used because they provide a higher volumetric productivity and they can be run in continuous mode. In this way, mechanical stress given by the stirring is eliminated, the products are very easily separated from the catalyst, and less side-reactions or product inhibition occur.\[398\] PBRs can have different configurations according to the format of enzyme used. In Fig. 11, a single-step packed-bed reactor is depicted in panel A. A two-step reaction with immobilized enzymes is carried out with two beds (B), with alternate multiple beds (C), with mixed beds (D) or with one bed of immobilized enzyme plus one soluble enzyme (E).\[395\] Nevertheless, the mass transfer can be inefficient and this type of reactor
is not appropriate for multi-phase reactions. The flow-through has to be finely controlled. Adjustment of pH and in situ product removal are also complicated. However, sequential PBRs can help to avoid these problems.\textsuperscript{[410]}

![Diagram of airlift reactor and continuous fluidized bed reactor (CFBR)](image)

**Fig. 10:** Scheme of a airlift reactor on the left and a continuous fluidized-bed reactor (CFBR) on the right.

![Diagram of continuous packed-bed reactor (CPBR)](image)

**Fig. 11:** Scheme of packed-bed reactors (PBRs). In A a single-enzyme PBR is depicted. In B, C, and D a two-enzyme process is carried out with two beds, alternate beds, and mixed bed, respectively. In D a bed of immobilized enzyme is combined to one soluble enzyme.

In multi-enzymatic processes, it is likely that the enzymes have different half-lives and become inactivated at different moments. Therefore it is necessary to replace the catalysts individually.\textsuperscript{[405]} To facilitate this operation immobilization carriers of different size should be used. The different catalysts can be separated by size exclusion through several sieves. Compartmentalization of the reactor allows confining each catalyst to a specific zone. The selective replacement of the catalysts can be easily accomplished by using sieves in case of immobilized enzymes, or ‘tea-bags’ with soluble enzymes. “Tea-bags” are pockets made of ultrafiltration membranes that are easily replaced when needed.\textsuperscript{[407]} A third method is to use a combination of solid and magnetic carriers for the immobilization of enzymes. The catalysts are separated and selectively replaced.
by using the combination of magnetic field and filters.\textsuperscript{[411]} In the continuous packed-bed reactors, the activity loss can also be compensated by reducing the inlet feed flow rate, with no effect on the conversion but with lower space/time yield.

Another important technical detail in the reactor design concerns the type of supply of substrates, control of intermediates, and product removal.\textsuperscript{[409]} Both a single feed at the start of the reaction or sequential addition of substrates at different stages of the reaction are possible. The choice depends on the inhibitory effect of the substrate and on the equilibrium of the reaction. In multi-enzymatic reactions it may be necessary to remove or concentrate the intermediates to overcome inhibition phenomena and optimize the equilibrium. If the first reaction is inhibited by the intermediate, it is necessary to dilute the mixture. If the second reaction is not favoured, the intermediates should be present at higher concentration. One way to achieve this is by kinetic controlled adsorption and release of intermediates using resins.\textsuperscript{[412]}

Separation of products and by-products is possible between each reaction step or in the downstream process. In-situ product removal helps to purify the product while optimizing the kinetic equilibrium of the reaction.\textsuperscript{[314]} Experimental work can help to determine the performance of each reactor setup, but the optimization of the kinetics of the conversion is far more complicated because many factors are involved. The optimization of multi-enzymatic processes is therefore rather complicated. Computational tools to simulate the behaviour of multi-enzyme processes under different conditions have been developed as well as integral kinetic models describing the reactions in different reactors. To optimize the process a model is built with the aid of mathematical expressions, which help in the decision-making and are validated using experimental data. The development of the model is carried out in a number of steps which can be grouped either as “considerations on the reaction” or “considerations on the process”, strictly connected to each other.\textsuperscript{[395-405]}

Considerations on the reaction include the identification of the chemico-physical properties of the reaction and its components, the reaction mechanism, kinetics, and inhibition phenomena. These data are organized in an interaction matrix, which helps to identify a set of constraints and eliminate many options. Afterwards, the model is built, evaluated, and reformulated if necessary. The selection of the reactor, the reaction mode (batch, semi-batch, continuous, etc...), the format of the enzymes (soluble or immobilized), possible assumption and simplifications, process optimization, and downstream processing are studied thanks to the “considerations on the process”. Possible inhibitory effects are considered when the reaction mode is analyzed.

Once developed the model, an “operating window” helps to visualize how the
performance metrics (reaction rate, biocatalytic productivity, costs) change when the parameters and their combination (stability and concentration of catalyst, substrates, temperature, pH, pressure, flow rate, agitation speed) are varied.\[393\] The operating window can be generated by mathematical modelling or by interpolation and extrapolation of experimental data. For single-enzyme processes it is important to set a threshold value below which the process is not feasible. For multi-enzymatic models the operating window is the overlap in the windows of individual enzymes and it is generally much smaller than for single-enzyme processes.\[405\] In this case the area of the operating window can change according to different operation modes, but it can be extended only by the modification of the intrinsic properties of the biocatalyst. Protein engineering in particular directed evolution represents the most powerful tools at this stage. Their main scope is not only the development of biocatalysts with needed properties, but also the improvement of existing characteristics. Process design demands more stable enzymes, or enzymes with optimal activity at different temperature or pH values, or with modified kinetic values. This can all be achieved by directed evolution and mutagenesis techniques.\[413-414\]

5. Outline of this thesis

The research described in this thesis was part of the IBOS project “Industrially relevant heterocycles through biocatalytic cascades” established between the universities of Amsterdam, Nijmegen, and Wageningen with the industrial support of DSM, Syncom, and MSD. The IBOS programme (Integration of Biosynthesis & Organic Synthesis), sponsored with a budget of \£ 13.6 by the Dutch government, NWO, and the Dutch chemical and life sciences industry aims at the integration of modern (bio)chemistry, molecular biology, biotechnology, and organic synthesis. The desired outcome is a change of strategy for the synthesis of fine chemicals by replacing conventional synthetic methods by nature-inspired processes. The project “Industrially relevant heterocycles through biocatalytic cascades” in particular deals with the development of novel, sustainable, and industrially viable synthetic pathways towards versatile enantioselectively pure heterocyclic building blocks. The integration of multiple enzymatic steps into one-pot cascade processes is the ultimate goal to convert cheap renewable sources into valuable compounds and limit the production of waste.

In this framework, this thesis describes the development of multi-enzymatic cascade reactions in one pot for the synthesis of natural and unnatural carbohydrates. Phosphorylation with acid phosphatase and C-C bond formation
with aldolases are the main catalytic steps in these routes. All cascades are based on the highly valuable dihydroxyacetone phosphate as key intermediate. Another part of this research is the implementation of these already established cascades with a more efficient process, mainly based on the immobilization of the enzymes and their use in different types of reactors. This allowed the switch from one-pot batch methods to the labour-free technology of continuous-flow synthesis with immobilized enzymes.

After a general background given in **Chapter 1, Chapter 2** describes a one-pot cascade reaction with four enzymes (acid phosphatase, oxidase/catalase, and DHAP-dependent aldolases). Achiral and cheap precursors like glycerol and aldehydes are converted into complex chiral compounds with high yields.

**Chapter 3** describes a new method to produce phosphorylated compounds on a gram scale in continuous flow. Acid phosphatase was immobilized and used in a packed-bed reactor. Among others, valuable compounds like glucose-6-phosphate and N-acetyl-D-glucosamine-6-phosphate were produced and prepared by this method.

A two-enzyme cascade reaction with immobilized enzymes in continuous mode is described in **Chapter 4**. Immobilization of the enzymes and the incorporation into a packed-bed reactor with three columns allow the synthesis of highly valuable compounds in continuous mode. This is one of the few examples of continuous-flow synthesis where one enzyme (acid phosphatase) compartmentalized in a column reactor drives the overall thermodynamic equilibrium of the cascade by catalyzing two opposite reactions (phosphorylation and dephosphorylation).

The use of transketolase as an alternative enzyme to aldolases is demonstrated in **Chapter 5**. This enzyme catalyzes in presence of hydroxypyruvate a C 2-ketol elongation of phosphorylated aldose sugars obtained with the method described in chapter 3 to yield phosphorylated elongated ketose sugars. Transketolase was also immobilized and a complex flow system was set up using a packed-bed flow reactor with two columns and three pumps to carry out this C 2-ketol elongation.

**Chapter 6** describes advantages and limitations of alkaline phosphatase as a substitute for acid phosphatase. By immobilization of alkaline phosphatase, the inhibition by phosphate observed with the soluble enzyme could be suppressed. As a result the enzyme could carry out efficient phosphorylation reactions but the use in cascade reactions did not result in further improvement compared the already established methodology with acid phosphatase.

The research results presented in this thesis show that in line with the original aim of the IBOS project it is indeed possible to connect classical enzymology to organic chemistry and process technology. As a result novel benign biocatalytic
procedures have been developed for which possible industrial applications exist.

6. Bibliography

Introduction

Chapter 1

**Introduction**


Chapter 1

Introduction


Chapter 1


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Introduction