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New challenges for artificial metalloenzymes based on the Biotin-(Strept)avidin technology

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Prof. Dr. Thomas R. Ward und Prof. Dr. Dennis Gillingham.

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<div align="right">

Dekan

Prof. Dr. Martin Spiess

</div>

« Ce n'est pas parce que les choses sont difficiles que nous n'osons pas,

c'est parce que nous n'osons pas qu'elles sont difficiles. »

Sénèque

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Ci kaw, Ci kanam!
Dieuredieuf.

List of abbreviations

⊂	included in
Ac	Acetyl
Avi	Avidin
B$_4$F	Biotin-4-fluorescein
BINAP	2,2'-bis(diphenylphosphanyl)-1,1'-binaphtyl
Biot	Biotinyl
Boc	*tert*-Butyloxycarbonyl
BSA	Bovine serum albumin
CD	Circular dichroïsm
CDMT	2-chloro-4,6-dimethoxy-1,3,5-triazine
COD	1,5-Cyclooctadiene
Conv	Conversion
DCC	*N,N'*-dicyclohexylcarbodiimide
DMAP	4-(Dimethylamino)pyridine
DMB	didodecyldimethylammonium bromide
DMF	Dimethylformamide
DMSO	dimethylsulfoxyde
ee	Enantiomeric excess
eq.	Equivalent
ESI	Electron spray ionization
GC	Gas chromatography
HABA	2-(4-Hydroxyphenylazo)benzoic acid
HG	Hoveyda-Grubbs
HPLC	High pressure liquid chromatography
i-Pr	*iso*-propyl

LC	Liquid chromatography
MES	2-(*N*-morpholino)ethanesulfonic acid
mes	Mesityl
MS	Mass spectrometry
NHC	*N*-heterocyclic carbene
NHS	*N*-hydroxysuccinimide
NMR	Nuclear magnetic resonance
PAGE	Polyacrylamide gel electrophoresis
PDB	Protein Data Bank
Ph	Phenyl
ppm	part per million
RCM	Ring closing metathesis
Rec-Avi	Recombinant avidin
RT	Room temperature
Sav	Streptavidin
SDS	Sodium dodecyl sulfate
SMPS	Slow migrating protein species
(strept)avidin	Either avidin or streptavidin
TEMED	Tetramethylethylenediamine
THF	Tetrahydrofuran
TLC	Thin Layer Chromatography
TON	Turn over number
Ts	toluene-4-sulfonyl
UV/vis	Ultraviolet/visible
WT	wild type

Abstract

Artificial metalloenzymes are designed to gather advantages of both homogeneous catalysts and enzymes. The biotin-(strept)avidin technology insures the incorporation of an active catalyst in a proteinic scaffold which provides a chiral environment. The combination of these two parts provides efficient artificial metalloenzymes applied in various asymmetric reactions such as hydrogenation, transfer hydrogenation, allylic alkylation, etc…

In this work, the properties of (strept)avidin were used to explore new challenges for artificial metalloenzymes.

Artificial hydrogenases were used as model reaction to demonstrate that artificial metalloenzymes can be more than a concept. With this goal in mind, the loading of the artificial hydogenase was reduced to 0.1% and the concentration of substrate increased to 60 mM with very small erosion of activity and selectivity. The scale of the reaction was increased 15 times without any impact on conversion and *ee*. Recycling of the artificial hydrogenase was investigated by immobilizing streptavidin using biotin sepharose. The resulting artificial metalloenzymes could be used twice without any loss of activity. Lyophilisation of the artificial hydrogenase allows a convenient storage of the artificial hydrogenase.

The versatibilty of the biotin-streptavidin technology was exploited to create and optimize artificial allylic alkylases. The thermostability of streptavidin and its high tolerance towards organic solvents resulted good conversions and *ee* values up to 97%.

The thermostability of streptavidin was further investigated as a potential rapid purification strategy towards screening heat-treated cell-free extracts.

With the aim of selective modification of tyrosine-residues, the potential of the artificial allylic alkylases was extended towards the use of phenol as nucleophile. The reaction was optimized to operate under physiological conditions. Detailed investigations of the reaction suggested self-alyllation of the tyrosine residues of the artificial metalloenzyme.

Artificial ligases were developed by incorporation of a biotinylated Hoveyda-Grubbs 2nd generation catalyst in (strept)avidin. The optimization of the artificial metalloenzyme gave good conversions for ring closing metathesis of diallyltosylamine under acidic pH and high salt concentration.

I.   Introduction

1.1. General

The distinction between chemistry and biology is ever increasingly becoming smaller and smaller. Advances in the fields of chemistry and biology allow the understanding of various processes involved in life. Bioinorganic chemistry is an example of a domain at the interface between chemistry and biology. Garner defined bioinorganic chemistry as an interdisciplinary field of science which draws on the strengths of the disciplines of inorganic chemistry and biological sciences and requires the application of advanced physical and theoretical methodologies [1]. The core of bioinorganic chemistry has focused on the study of metal sites in biological processes.

The nature of the metal involved is linked to its function in the organism. Group I and II metals such as sodium, potassium or calcium are found in charge regulation processes or as structural elements [2]. The function of transition metals can be varied. Metals existing in a single oxidation state can function either as structural elements or lewis acids. As an example, zinc fingers are protein domains in which zinc chelates residues (cysteines, histidines,...) to contribute to the stability of the protein. Elements that exist in different oxidation states like iron or copper have roles in electron transfer, oxygen transport or redox chemistry. Catalytic activity of metalloenzymes is most often ensured by transition metals, for example, cobalt is found in vitamin B12, an essential cofactor of enzymes involved in protection of the nervous system or the synthesis of methionine (Figure 1) [3, 4].
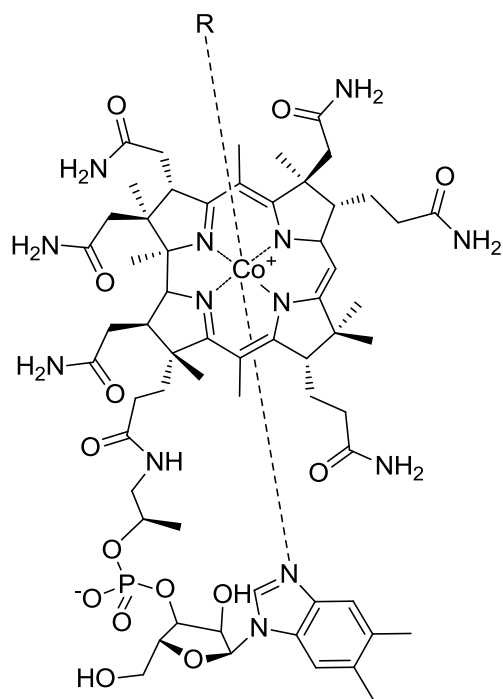
Figure 1. Coenzyme B12.

1.2. Metalloenzymes

Metalloenzymes are biological catalysts constituted of a proteinic part into which a metal is incorporated, providing the catalytic activity [5].

1.2.1. Definition of catalyst.

A catalyst can be defined as a species that increases the rate of a chemical reaction. The catalyst is regenerated during the reaction and that allows using a small quantity of this substance [6].
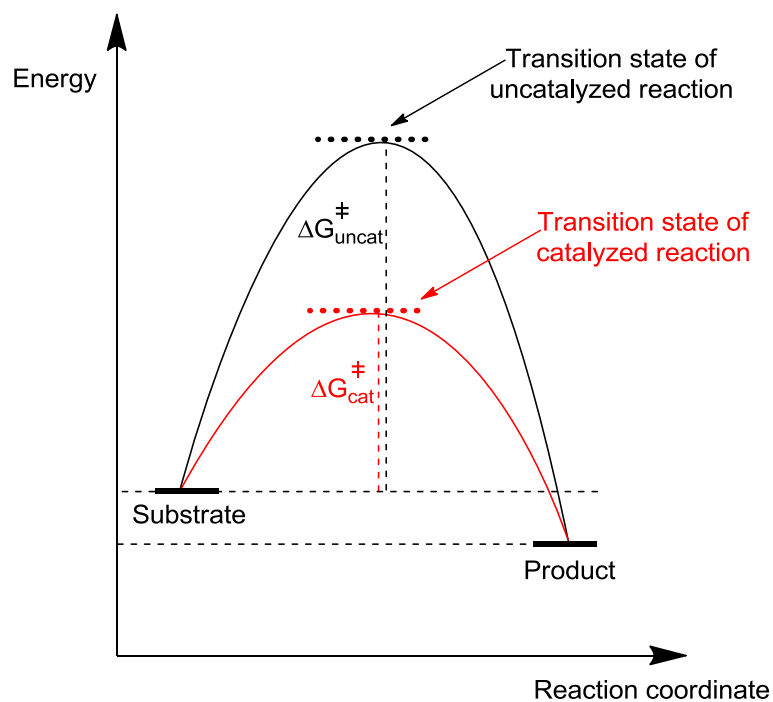
Figure 2. Energetic diagram of a catalyzed reaction

The thermodynamics and the equilibrium of the overall reaction are not affected by the catalyst; however, the catalyst changes the Gibbs-free energy of the transition state (Figure 2). It reduces the highest energy barrier by stabilizing the transition state observed in the uncatalyzed reaction.

1.2.2. Examples of efficiency of metalloenzymes.

1.2.2.1. Nitrogenases.

Nitrogenases are metalloenzymes able to generate ammonia from N_2. These metalloenzymes are found in some prokaryotic organisms. Fixed nitrogen is indispensable to life as it is required for the biosynthesis of amino acids and nucleic acids [7-9].
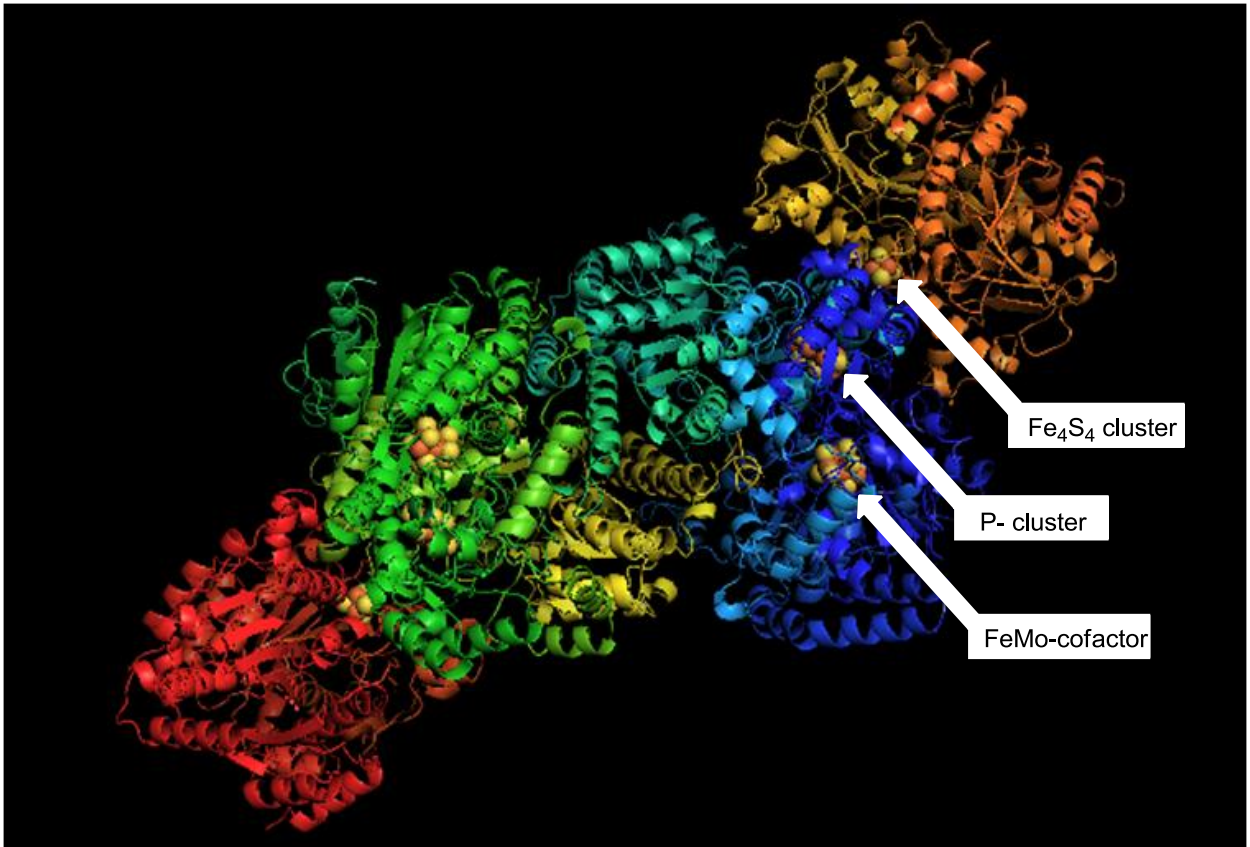
Figure 3. Nitrogenase. (PDB: 1N2C)

This enzyme consists of two different subunits, the Fe-protein and the MoFe-protein (Figure 3). The MoFe-protein contains two clusters: a Fe-S cluster named the P-cluster and a second cluster containing iron, sulfur and another metal such as molybdenum, vanadium or iron. In most of the cases, the cofactor contains molybdenum and is called FeMoco.

This MoFe-protein is associated with a Fe-protein which is bridged to the MoFe-protein by an additional Ferredoxin Fe-S cluster (Figure 4).
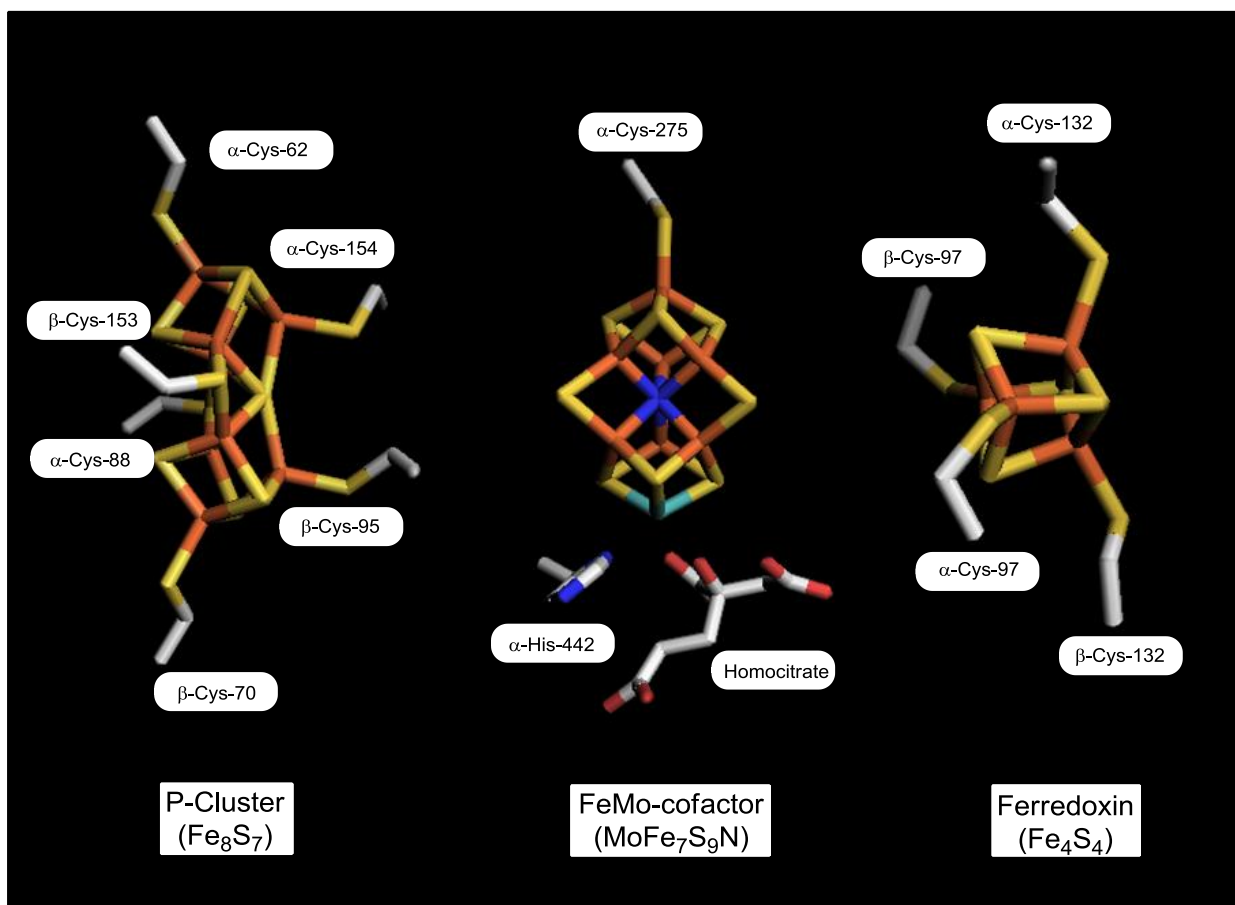
Figure 4. Representation of the metalloclusters in nitrogenase. (Adapted from PDB files 1M1N and 2NIP). Iron is represented in orange, sulfur in yellow, nitrogen in blue, molybdenium in sky blue, oxygen in red and carbon in grey.

The mechanism of nitrogen reduction is not fully elucidated but several points are widely accepted (Scheme 1) [10-12]. At the first step, the Fe-protein and the MoFe-protein associate to form the complex triggering an electron transfer from the Ferredoxin to the P-cluster and then further to the FeMoco. In the same time, two ATP molecules which were bound to the Fe-protein are hydrolyzed to ADP and phosphate salts. At this point, the Fe-protein and the MoFe-protein dissociate and the cycle starts again. During this cycle, one electron is transferred to the active site. In order to reduce one N_2 molecule, this cycle has to run eight times.

2 ADP , 2 HPO$_4^{2-}$

2 ATP

Fe protein

Fe protein

Fe protein

Fe protein

Fe protein

e$^-$, H$^+$

FeMo protein

FeMo protein

FeMo protein

FeMo protein

FeMo protein

H$_2$

8 times

N$_2$

2 NH$_3$

$$N_2 \;+\; 8\,H^+ + 8\,e^- \;+\; 16\,ATP \longrightarrow 2\,NH_3 \;+\; H_2 + 16\,ADP \;+\; 16\,HPO_4^{2-}$$
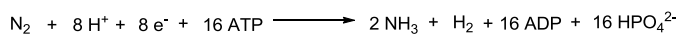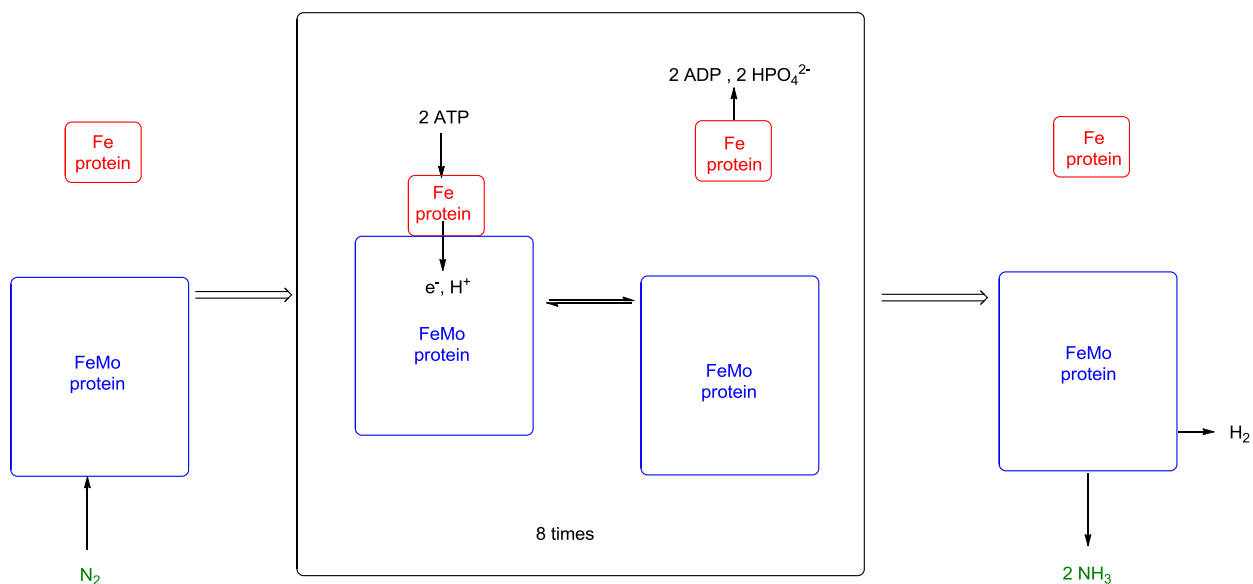
Scheme 1. Mechanism of reduction of N$_2$ with nitrogenase

Nitrogenases illustrate the cooperation between inorganic complexes and proteinic scaffolds. The interactions of the different clusters with amino acid residues of the proteinic scaffold, for example, cysteines allow them to be so efficient. Many attempts to isolate the FeMoco have been made. However, it has not yet been possible to catalyze ammonia synthesis in comparable yields with the isolated FeMoco or a synthetic analogue [13-17]. Until now, the Haber-Bosch process is the unique way to produce ammonia industrially from N$_2$ and H$_2$ in presence of an iron catalyst (Scheme 2) [18, 19].

$$N_2 \quad + \quad 3\,H_2 \qquad\longrightarrow\qquad 2\,NH_3$$

N$_{2\,(g)}$, H$_{2\,(g)}$
NH$_{3\,(g)}$

REACTOR

N$_{2\,(g)}$, H$_{2\,(g)}$

Magnetite (Fe$_3$O$_4$)
K$_2$O, CaO, Al$_2$O$_3$

100 - 250 bars
200 - 600°C

COOLING

NH$_{3\,(l)}$

N$_{2\,(g)}$, H$_{2\,(g)}$
recycling
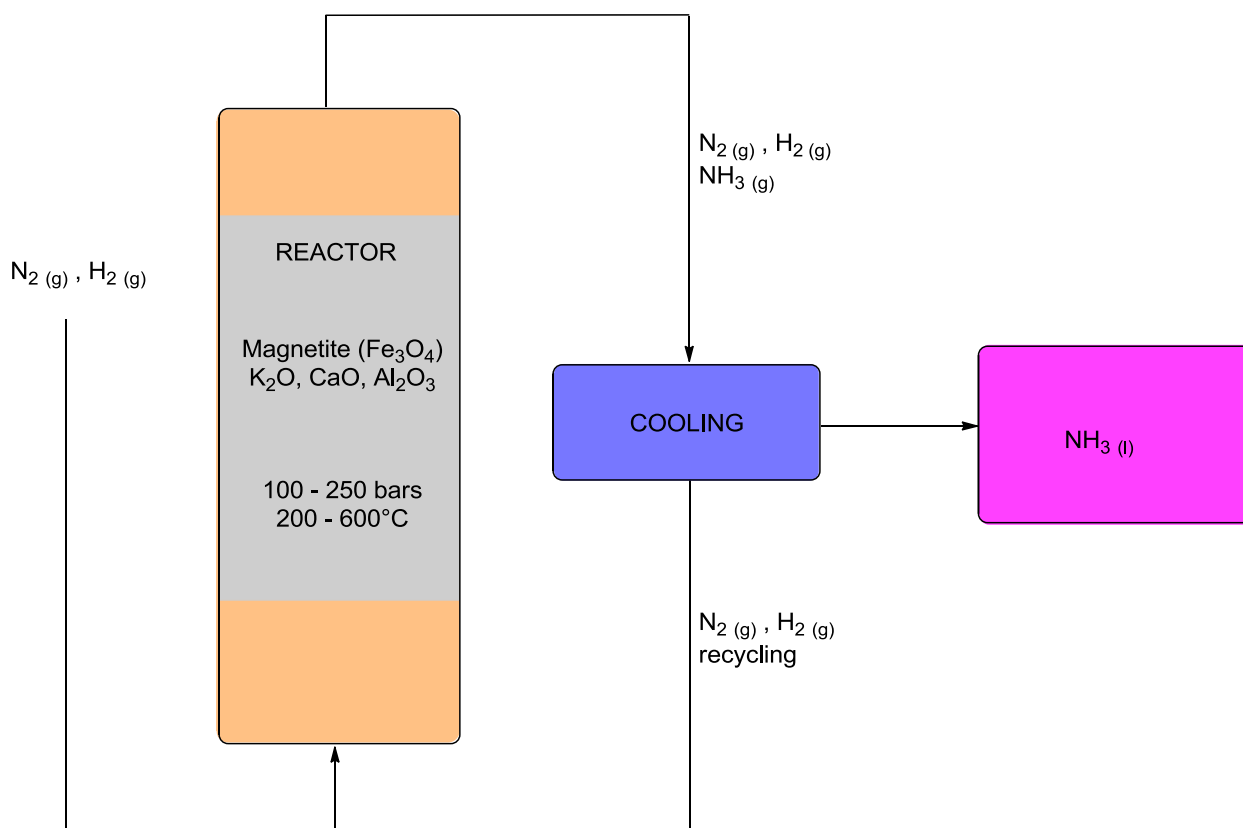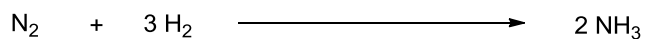
Scheme 2. Haber-Bosch process.

However, this process requires high temperatures and pressures. This is due to the fact that in the process, atomic nitrogen binds to the surface of the catalyst. Although the equilibrium is shifted towards the reactants, this phenomenon can be avoided by increasing the temperature. Additionally, the pressure must be increased in order to move the equilibrium in favor of product formation.

1.2.2.2. Cytochrome P 450.

Cytochrome P450s are another illustration of the importance of the interactions between a proteinic scaffold and the active metal [20-23]. Cytochrome P450 transfers one atom of oxygen from molecular oxygen to a substrate in the presence of a reducing agent.

Cytochrome P450s are metalloporphyrins consisting of an iron porphyrin with a cysteine acting as an axial ligand. The other axial position is used for the activation site of O_2.

Figure 5. Comparaison between cythochrome P450 (left) and hemoglobyn (right). (PDB : 1JPZ and 3AQ5)
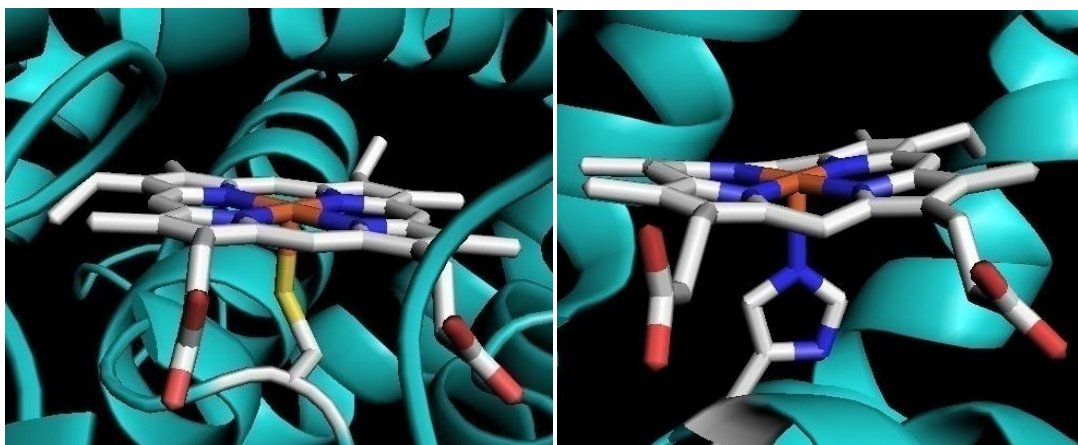
The amino acid acting as an axial ligand is the unique difference in the first coordination sphere of iron between cytochrome P450 and others metalloproteins such as hemoglobin or myoglobin (Figure 5). In the case of hemoglobin or myoglobin, the position is occupied by a histidine residue. Hemoglobin and myoglobin ensure the transport of dioxygen in blood by forming a reversible Fe-O-O complex. The difference in activity of hemoglobin vs. cytochrome P450 is explained by the effect of the ligand in *trans* of the fixation site of molecular oxygen [24], as well as subtle differences in the second coordination sphere. These latter are particularly hard to control in small bio-inorganic model compounds. This may well be one of the reasons why such model compounds rarely rival with the versatility of metalloenzymes.

1.3. Artificial metalloenzymes.

1.3.1. Concept of artificial metalloenzymes.

Many reactions can be improved in the presence of a catalyst or an enzyme (Table 1) [25]. In most cases, conditions required with enzymes are milder than those required with either homogeneous or heterogeneous catalysts. Each system however has its own advantages and disadvantages (Table 2).

Table 1. Comparison of chemical and biological systems.

Reactions	Chemical system	Biological system
Methane hydroxylation	$CH_4 + H_2O \rightarrow CO + 3\ H_2$ (Ni catalyst, 700-900°C, 1–25 bar) $CO + 2\ H_2 \rightarrow CH_3OH$ (Cu/Zn catalyst, 250-280°C, 70-110bar)	$CH_4 + O_2 + NADH + H^+ \rightarrow CH_3OH + H_2O + NAD^+$ (methane monooxygenase)
CO oxidation	$CO + H_2O \rightarrow CO_2 + H2$ (Fe/Cu catalyst, >200°C)	$CO + H_2O \rightarrow CO_2 + 2\ H^+ + 2e^-$ (CO dehydrogenase)
CO insertion	$CH_3OH + CO \rightarrow CH_3COOH$ $[Rh(I)I_2(CO_2)]^-$, 120°C, 30 atm	$CH_3-[M] + CO + HS-CoA \rightarrow CH_3(CO)-S-CoA + H^+ + [M]^-$ [a] (acetyl-CoA synthase)

[a]CH3−[M] is a corrinoid-iron-sulfur protein that acts in the reaction as a methyl group donor; HS-CoA is the coenzyme.

For example, transition metal catalysts are cheaper and easier to synthesize. They are also well characterized and more resistant to harsh conditions of temperature and pressure. On the other hand, enzymes are very selective and have a higher tolerance to other functionalities. In addition, the environment around the active site ensures a high stability and often enables high turnover numbers. Finally, enzymes are active under physiological conditions.

Table 2. Comparison between homogeneous and enzymatic catalysis.

Parameters	Homogeneous catalysis	Enzymatic catalysis
Substrate scope	Large	Limited
Enantiomers	2 enantiomers	1 enantiomer
Tolerance to organic solvents	Excellent	Limited
Substrate concentration	High	Low to moderate
TON/TOF	Average	High
Optimization	Chemical synthesis	Genetic
Repertoire of reaction	Large	Restricted
Second sphere coordination	Ill-defined	Well-defined
Reactions condition	High temperature and pressure	Room temperature and atmospheric pressure
Environmental impact	Hazardous	Friendly

Artificial metalloenzymes are aimed at combining the advantages of transition metal catalyzed reactions with those of enzymes [26-28]. This results from the incorporation of a metal complex with a catalytic activity in a macromolecular host which provides a defined environment around the metal.

1.3.2. Design of artificial metalloenzymes.

The three key parameters in the design of an artificial metalloenzyme are the transition metal catalyst, the biomolecular scaffold and the mode of attachment of the transition metal catalyst to the biomolecular scaffold.

1.3.2.1. Transition metal catalyst.

The transition metal is responsible for the activity of the artificial metalloenzyme. Recent advances in the field of transition metal catalyzed reactions considerably enlarge the reaction scope where such a system can be applicable [29]. One critical limitation of organometallic catalyst is water compatibility. Another point that should be taken into consideration is the compatibility of the reaction conditions (temperature, pressure,…) or reagents (surfactants, oxidizing agents,…) with the stability of the biomolecular scaffold.

1.3.2.2. Biomolecular scaffold.

The biomolecular scaffold provides the second coordination sphere around the metal. Most often, the biomolecular scaffold is selected based on the presence of an existing active site that can be modified in order to incorporate a new functionality.

Kaiser, one of the pioneer in this field, developed a Cu(II) carboxypeptidase that catalyses the oxidation of ascorbic acid [30].

Ueno, Watanabe and co workers described the *in situ* synthesis of a palladium cluster incorporated in apo-ferritin, an iron storage protein, and performed size-selective olefin hydrogenation [31-33].

An alternative is to use a biomolecular scaffold where the active site is not present but entirely created by the introduction of new functionality. This strategy offers the advantage of enlarging the scope of biomolecular scaffolds [34].

Distefano used iodoacetamido-1,10-phenanthroline to modify a unique cysteine residue in adipocyte lipid binding protein (ALBP) to produce the conjugate ALBP-Phen. ALBP-Phen-Cu(II) catalyzed the enantioselective hydrolysis of several unactivated amino acid esters [35-37].

Mahy and coworkers presented new anionic metalloporphyrin–estradiol conjugate which was associated to a monoclonal anti-estradiol antibody 7A3, to generate new artificial metalloenzymes used in oxidation reactions [38-40].

A new approach was introduced by using DNA or peptides as scaffold for the creation of artificial metalloenzymes [41, 42].

Franklin and coworkers designed a chimeric Cu-peptide for DNA recognition [43-47]. Komiyama created an artificial restriction DNA cutter using two oligonucleotides bearing a monophosphate group combined to a Ce(IV)/EDTA complex [48-51]. In the same spirit, Krämer presented a peptide nucleic acid conjugated with a Zr(IV) complex for DNA scission [52].

Roelfes and Feringa used DNA as a scaffold for inducing selectivity and rate acceleration in several reactions such as Diels-Alder reactions, Michael reactions or Friedel-Crafts reactions [53-58].

1.3.2.3. Anchoring strategy.

The anchoring of the transition metal in the biomolecular scaffold is a key issue as random localization of the transition metal within the biomolecular scaffold must be avoided. There are three strategies for anchoring: covalent, dative and supramolecular (Scheme 3) [59].
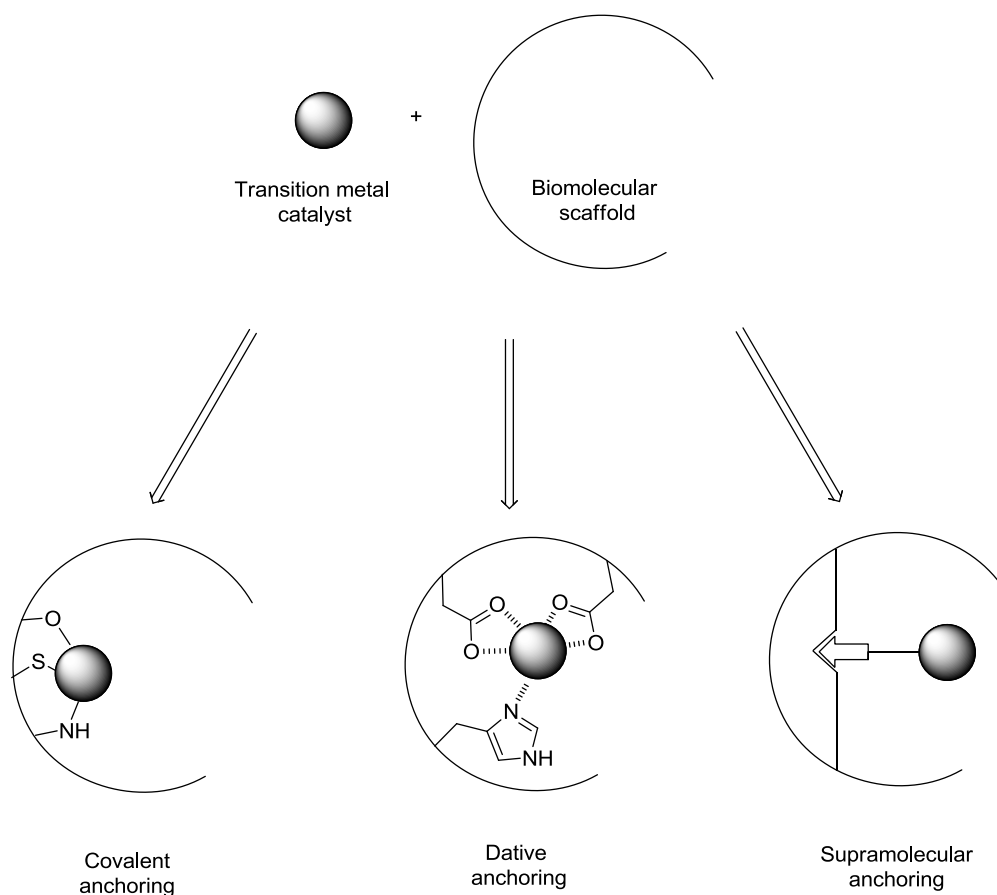
Scheme 3. Anchoring strategies for the creation of artificial metalloenzymes.

The covalent anchoring strategy relies on the creation of a covalent bond between one or several amino acids of the biomolecular scaffold (for example, cysteines) and a ligand of the transition metal catalyst. Many papers have reported the covalent anchoring of the cofactor to biomolecular scaffolds. As an example, Lu reported the anchoring of a Mn salen complex to myoglobin by the creation of two new covalent bonds between the ligand and the protein (Scheme 4) [60].

Kamer and co workers reported covalent anchoring of various phosphine ligands on photoactive yellow protein [61-63].
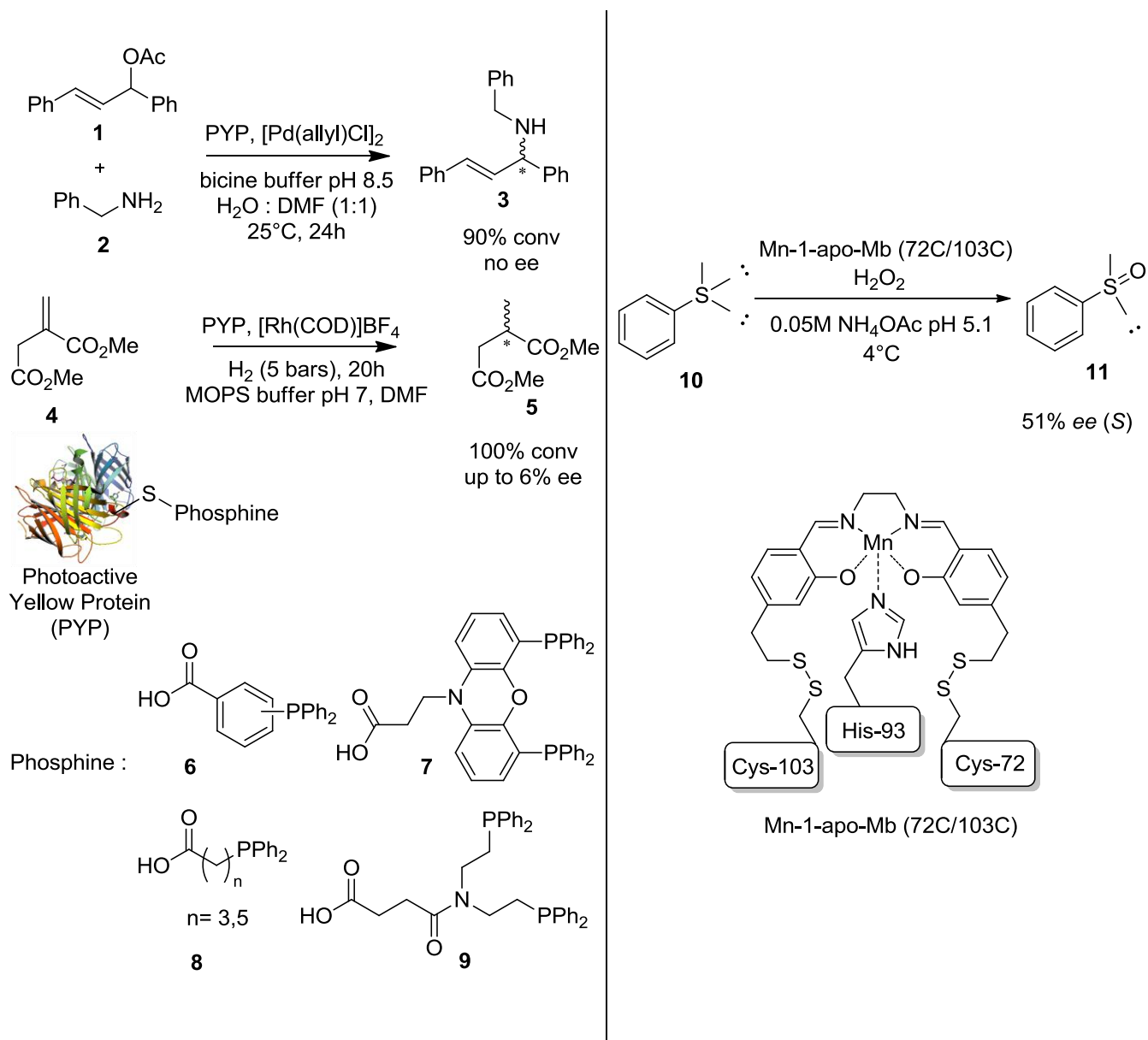
Scheme 4. Example of covalent anchoring: synthesis of phosphine-protein conjugates developed by Kamer (left) and double anchoring of Mn-salen on apo-Myoglobin developed by Watanabe (right).

In the dative anchoring strategy, one amino acid of the biomolecular acts as a ligand for the metal complex. Kokubo et al. demonstrated that a 1:1 complex of an osmate ester and bovine serum albumin (BSA) functions as a catalyst in the cis dihydroxylation of alkenes (up to 68% *ee*) [64]. Kazlauskas substituted zinc by rhodium in carbonic anhydrase to yield an artificial metalloenzyme for hydrogenation and hydroformylation (Scheme 5) [65, 66].

Using the same principle, Soumillion introduced manganese in carbonic anhydrase for selective epoxidation of alkenes [67, 68].
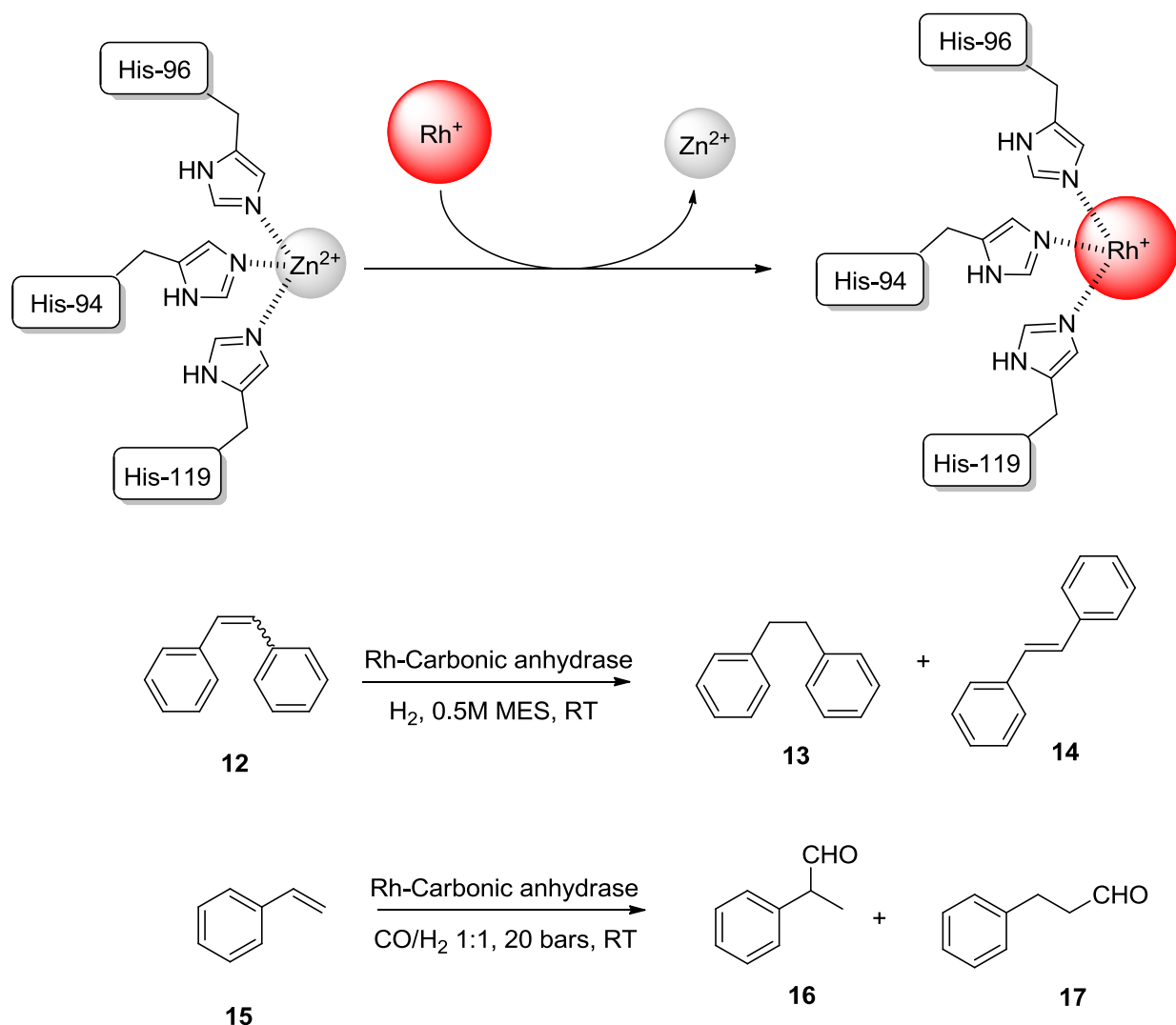
Scheme 5. Artificial metalloenzyme developed by Kazlauskas using dative anchoring.

The supramolecular anchoring strategy relies on weak the interactions between small molecules and the biomolecular scaffold. The crucial point of this strategy is the affinity of the guest molecule for the host biomolecular scaffold. Harada used the high affinity of antibodies for the creation of an artificial hydrogenase [69]. In the same spirit, Keinan presented an antibody-metalloporphyrin assembly that catalyzed enantioselective oxidations

[70, 71]. In his early report, Whitesides described the creation of an artificial metalloenzyme based on very high affinity of biotin for avidin [72].

1.4. Artificial metalloenzymes based on the biotin –(strept)avidin technology.

1.4.1. Biotin-(strept)avidin technology.

Avidin is a glycosylated protein that is naturally present in egg white [73-75]. Streptavidin is secreted by *Streptomyces* bacteria. This protein is expressed in *Escherichia coli* with relatively high yield [76]. In contrast to avidin, streptavidin is not glycosylated.
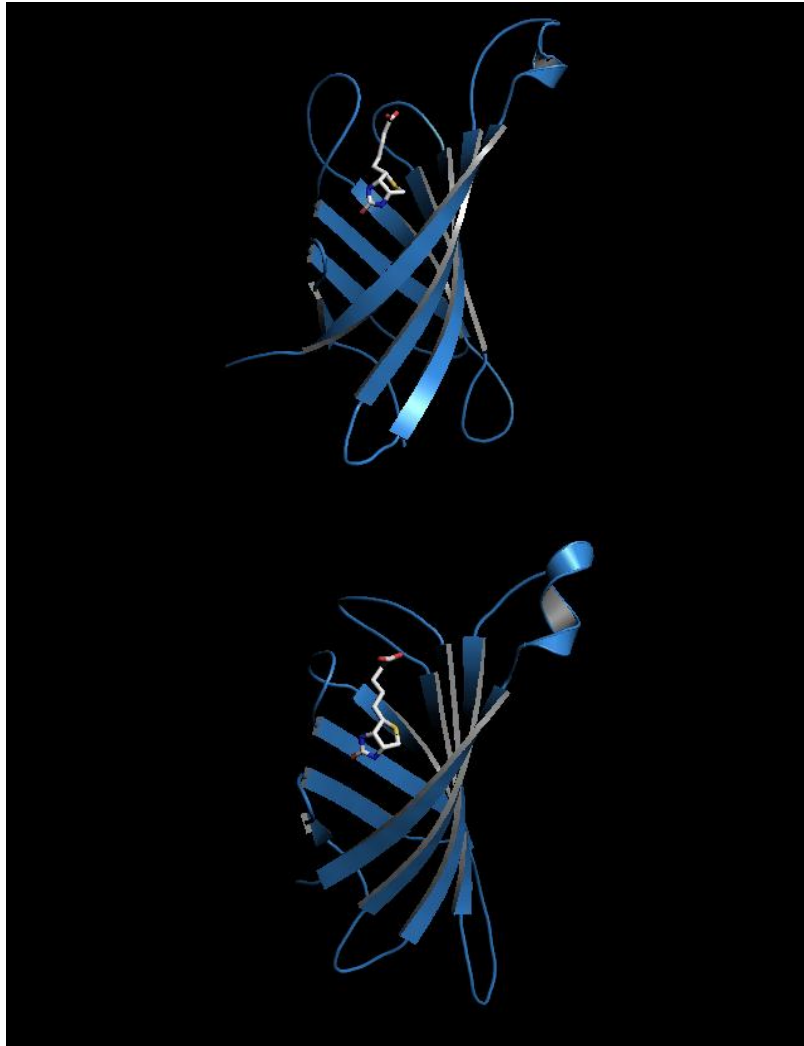
Figure 6. Representation of avidin monomer (top) and streptavidin monomer (bottom) with biotin bound. (PDB: 1AVD and 1SWE)

Avidin as well as streptavidin are tetrameric eight stranded β-barrel proteins which bind up to four biotins with high affinity. They display 31% sequence identity (41% homology) (Figure 6) [77-81].

These proteins are extremely robust and stable at high temperatures [76], at extreme pH [82-84] and high concentrations of denaturing agents [83, 85]. As an example, avidin does not denature with 6M guanidinium at pH 1.5 [85]. The robustness of these proteins allows the use of these scaffolds for a wide range of applications.

Biotin-(strept)avidin is often described as molecular Velcro [86, 87]. The interaction between biotin and (strept)avidin is extremely tight with a $Ka = 1.7*10^{15}$ M^{-1} for avidin and $Ka =$

$2.5*10^{13}$ M^{-1} for streptavidin [75]. These high values ensure a quasi irreversible anchoring of a biotinylated compound into the protein pocket as a result of numerous interactions between biotin and the protein such as hydrophobic interactions [77, 79, 88-92], Van der Waals interactions and hydrogen bonding (Figure 7) [88, 93-98]. All applications of the biotin-(strept)avidin technology rely on the fact that the valeric acid side chain of biotin can be derivatized with little influence on the remarkable affinity of the biotin-(strept)avidin couple [99-110].
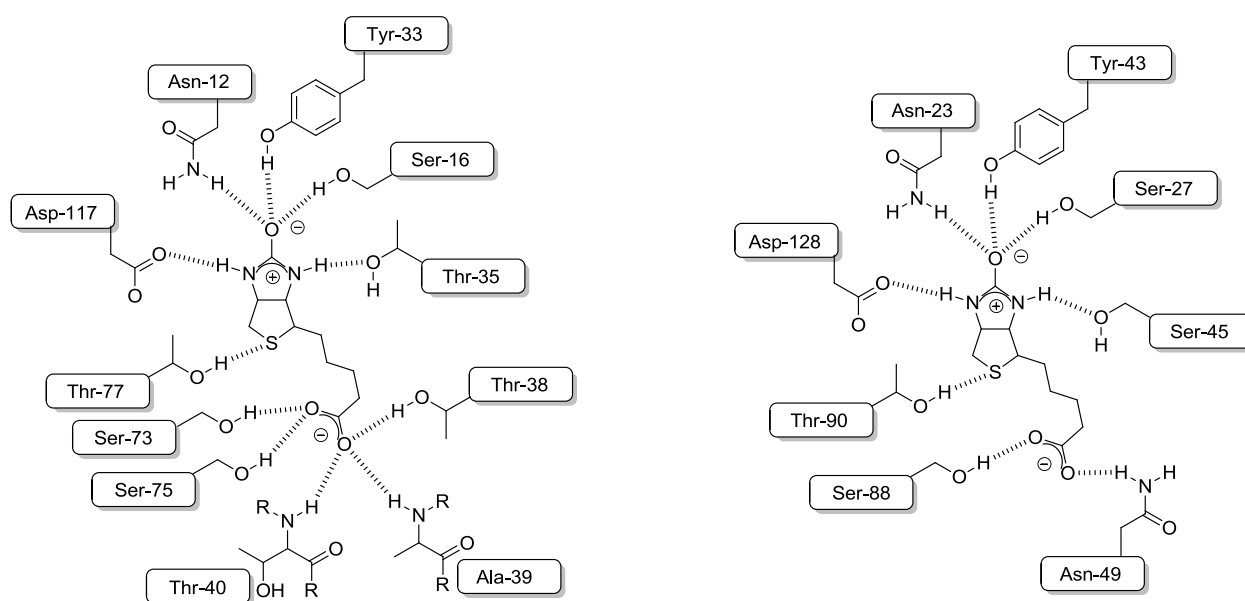
Figure 7. Representation of the interactions biotin-avidin (left) and biotin-streptavidin (right)

Since 2003, the Ward group has intensively explored the biotin (Strept)avidin technology for the creation of artificial metalloenzymes [111-114].

The presence of the biomolecular scaffold offers an additional advantage for optimization of the artificial metalloenzyme (Scheme 6). While chemical optimization can be achieved by modifiying the ligand or by introducing a spacer between the biotin anchor and the metal, the biomolecular scaffold can be genetically modified in order to influence the environment around the metal. By identification of the residues that are near to the active site, saturation

mutagenesis allows one to exchange the selected amino acid with the remaining 19 natural amino acids.
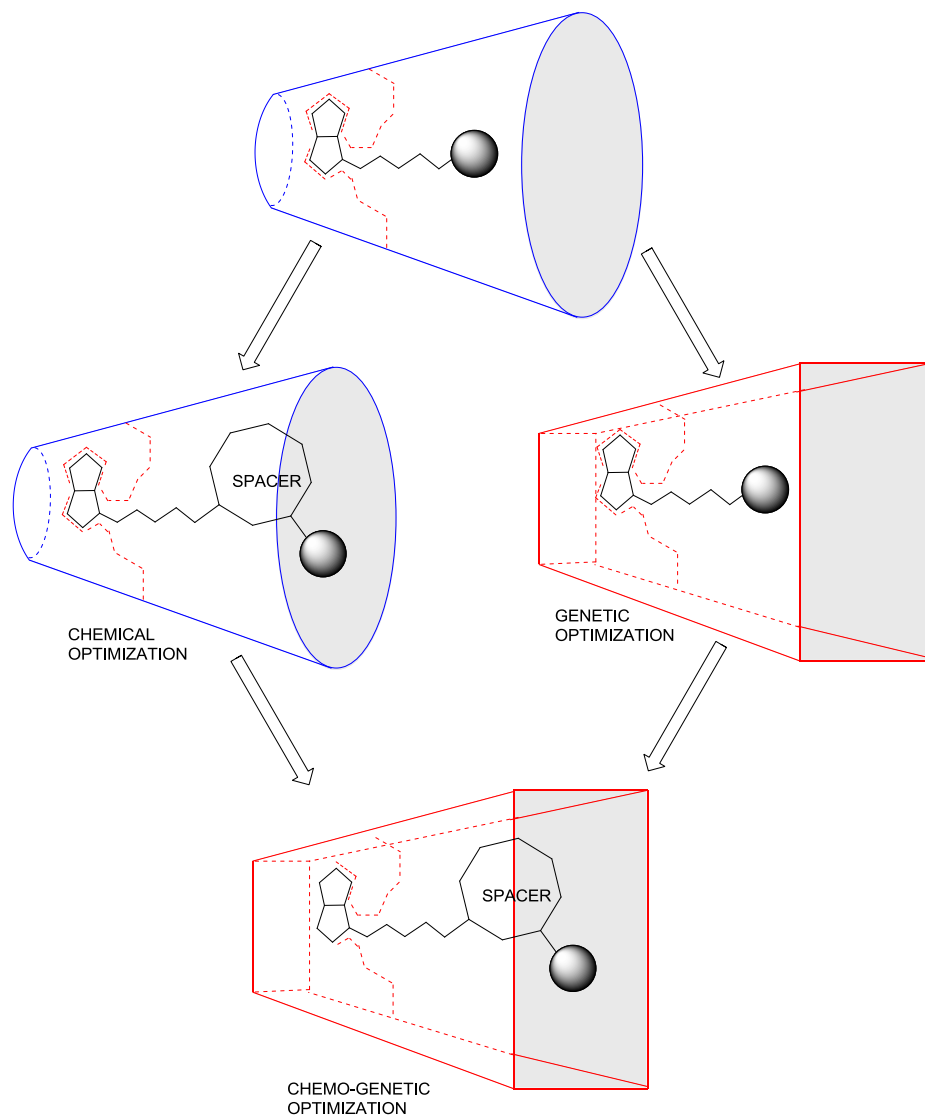
Scheme 6. Chemogenetic optimization of the artificial metalloenzyme.

1.4.2. Enantioselectivity: influence of the second coordination sphere for asymmetric hydrogenation.

Important improvements have been achieved in asymmetric hydrogenation since the initial work of Knowles and Horner (Figure 8) [115-158]. In 2001, Noyori and Knowles received the Nobel prize for their work in this field [159-163]. Nowadays, this reaction is well developed and used at industrial scale for the synthesis of various compounds.
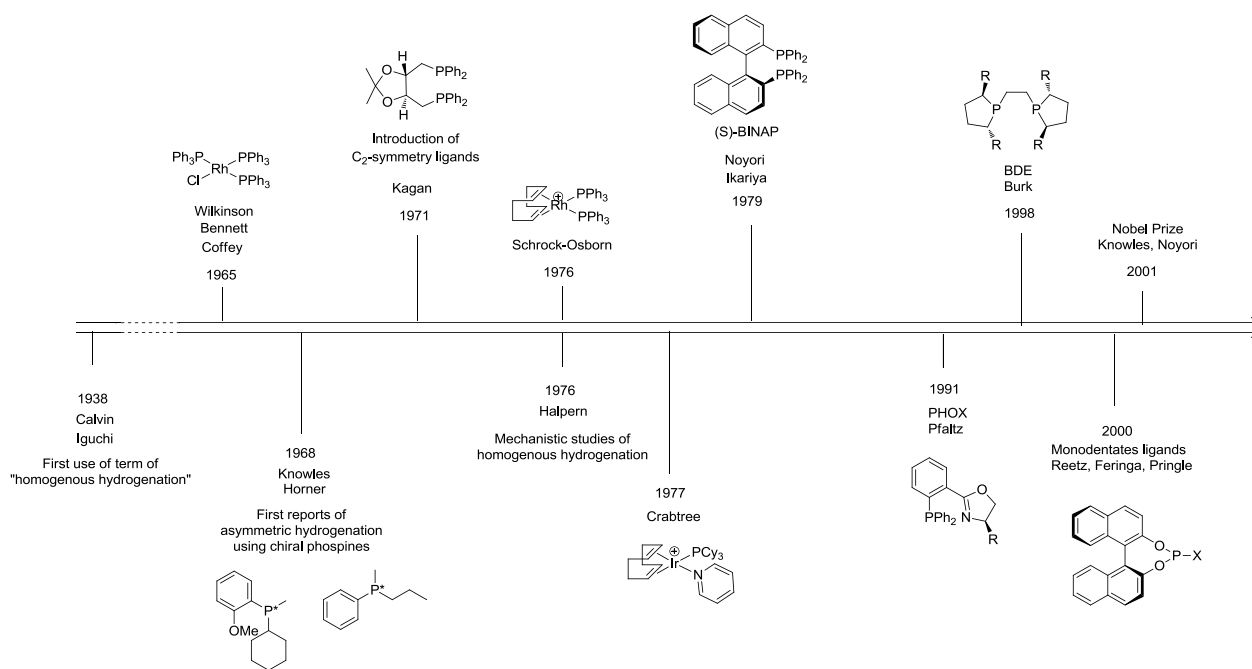
Figure 8. Overview of the development of hydrogenation.

The first coordination sphere refers to the ligand directly coordinated to the metal. The complex, consisting of the metal and the chiral ligand, can form with the substrate two diastereomeric intermediates.

The origin of the enantiomeric excess resides in the difference in energy between the two transition states leading from the diastereomeric intermediates to the enantiomeric products (Figure 9) [164-170]. The ligand is optimized to discriminate one transition state over the other. This can be rationalized with the quadrants illustrated for Rhodium BINAP hydrogenation of olefins (Scheme 7) [125, 171].
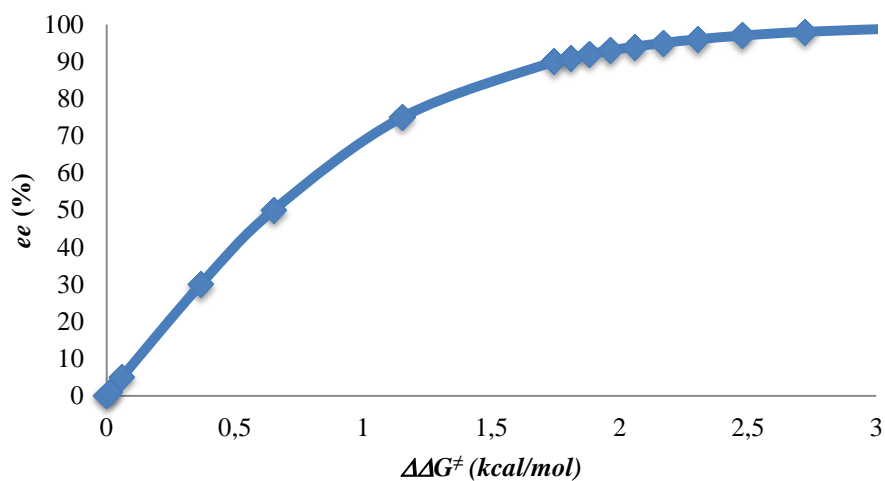
$$\Delta\Delta G^{\neq} = -RT * Ln\left(\frac{1-ee}{1+ee}\right)$$
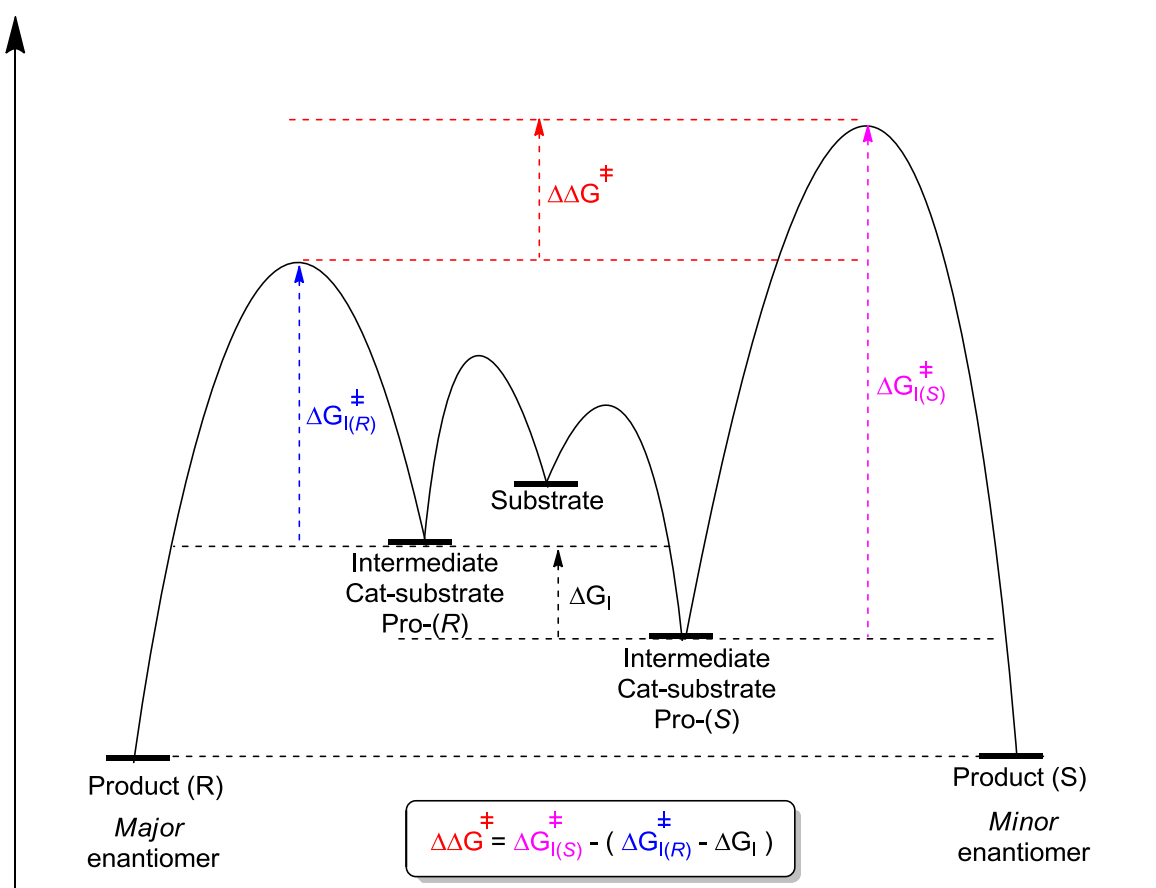
Figure 9. Influence of the $\Delta\Delta G^{\neq}$ on enantiomeric excess (up) and energetic diagram of an asymmetric hydrogenation reaction (down).

The position of the phenyl groups on the phosphorous can affect the coordination of the substrate to the metal. The phenyl group in the equatorial (position face on) has a higher steric hindrance compared to the phenyl in the axial (position edge on). The configuration of the ligand dictates the position of the phenyl groups. As illustrated in scheme 7, interactions of the carboxylic group of the enamide substrate bound to the catalyst and the phenyl group on phosphorus lead to the discrimination of the two diastereotopic intermediates. In the case of hydrogenation, the less stable intermediate is the most reactive for addition of hydrogen and provides the major enantiomer.
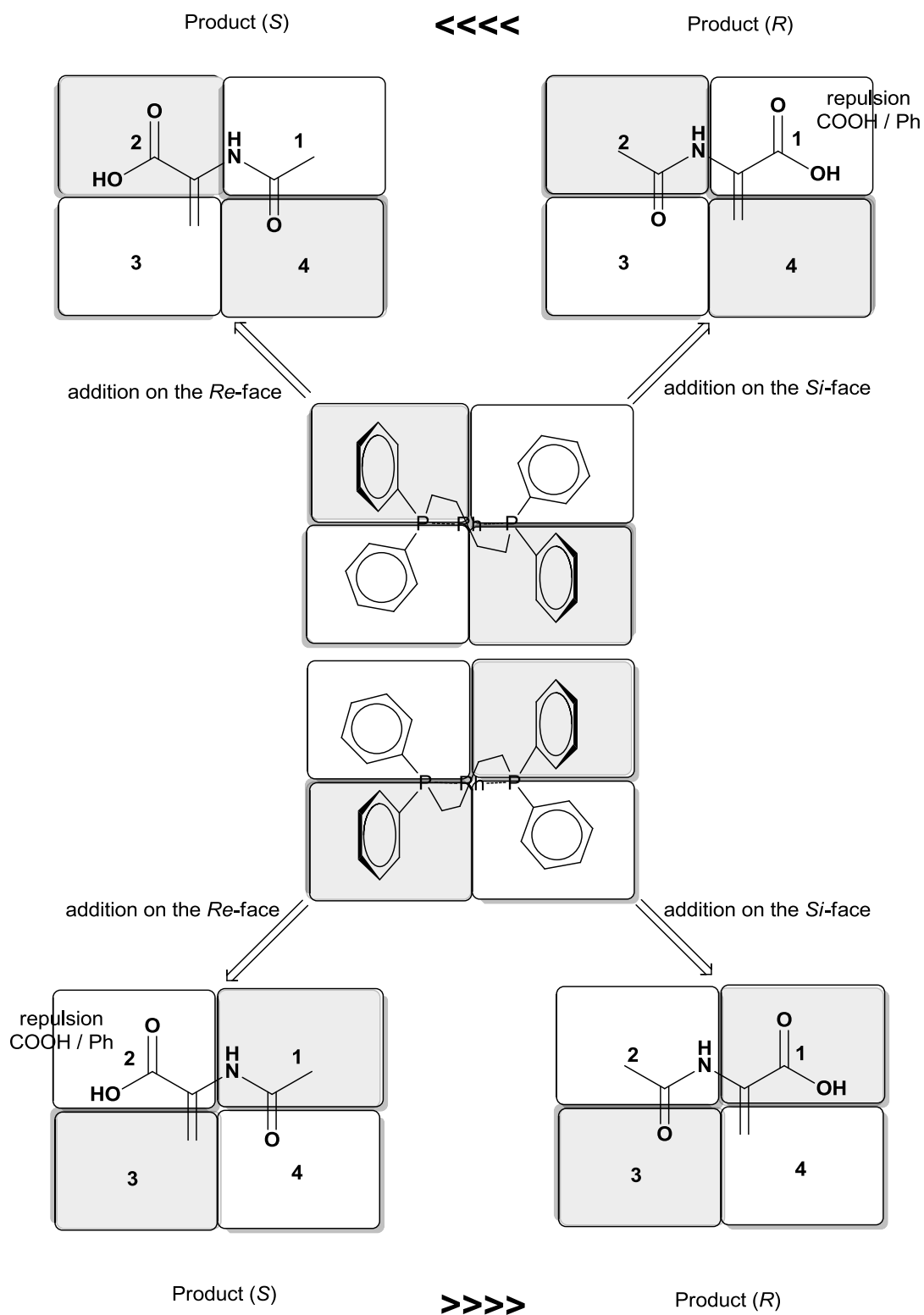
Scheme 7. Quadrants illustrated with Rh BINAP and *N*-acetamido acrylic acid. Grey squares represent phenyls in edge-on positions; white squares represent phenyls in face-on position. For clarity, BINAP is simplified.

The second coordination sphere can have an influence on the discrimination between the transition states. In the case of artificial metalloenzymes, the residues around the metal can interact with the metal or influence the incoming of the substrate in order to discriminate one of the two transition states. In his early report, Whitesides used biotin-avidin technology to control the stereoselective addition of dihydrogen to *N*-acetamidoacrylic acid (Scheme 8). By incorporation of an achiral [Rh(diphosphine)(norbordiene)]$^+$ complex into avidin, he obtained a moderate *ee* (40% *ee S*) [72].
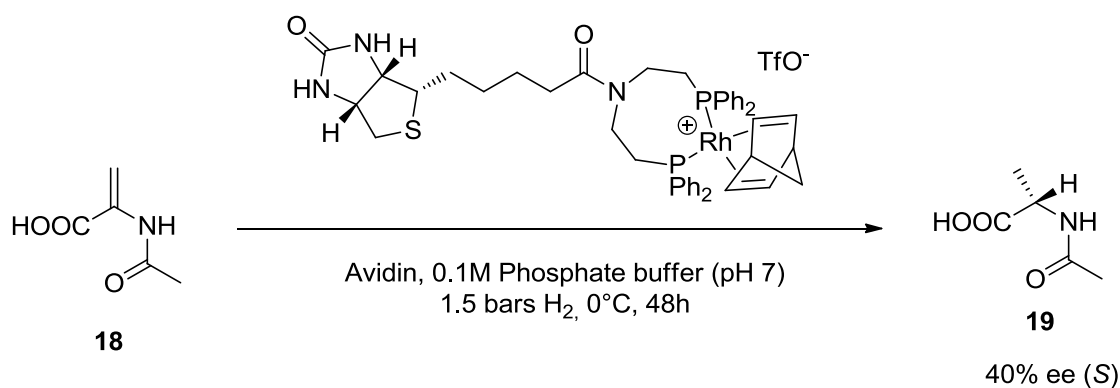
Scheme 8. Artificial hydrogenase developed by Whitesides.

Inspired by this work, many advances have been made in the development of artificial hydrogenases using this technology, mainly in the groups of Chan and Reetz [172, 173].

1.4.3. Influence of the second coordination sphere in asymmetric allylic substitution.

Allylic substitution has become a powerful tool for the creation of C-C bonds [174-183]. The allylic substrate is activated by a catalyst and the intermediate attacked by a nucleophile leading to the creation of a new bond. The enantiomeric excess is mainly influenced by two parameters (Scheme 9).
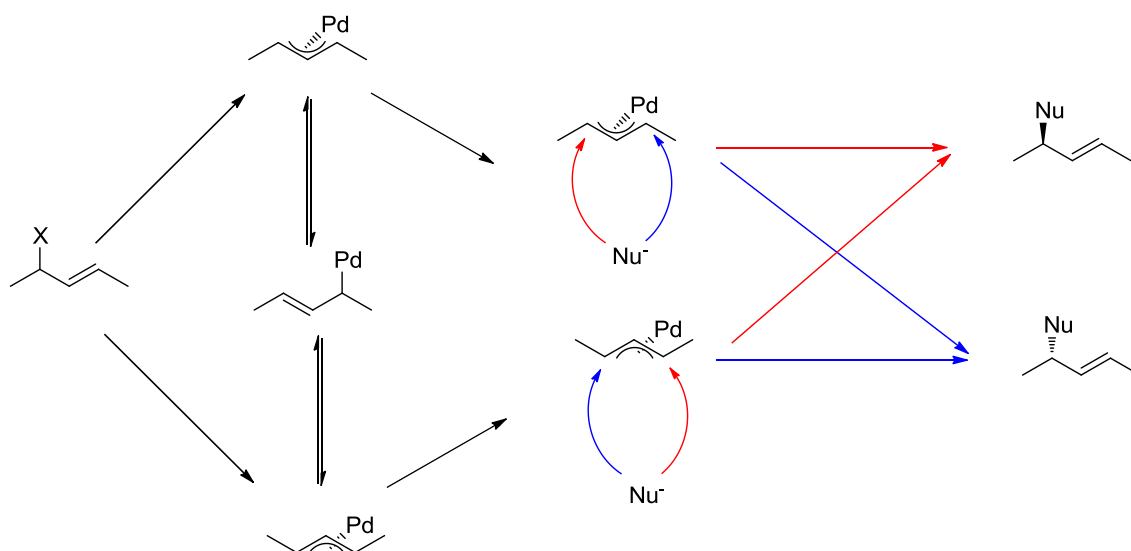
Scheme 9. Representation of the origin of enantioselectivity in asymmetric allylic alkylation.

- The isomerisation of the allyl intermediate. Once activated, the allyl intermediate can undergo rearrangements via η^3 to η^1 interconversions.This leads to scrambling of the two allyl termini

- The attack of the nucleophile. The allyl moiety offers two possible sites for attack of the nucleophile. The configuration on the asymmetric carbon on the final product is determined by the site of attack by the nucleophile.

 Ligands were designed to influence the site of attack of the nucleophile the allyl-metal intermediate and to influence the isomerisation rate of the allyl intermediate. An artificial metalloenzyme may create an ideal environment in order to direct the attack of the nucleophile by introducing steric hinderance. The rate of isomerisation of the allyl electrophile can be influenced by generating a confined environment around the metal [184-186].

Some examples in literature reported the use of peptides for asymmetric allylic alkylation [187-192].

Jäschke reported the creation of an artificial metalloenzyme for allylic amination by incorporation of an iridium catalyst to DNA (Scheme 10)[193].
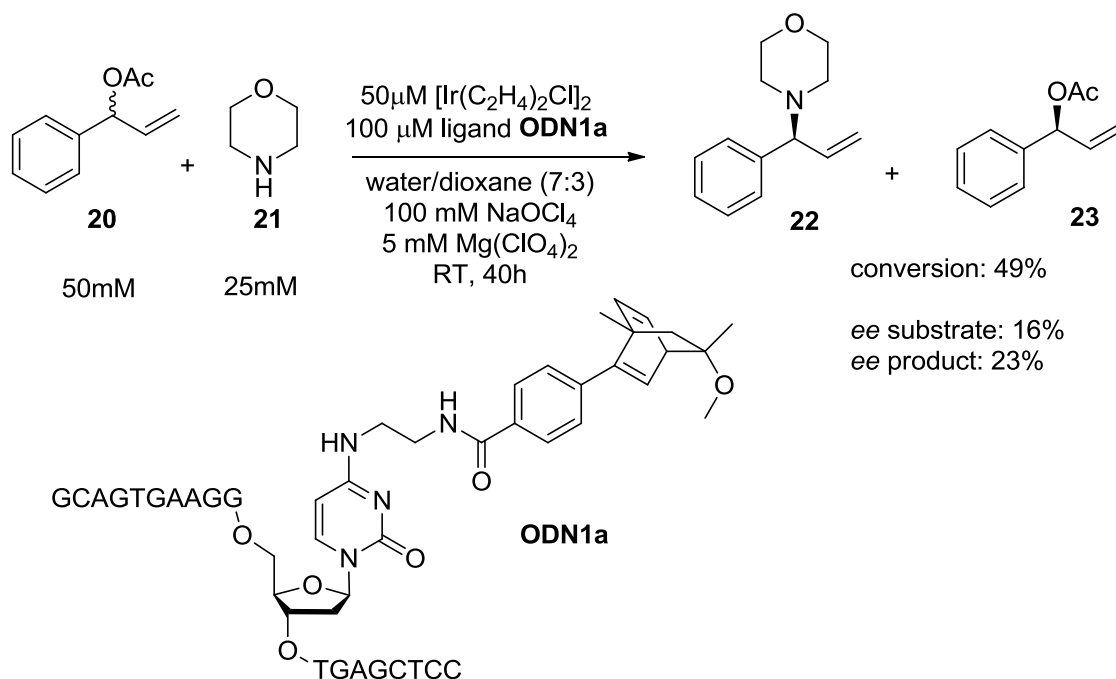
Scheme 10. Allylic amination using a iridium catalyst incorporated in DNA developed by Jäschke.

1.4.4 Molecular recognition: site selective modifications of proteins.

Transition metals are efficient tools for protein modification. Many catalyzed reactions are widely used for this purpose [194-196].

Based on the studies of Ogo and co workers, Francis reported a method for the reductive alkylation of lysines residues at room temperature and neutral pH (Scheme 11) [197-199]. Lysozyme has six lysines at its surface and these react in presence of an aldehyde to form imines which are reduced by transfer hydrogenation using the water stable [Cp*Ir(bipy)(H2O)]SO4.

37
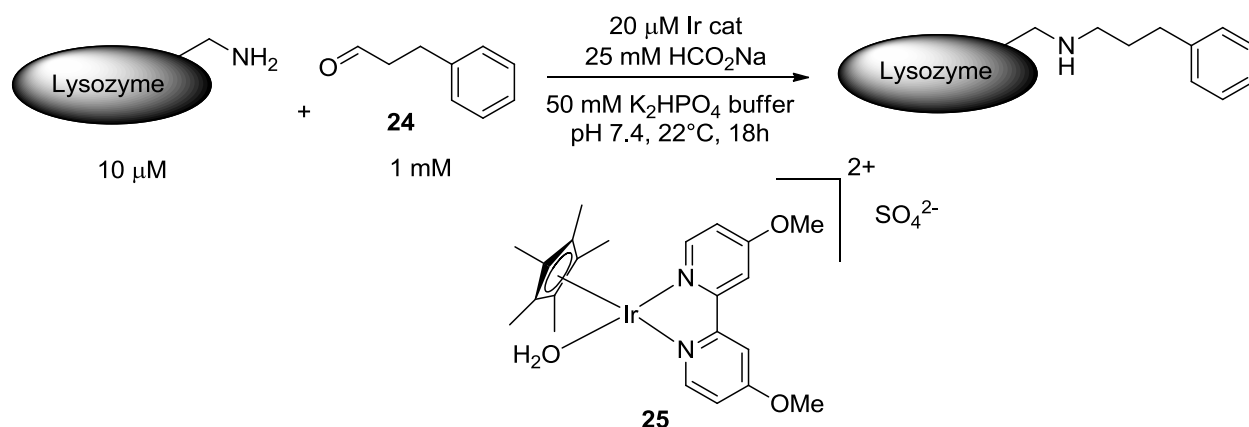
Scheme 11. Reductive alkylation of lysines developed by Francis.

Recently, Valencia, Barluenga and co workers reported the arylation of tyrosine and phenylalanine side chains using the Suzuki-Miyaura cross-coupling reaction (Scheme 12) [200]. A peptide sequence containing a tyrosine was previously iodinated and then coupled with an aryl trifluoroborate salt under Suzuki-Miyaura conditions.
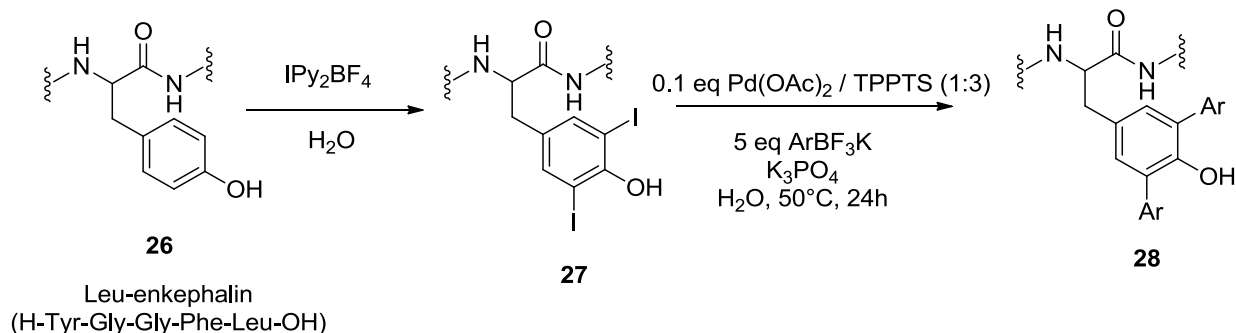
Scheme 12. Arylation of tyrosines and phenylalanine residues using Suzuki-Myaura cross-coupling.

In the same spirit, Davis recently reported a Suzuki-Miyaura coupling to a protein at a genetically incorporated aryl halide (Scheme 13) [201]. Based on the work of Schultz [202], he introduced unnatural *p*- halophenylalanine amino acid residues in a maltose binding protein.
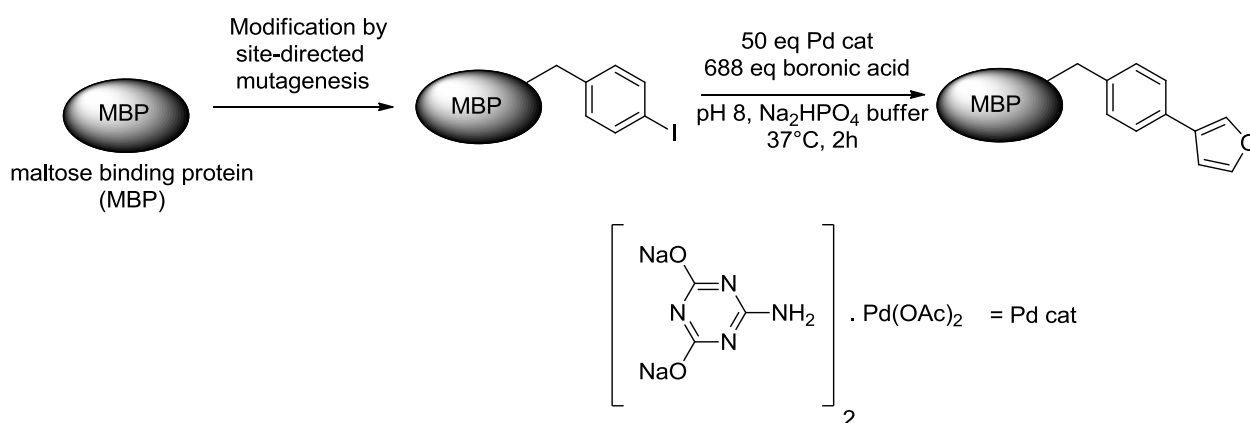
Scheme 13. Modification of genetically modified protein using Suzuki-Myaura cross-coupling.

Recently, Davis reported an interesting way to modify proteins using olefin metathesis (Scheme 14) [203-207]. A cysteine group on the protein surface is eliminated with *O*-mesitylenesulfonylhydroxylamine (MSH) to provide a dehydroalanine residue, which can undergo conjugate addition reactions with allylmercaptan to obtain an allylsulfide group on the protein surface. He reported that allylsulfide groups are effective substrates for the cross-metathesis reaction in the presence of the commercially available Hoveyda-Grubbs II catalyst under aqueous conditions.
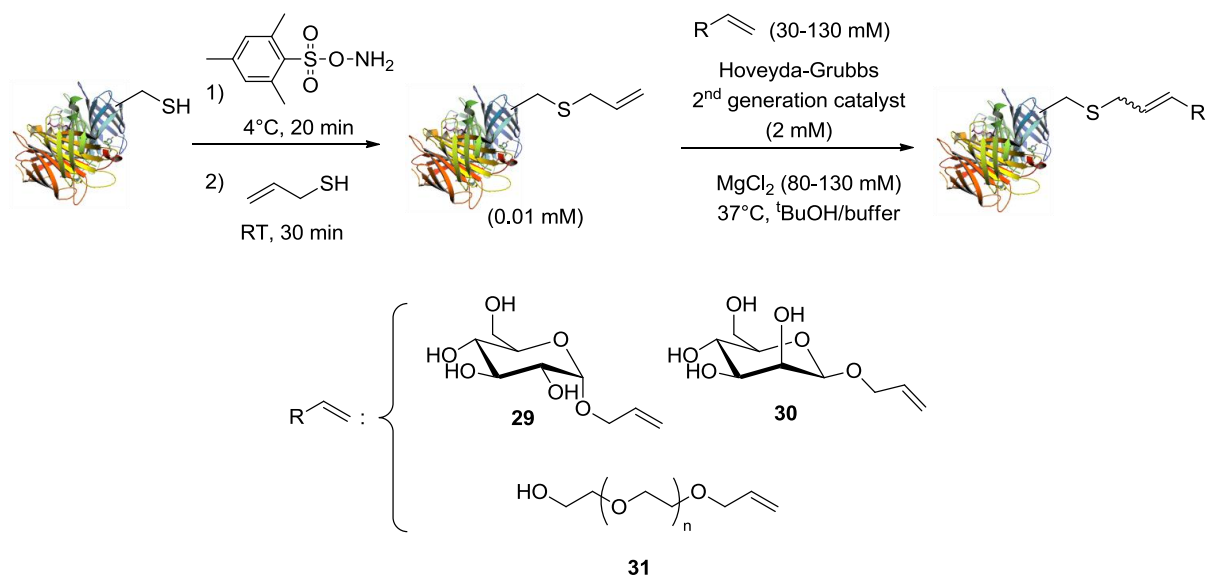
Scheme 14. Selective cystein modification using olefin metathesis.

Olefin metathesis offers the advantage to be compatible with the presence of other functionalities on the protein. From its discovery, olefin metathesis became one of the most useful reactions catalyzed by transition metal complexes. Many research groups contributed to develop this reaction and expand its potentiality (Figure 10) [208-245]. Schrock, Grubbs and Chauvin were awarded in 2005 with the Nobel Prize for this reaction [246-248]. Ring closing metathesis widely contributed to the success of this reaction, finding wide use in total synthesis [249-252].
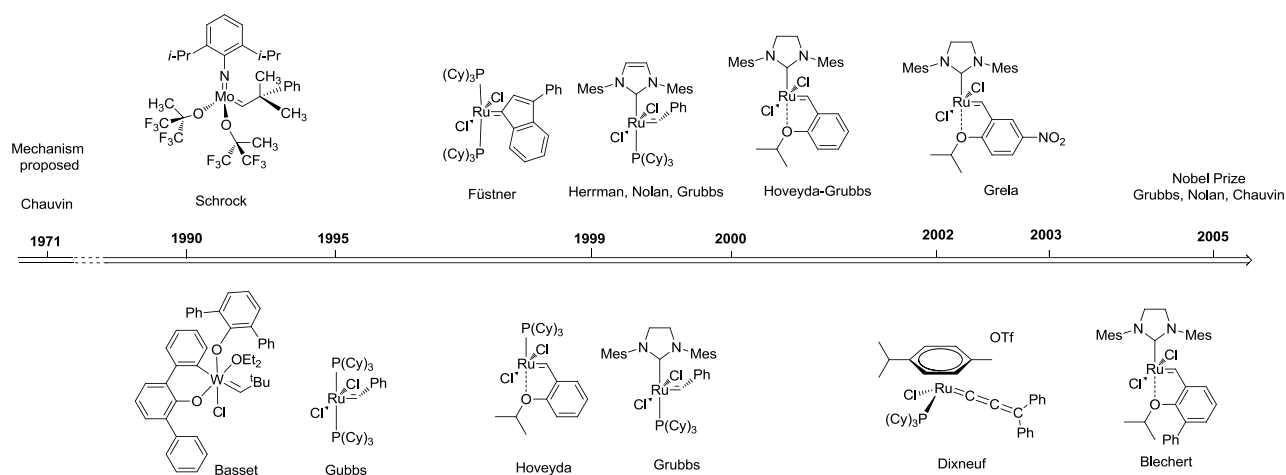
Figure 10. Overview of the development of olefin metathesis.

Since the first mechanistic hypothesis of Chauvin, the mechanism of olefin metathesis is now widely accepted [253-258]. After an initialization step, the mechanism occurs by a series of [2+2] cycloaddition–retrocycloaddition that release the product and ethylene (Scheme 15).
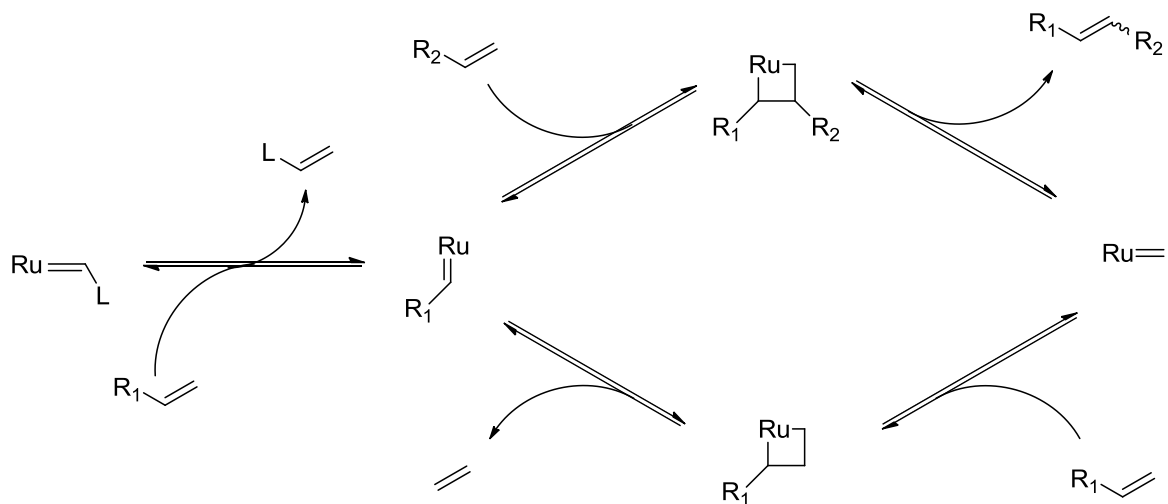
Scheme 15. Mechanism of olefin metathesis.

The potential of metathesis for protein modification is very large. However, in most cases, a high catalyst loading is necessary. A biomolecular host protein may result in reducing the loading of the catalyst, since the interactions between the two macromolecules may be important.

Ball described peptide modification using dirhodium metallopeptide catalysts combining peptide molecular recognition with the selective modification of aromatic residues (Scheme 16) [259, 260].
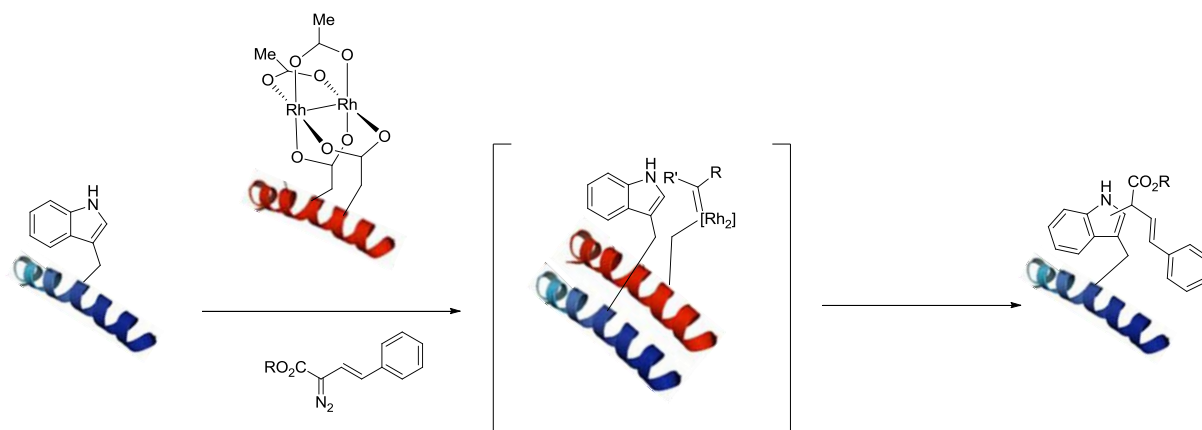
Scheme 16. Peptide molecular recognition and residue selective modification of tryptophane developed by Ball.

In his review, Francis underlined the problem of non specific metal binding to proteins when transition metal catalyzed couplings were used for protein modification. This problem can be overcome in the case of artificial metalloenzymes since the localization of the metal into the protein is ensured by the specific anchoring [261].

The presence of the biomolecular scaffold is important for *in vivo* bioconjugation. Cellular media is composed of many elements that can poison the free catalyst and the biomolecular scaffold can act as a shield. Recently, Bradley reported that palladium nanoparticles trapped within polystyrene microspheres can enter cells and mediate a variety of reactions such as allylcarbamate cleavage and Suzuki-Miyaura cross coupling (Scheme 17) [262].
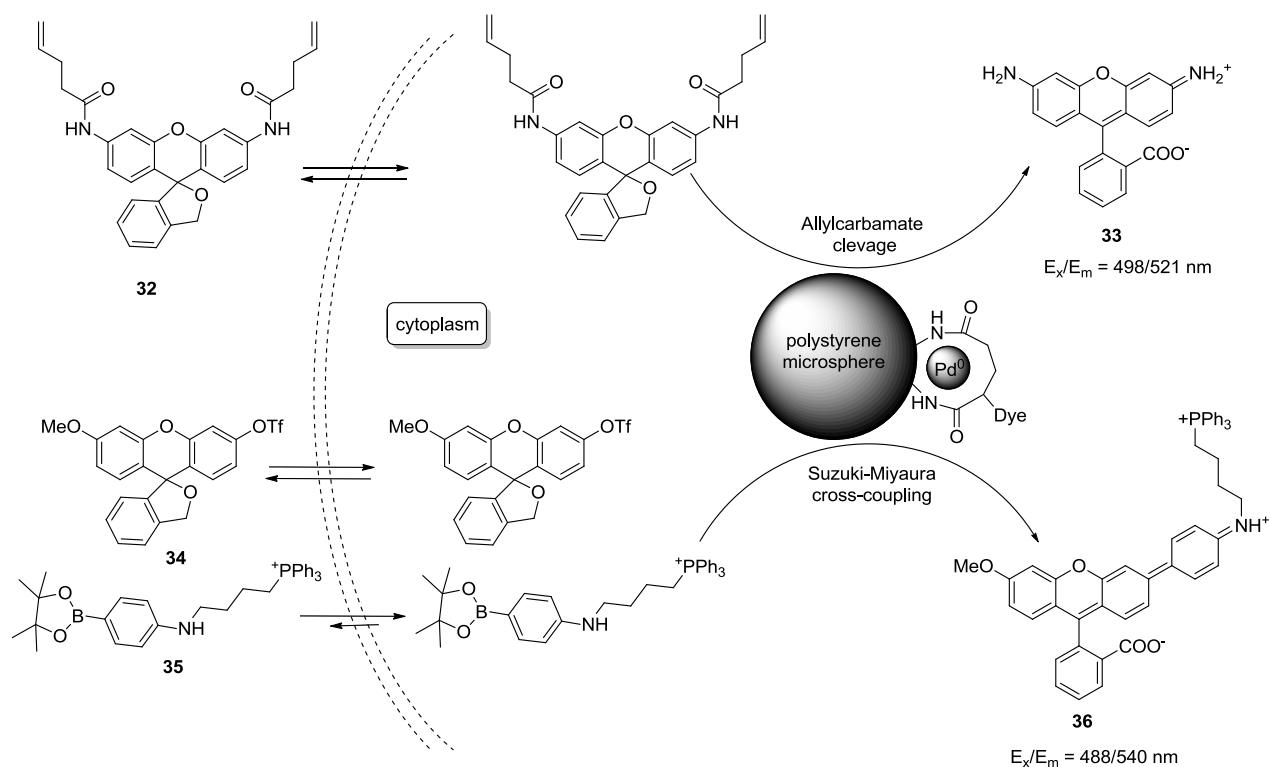
Scheme 17. Cell mediated reactions using trapped palladium nanoparticles developed by Bradley.

1.5. Aim of this thesis

The high stability of (strept)avidin combined with its high affinity towards biotinylated compounds offer a large number of promising and challenging applications. In this thesis, we focused our efforts on exploiting the potential of streptavidin as a biomolecular scaffold to enlarge the scope of artificial metalloenzymes based on the biotin (strept)avidin technology.

In the first part of this work, it was demonstrated that artificial metalloenzymes can be more than a concept. The scope of the system in the case of artificial hydrogenases was determined and then the properties of streptavidin were employed to find solutions in order to make this system an attractive tool for synthesis. The stability of streptavidin was illustrated in the improvement of the allylic alkylation reaction. In parallel, a thermal treatment of cell free

extracts was tested to rapidly purify streptavidin isoforms with the aim of implementing directed evolution protocols for the optimization of artificial metalloenzymes.

Until now, streptavidin has been used to generate a chiral environment in asymmetric reactions. A new application of the biomolecular scaffold is presented in the second part of this thesis. Based on the principle that the biomolecular scaffold may provide molecular recognition, artificial metalloenzymes were designed for site selective modification of proteins by investigating suitable reactions to perform the modification. Artificial alkylases were tuned for allylic tyrosination of proteins. Finally, an artificial metalloenzyme for olefin metathesis was developed.

II.   Results and discussion.

2.1. Artificial hydrogenase based on biotin-streptavidin technology.

2.1.1. Introduction to artificial hydrogenases.

Since 2001, Ward reported on the potential of improvement of artificial hydrogenases by chemogenetic optimization (Scheme 18).

Scheme conditions:

1 mol % ligand
0.9 mol % [Rh(COD)$_2$]BF$_4$
0.33 mol % protein

0.1 M MES buffer (pH 5.5)
5 bars H$_2$, 16h, RT

R = H : *N*-acetamidoacrylic acid

R = Ph : *N*-acetamidocinnamic acid

R = H : *N*-acetamidoalanine
      N-AcAla

R = Ph : *N*-acetamidophenylalanine
       N-AcPhe
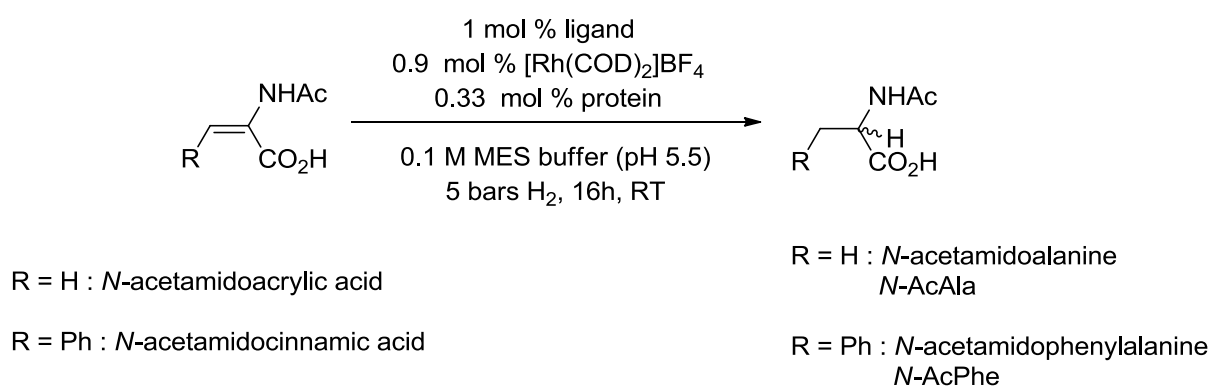
Scheme 18. Reduction of *N*-acetamidodehydroaminoacids using artificial metalloenzyme based on the biotin-streptavidin technology.

A first generation of artificial hydrogenases was obtained by introduction of an achiral rhodium complex in streptavidin mutants in a similar manner to Whiteside's original approach [263, 264]. This first generation contained achiral spacers between the biotin and the diphosphine moiety (Figure 11). Chemical optimization was performed by introducing an alkyl or an aromatic spacer, which led to significantly altered selectivity and activity of the hybrids (Table 3).

...-3^n-...

n= 1, 2, 3, 4

...-4^q-...

q= ortho, meta, para

...-...-1

...-...-2

Biot-...-...

Anchor

Spacer

Diphosphine
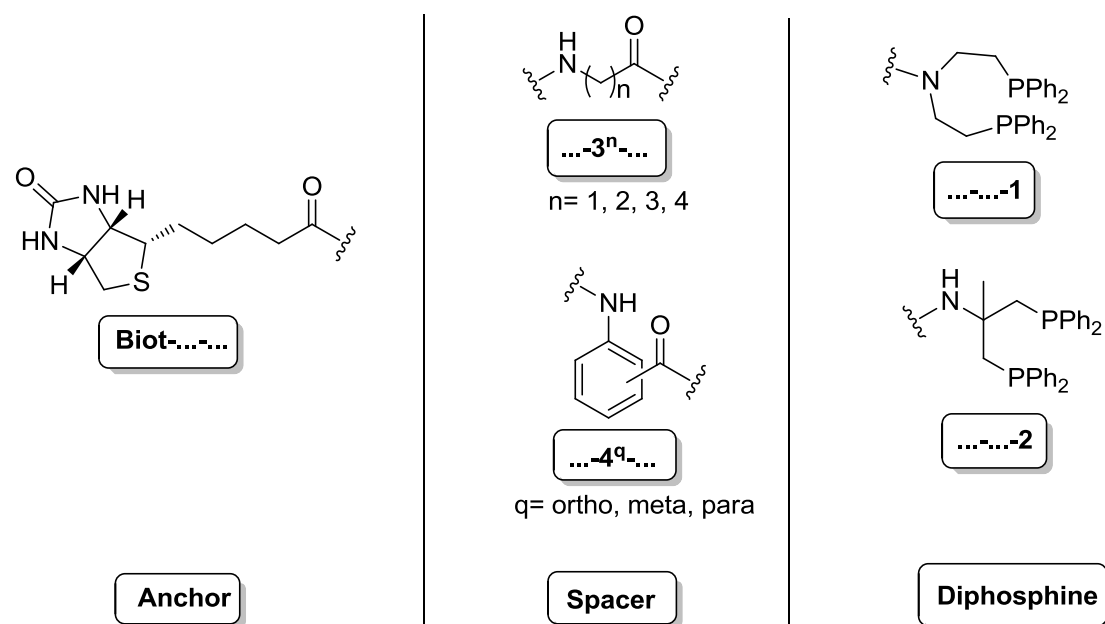
Figure 11. First generation of artificial hydrogenases using an achiral spacer.

Table 3. Selected results of first generation artificial hydrogenases.

Entry	Ligand	Protein	N-AcPhe conv[a]	N-AcPhe ee [b]	N-AcAla conv[a]	N-AcAla ee [b]
1	Biot-1	Sav WT	84	93	quant. [c]	94
2	Biot-4^meta-1	S112K	89	-88	quant. [c]	-63
3	Biot-3^1-2	S112F	20	-36	quant. [c]	-64
4	Biot-3^4-2	S112Q	77	92	quant. [c]	87

[a] conversion in %, [b] ee in %, positive ee values in favor of the (R)-enatiomer, negatives values in favor of the (S)-enatiomer, [c] quantitative; N-AcPhe: N-acetamidophenylalanine; N-AcAla: N-aceamidoalanine

A new class of artificial metalloenzyme for hydrogenation was generated by introduction of an enantiopure amino acid spacer instead of an achiral amino acid (Figure 12) [265, 266]. Dr Myriem Skander and Dr Edy Untung Rusbandi selected phenylalanine and proline as chiral spacers. Phenylalanine was selected to increase the hydrophobic interactions between the aromatic part of the amino acid and the aromatic residues in the pocket of streptavidin. Proline was selected with the aim of generating a more rigid structure. Screening of these new ligands with the isoforms of streptavidin afforded interesting results. In most cases, the chirality of the product was inverted by changing the chirality of the spacer (Figure 13). The

second generation catalysts using the proline spacer provided a high stability and tolerance toward organic solvents. Catalyses were performed with 45% DMSO as miscible organic solvent and ethyl acetate (biphasic system) and only small erosion in conversion and ee were observed.
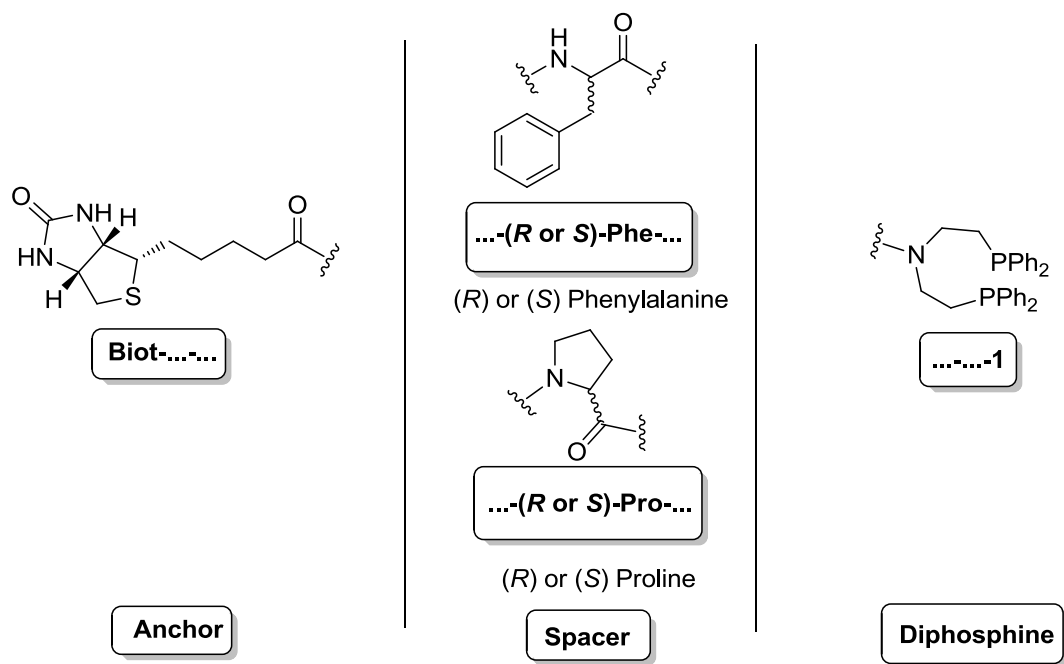
Figure 12. Second generation of artificial hydrogenase using amino acid spacer.
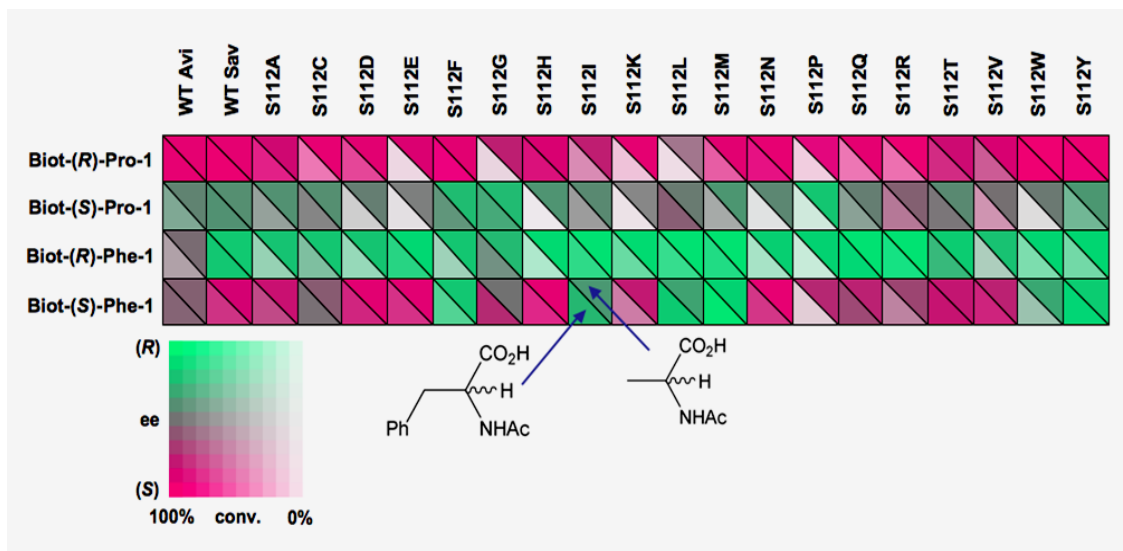
Figure 13. Fingerprint display of the result of the chemogenetic optimization of the second generation of artificial hydrogenase.

2.1.2. "Large scale application" of artificial hydrogenase.

Biocatalysts offer the advantage to be ecologically friendly since little or no toxic chemicals are involved in their production. Besides, they are also very selective compared to common small catalysts. They can be considered as an alternative for small molecule catalysts for application in industry. However, despite these advantages, the cost of production and the engineering of these systems can limit their use for large scale applications. The limited range of reactions that can be performed with biocatalysts fulfilling the industrial requirements of activity, stability and selectivity restricts their application. Artificial metalloenzymes with their complementary properties could potentially provide a useful addition to the catalyst portfolio. Artificial metalloenzymes strive to combine the structural diversity of biomacromolecules with the wealth of transition metal catalyzed reactions.

Roelfes discussed in a recent review several criteria that artificial metalloenzyme would have to fulfil to be applicable in a large scale reaction, most importantly: a high activity and an easy preparation and use of the system [267]. Another point can be addressed: the cost of the process. Most often, the catalyst is a high value species. It is therefore desirable to recover the catalyst and use it several times. Up to now, only few examples of the application of artificial metalloenzymes on a large scale have been reported [268].

Several experiments were carried out with artificial hydrogenases to determine if the system may be amenable for an application on a larger scale. The investigation of the second generation of artificial hydrogenases has demonstrated that artificial metalloenzymes can be used in presence of large amounts of organic solvent. This is desirable as the solubility of substrates is most often a limiting factor for aqueous catalysis. In the following, the results of an investigation into the suitability of artificial hydrogenases based on the biotin-streptavidin technology for the larger scale applications are presented.

2.1.3. Activity of the artificial metalloenzyme.

First, the activity of the artificial metalloenzyme was tested. For this purpose, the ratio between the substrate and the catalyst was varied from 100 to 1000.

The amount of the catalyst and the total volume were maintained constant. Accordingly, the concentration of the substrate was varied from 6 mM to 60 mM . At concentrations over 60 mM, the substrate was not fully soluble.

Table 4. Effects of an increase of the ratio between substrate (*N*-acetamidoacrylic acid) and catalyst ([Rh(COD)Biot–1]$^+$ ⊂ streptavidin WT).

Entry	[substrate] [a]	Ratio substrate/catalyst [b]	*N*-AcAla Conv [c]	*N*-AcAla *ee* [d]
1	6	100	quant. [e]	94
2	12	200	quant. [e]	94
3	18	300	quant. [e]	94
4	30	500	quant. [e]	94
5	60	1000	85	90

[a] concentration in mM, [b] equivalent of substrate (*N*-acetamidoacrylic acid) compared to artificial metalloenzyme, [c] conversion in %, [d] *ee* in %, values in favor of the (*R*)-enatiomer, [e] quantitative; 5 bars H$_2$, 16h, room temperature; *N*-AcAla: *N*-acetamidoalanine.

The conversion was quantitative down to a catalyst to substrate ratio of 500 and the *ee* remained constant. (94 % *ee*, entries 1-4, table 4). For a catalyst to substrate ratio of 1000, a small erosion in activity and selectivity was observed. (85% conversion 90% *ee*, entry 5, table 4).

Analytical scale reactions in the laboratory are often carried out on a milligram scale but when the scale is increased to the gram scale, differences in conversion and *ee* are sometimes

observed. In the case of artificial metalloenzymes, the amount of employed material is very small since the material is expensive and the yield in the production of the protein is often low. Thus, the experiments are performed in general at micromolar scale.

The artificial metalloenzyme described above containing a proline spacer showed a high stability. To investigate if this higher stability observed in small scale reactions would also be benifical for the upscaling of the reaction, the amount of the reactants was multiplied by 15 for this experiment. Streptavidin WT was selected as the protein moiety since this protein was available in large amounts and gave good ee's.

Table 5. Large scale catalysis using [Rh(COD)Biot–(R)-Pro–1]$^+$ ⊂ streptavidin WT.

Entry	Protein amount [a]	Ratio substrate/catalyst [b]	N-AcAla Conv [c]	N-AcAla ee [d]
1	1.67	100	quant. [e]	87
2	25	100	quant. [e]	87
3	25	2000	89	40

[a] in mg, [b] equivalent of substrate (N-acetamidoacrylate) compared to artificial metalloenzyme, [c] conversion in %, [d] ee in %, values in favor of the (S)-enatiomer, [e] quantitative; 5 bars H$_2$, 16h, room temperature; N-AcAla: N-acetamidoalanine.

Upon increasing the scale by a factor 15 while maintaining the catalyst loading constant, the conversion and the ee were unaffected (entry 2, table 5). This result suggests that the artificial hydrogenase does not lose its activity or selectivity upon upscaling the reaction. However, the substrate/catalyst ratio as increased to 2000, at constant catalyst concentration, a heterogeneous mixture was obtained. The conversion remained high but the ee dramatically decreased to 40% (entry 3, table 5).

2.1.4. Recycling of the artificial metalloenzyme.

Generally, the catalyst is a high value species due to multi step synthesis of the ligand and expensive reagents such as precious metals. Very active and operationally stable catalysts allow a lower loading but it would still be desirable to recover the catalyst after a catalytic run.

For the purpose of immobilization, Dr Edy Untung Rusbandy developed a strategy relying on the high affinity of the biotinylated sepharose for streptavidin (Scheme 19).

= Biotin-Sepharose	= Tetrameric (strept)avidin	= Biotinylated-Rh complex
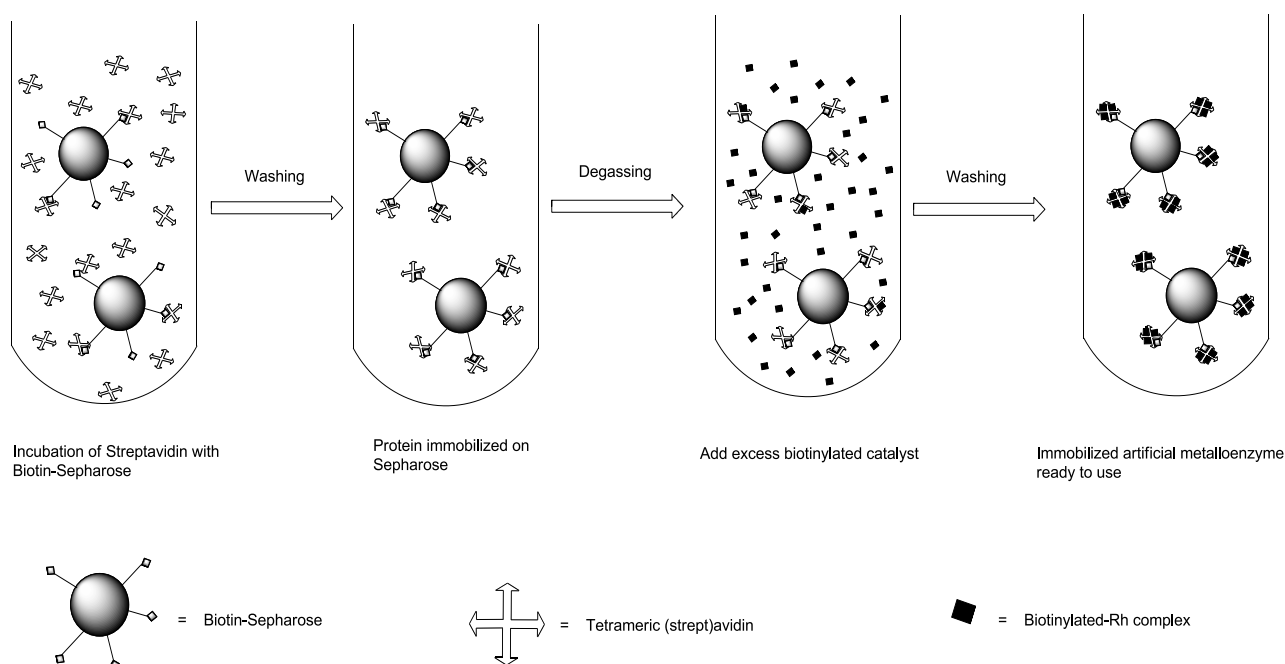
Scheme 19. Principle of immobilization of the artificial metalloenzyme.

An excess of (strept)avidin is added to the biotin sepharose followed by washing to remove the excess protein. The tetrameric protein is fixed on the polymer using one of its four binding sites. The remaining (up to three) free binding sites are then employed to bind to the biotinylated catalyst. The excess catalyst is then washed off. The immobilized artificial hydrogenase is then used in catalysis. This principle of immobilization was exploited to recycle the precious artificial hydrogenase. For this experiment, we selected the most versatile artificial metalloenzyme based on [Rh(COD)**Biot–(R)-Pro–1**]$^+$ ⊂ S112W.

Table 6. Recycling of the artificial metalloenzyme [Rh(COD)Biot–(R)-Pro–1]$^+$ ⊂ S112W.

Entry	Run	N-AcAla Conv [a]	N-AcAla ee [b]
1	1st	quant. [c]	96
2	2nd	quant. [c]	96
3	3rd	25	30

[a] conversion in %, [b] ee in %, values in favor of the (S)-enatiomer, [c] quantitative; substrate: N-acetamidoacrylate, 5 bars H_2, 16 h, room temperature; N-AcAla: N-acetamidoalanine.

The use of the immobilized artificial metalloenzyme led to similar results compared to the system "in solution" (entry 1, table 6). After the first run, the catalyst was recovered by filtration and washed. A solution containing the substrate was added to the immobilized artificial metalloenzyme for a second run under the same conditions (5 bar H_2, 16h, room temperature) that led to the same result (entry 2, table 6). A third run, following the same procedure, displayed a dramatically reduced activity and selectivity of the catalyst (entry 3, table 6). It was concluded that the immobilization on biotin sepharose allows recycling the artificial hydrogenase at least once.

2.1.5. Lyophilisation of the artificial metalloenzyme.

With the aim of simplifying manipulation and use of artificial metalloenzymes, their lyophilisation was investigated (Scheme 20).
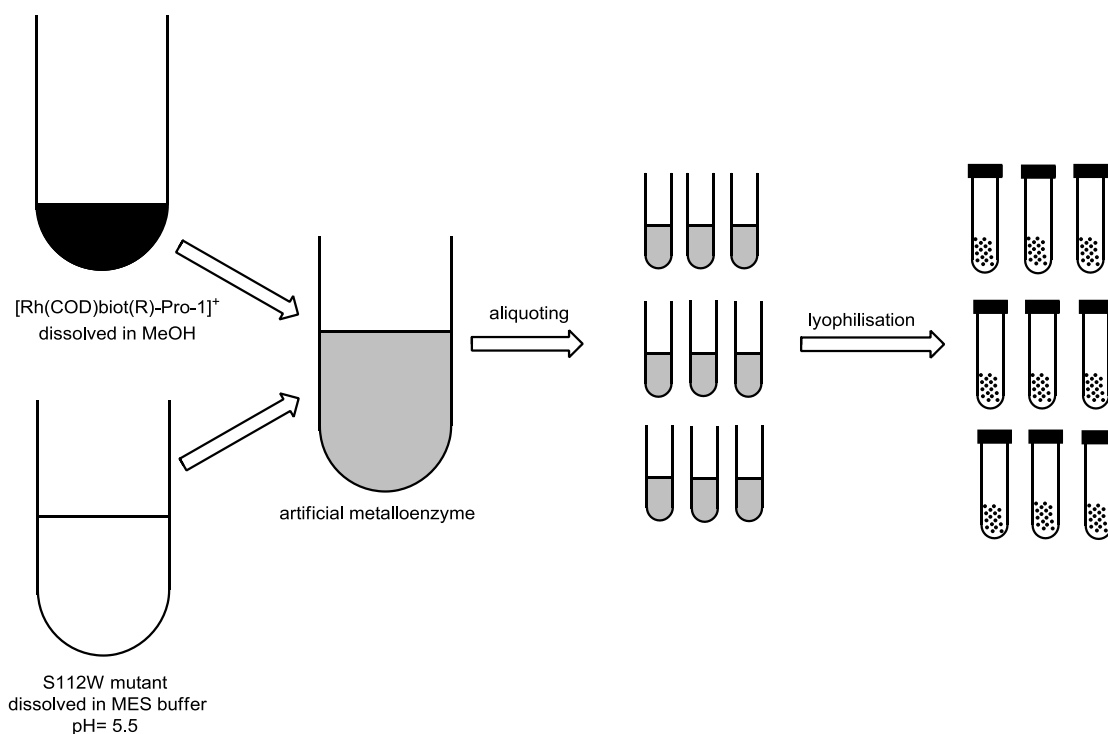
Scheme 20. Principle of the lyophilization of the artificial hydrogenase.

A large amount of the artificial metalloenzyme was prepared and then aliquoted into several small flasks. Each flask was lyophilyzed and the powder could be easily stocked and handled. Once water was added, the artificial metalloenzyme was ready for use.

An example was carried out with the best artificial hydrogenase, [Rh(COD)**Biot–(R)-Pro–1**]$^+$ ⊂ Sav S112W.

After lyophilisation, the artificial metalloenzyme was dissolved in water in the presence of the substrate under 5 bars of hydrogen. The activity and the selectivity of the catalyst remained unchanged compared to the non-lyophilized catalyst under the standard screening conditions (full conversion, 96% *ee* in favour of the *S* enantiomer).

2.2. Artificial alkylase based on the biotin-streptavidin technology.

2.2.1. Introduction to artificial alkylases.

 In the Ward group, Dr. Julien Pierron developed an artificial metalloenzyme for the formation of new C-C bonds in water using the principle of allylic alkylation [269]. A palladium complex was incorporated into streptavidin using a biotinylated diphosphine ligand. The protein provided an adequate environment in order to discriminate the attack of the nucleophile on the allyl moiety (Scheme 21). The principle of artificial metalloenzyme was tested in the allylic alkylation of diphenylacetate (Scheme 22).
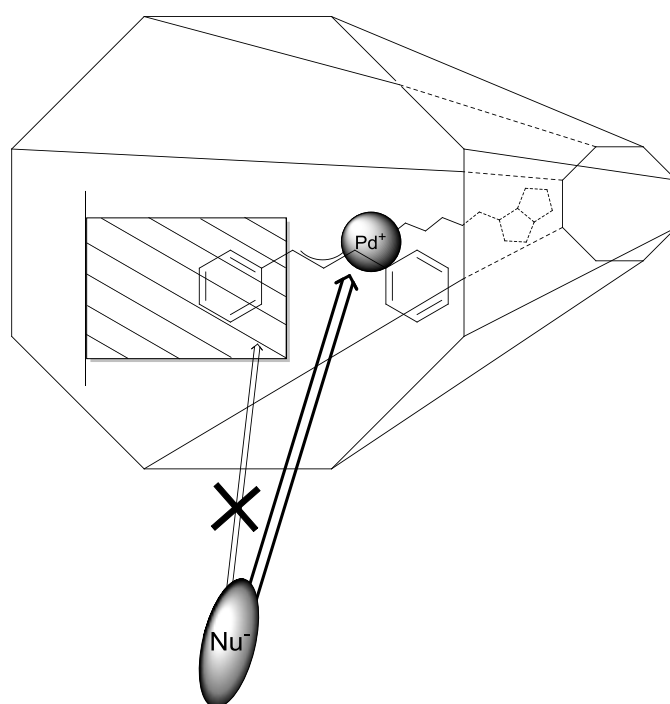
Scheme 21. Schematic representation of the influence of streptavidin in asymetric allylic alkylation.

 As previously demonstrated for the optimization of artificial hydrogenases (see Figure 13). The nature of the artificial metalloenzyme allows two kinds of optimization, chemical and genetic. Chemical optimization was obtained by modification of the diphosphine or

introduction of a spacer between the biotin and the diphosphine moiety. Genetic optimization was obtained by point mutation of an amino acid at selected positions. The chemogenetic optimization allowed Julien Pierron to obtain good conversions and good enantiomeric excesses (Figure 14). Through the screening of different ligands and proteins, he determined that the association of the ligand **Biot-4^ortho-1** and the mutant S112A gave the best result (90% *ee*, 90% conversion). Both chemical and genetic strategies can be pursued to improve the performance of artificial allylic alkylases.
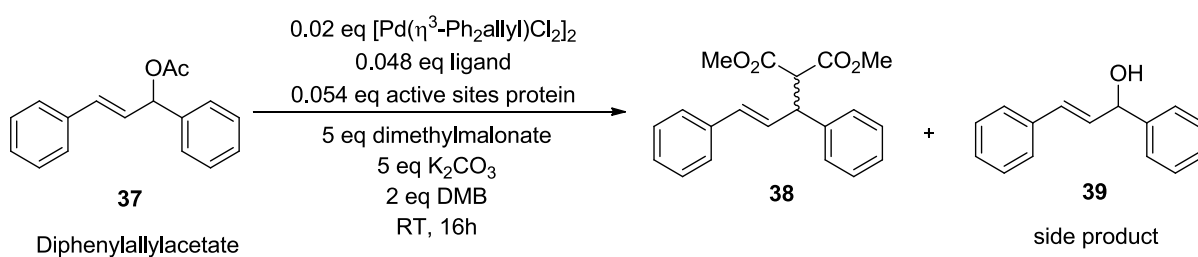
Scheme 22. Asymetric allylic alkylation of diphenylallylacetate using artificial metalloenzyme based on the biotin-streptavidin technology.
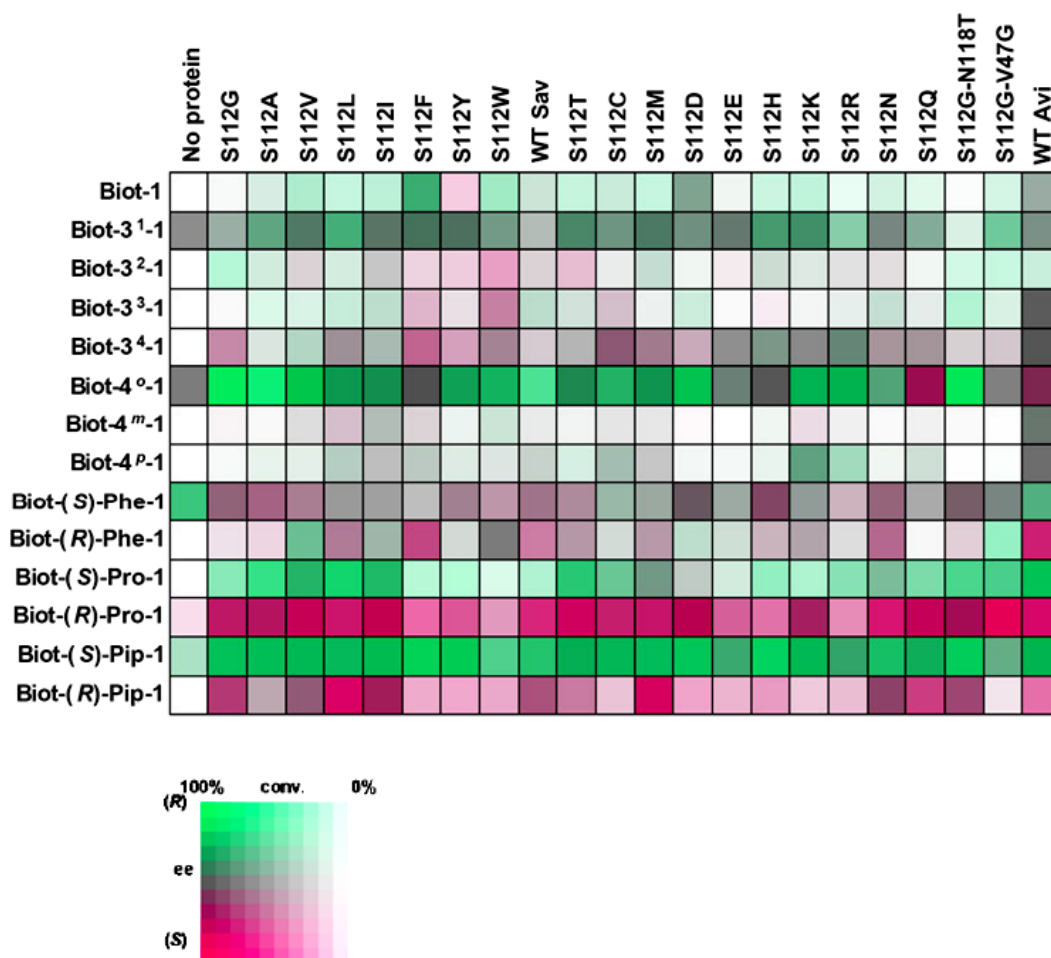
Figure 14. Fingerprint display of the result of the chemogenetic optimization of the artificial metalloenzyme for asymetric allylic alkylation.

A major difficulty encountered in the development of the allylic alkylation of 1,3-diphenylpropenylacetate was the competing hydrolysis of the substrate under the basic conditions required during catalysis. Without any additive, the hydrolysis product is formed preferentially over the alkylation product.

In order to limit this side reaction, Dr Julien Pieron investigated the effect of detergents such as DMB which significantly increased the solubility of the reagents. Better results were obtained in term of conversion but the *ee* dropped from 95% to 90%. Others parameters were investigated in order to increase the rate of the alkylation reaction without erosion of selectivity.

2.2.2. Influence of temperature on the reaction outcome.

The effect of increased temperature on the system in the presence of DMB was tested.

Table 7. Effects of the temperature on the activity of the artificial metalloenzyme.

Entry	Temperature	Conv [b]	*ee* [c]
1	0	41 [a]	65
2	5	49 [a]	85
3	25	77 [a]	89
4	40	86 [a]	92
5	60	75 [a]	91

[a] only product and hydrolysis product was observed, [b] conversion in %, [c] *ee* in %, values in favor of the (*R*)-enantiomer; conversion and *ee* determined by HPLC; substrate: 1,3-diphenylpropenylacetate, artificial metalloenzyme: ([Pd(Ph$_2$allyl)**Biot-4^{ortho}-1**]$^+$ ⊂ S112A), nucleophile: dimethylmalonate, base: K$_2$CO$_3$, surfactant: DMB.

The activity and selectivity increased with increasing temperature. At 0°C, the conversion and the *ee* were lowest (entry 1, table 7). The conversion and the *ee* were highest at 40°C (entry 4, table 7) but decreased slightly in comparison at 60°C (entry 5, table 7). The time course of the reaction at room temperature was compared to a reaction performed at 40°C (Figure 15). The rate of product formation increased at 40°C and the *ee* remained constant for the period monitored for both reactions.
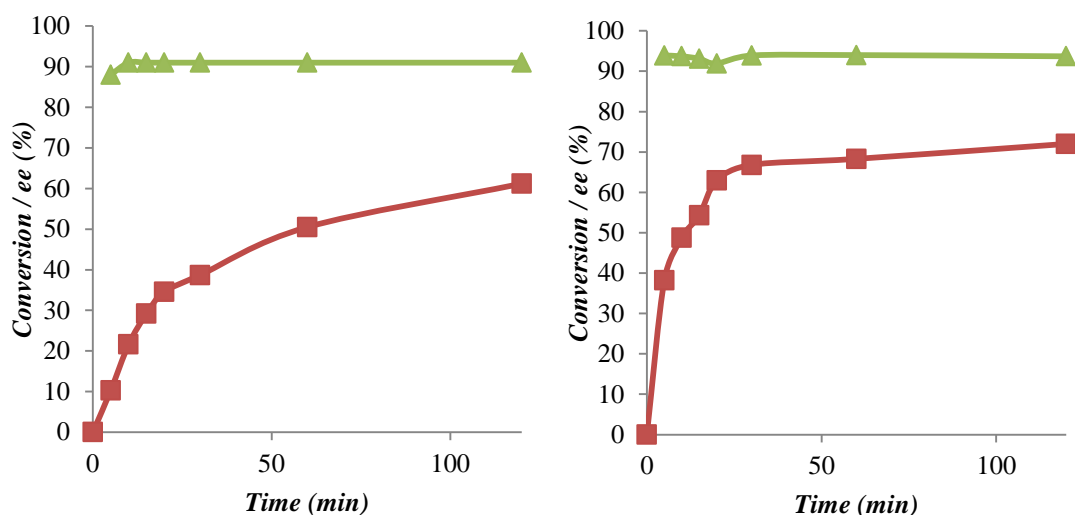
Figure 15. Conversion (in red) and *ee* (in gr*ee*n) profiles of the artificial alkylase at 25°C (left) and 40°C (right).

In conclusion, temperature had a positive effect on the reaction. Importantly, an increase of the temperature increases the rate of the reaction but also leds to an increase of the *ee*.

2.2.3. Influence of organic solvent on artificial alkylase.

Within this work, it was demonstrated that certain organic solvents are tolerated by streptavidin. The effect of an increased proportion of DMSO as a substitute for the chaotropic detergent in catalysis was thus investigated.

Table 8. Effects of DMSO on the activity and selectivity of the artificial metalloenzyme.

Entry	% DMSO	Additive	Conv [b]	*ee* [c]
1	9	--	20 [a]	95
2	9	DMB	95 [a]	90
3	25	--	37 [a]	96
4	50	--	90 [a]	95

[a] only product and hydrolysis product was observed, [b] conversion in %, [c] *ee* in %, values in favor of the (*R*)-enantiomer; conversion and *ee* determined by HPLC; substrate: 1,3-diphenylpropenylacetate, artificial metalloenzyme: ([Pd(Ph$_2$allyl)**Biot-4^{ortho}-1**]$^+$ ⊂ S112A), nucleophile: dimethylmalonate, base: K$_2$CO$_3$, room temperature.

It was observed that both the conversion and the *ee* increased when the proportion of DMSO was increased. With 25% DMSO, the conversion was lower than with DMB as an additive but the *ee* was significantly higher (entry 3, table 8). When the amount of DMSO was further increased to 50%, the conversion approached the results with DMB and the ee increased substantially (entry 4, table 8). Very little erosion of *ee* occurred.

SDS page was used to verify the stability of Steptavidin under these modified conditions (Figure 16): the protein is stable with 25% or 50% DMSO and maintains its ability to bind biotin. According to SDS page, only a small proportion of the protein is denatured with 50 % DMSO. Under these conditions, a band is observed with a mass that corresponds to the the monomer but with no binding to *Biotin*-4-fluorescein. *Biotin*-4-fluorescein (B$_4$F) was added to the sample prior to its loading on the gel. As B$_4$F has a higher affinity for (strept)avidin than the biotinylated catalyst, this latter is therefore displaced [270, 271]. The fluorescent nature of B$_4$F is used to visualize the functional (biotin-binding) proteins.
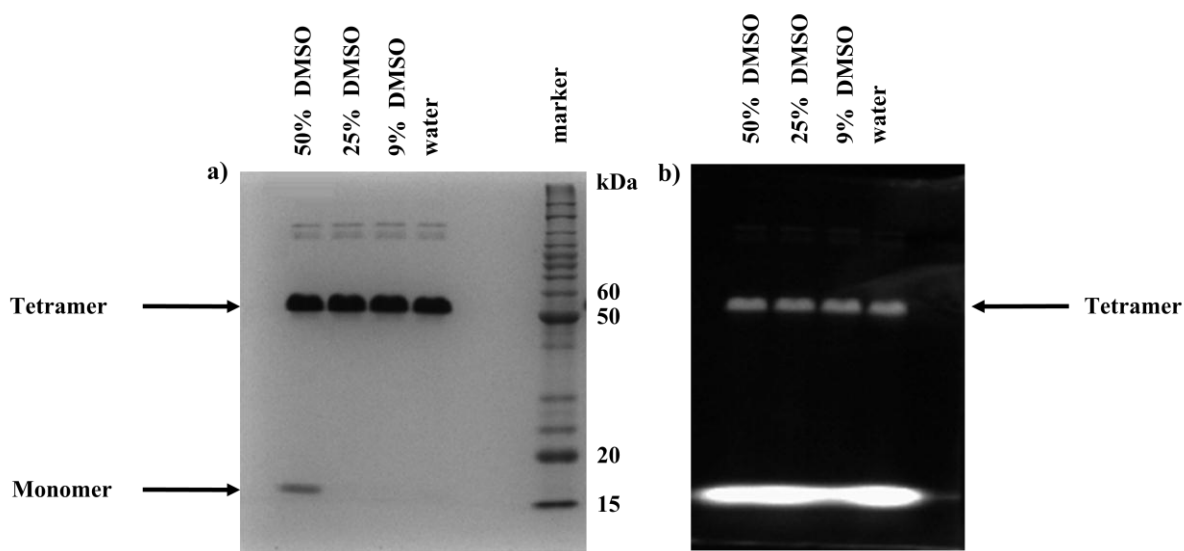
Figure 16. Stability of the protein to an increasing amount of organic solvent monitored by SDS-PAGE. a) Commasie blue stained gel; b) UV picture of B$_4$F binding activity of protein.

2.2.4. Combined effects of temperature and organic solvent.

Since an increase of the proportion of DMSO and an increase in temperature both had a positive effect on the catalysis results, we investigated of the simultaneous variation of the two parameters.

Table 9. Effects of temperature and DMSO content on the performance of artificial allylic alkylases.

Entry	Additive	Temperature	Conv [b]	*ee* [c]
1		20	33 [a]	96
2	25% DMSO	30	43 [a]	96
3		40	56 [a]	97
4		20	87 [a]	94
5	50% DMSO	30	90 [a]	95
6		40	87 [a]	96

[a] only product and hydrolysis product was observed, [b] conversion in %, [c] *ee* in %, values in favor of the (*R*)-enantiomer; conversion and *ee* determined by HPLC; substrate: 1,3-diphenylpropenylacetate, artificial metalloenzyme: ([Pd(Ph$_2$allyl)**Biot-4^{ortho}-1**]$^+$ ⊂ S112A), nucleophile: dimethylmalonate, base: K$_2$CO$_3$.

With 25% DMSO , the enantioselectivity increased from 95% to 97% and the conversion increased from 33 % to 56% (entries 1,2 and 3, table 9). A comparable effect was observed with 50% DMSO. The *ee* was increased from 94% to 96% (entries 4,5 and 6, table 9).

the high stability of streptavidin towards organic solvents and its thermostability was used to improve the output of the reaction. In the same spirit, these properties of streptavidin can be applied at the protein purification process for a high throughput screening of streptavidin and its mutants.

2.2.5. High throughput screening exploiting the thermostability of streptavidin.

In order to perform a quick screening of protein variants, Dr Alessia Sardo developed a new stategy to purify streptavidin [272]. The purification is the major bottle neck in the production of streptavidin and restricts the screening to small library sizes. Alessia Sardo exploited the thermostability of streptavidin in order to shorten the process and adapt it to a quick screening protocol by means of parallel heat treatment of cell free extracts. It was shown that streptavidin was stable at high temperature (85°C for several minutes in absence of biotin) and this stability can be increased in presence of biotin. First, the effect of the temperature on the protein was investigated. We selected streptavidin WT and two other mutants namely S112A which gave the highest selectivity in favor of the *S* enantiomer and S112Q which preferentially gave the *R* enantiomer. These proteins were heated for 5, 10 and 30 min and were subsequently analyzed on SDS page (Figure 17).
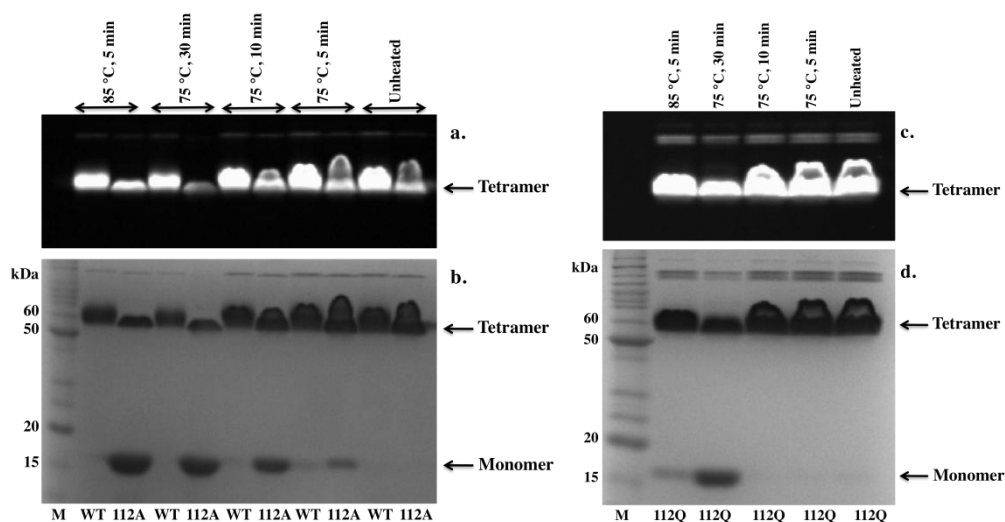
Figure 17. Thermostability of the streptavidin WT and two mutants S112A and S112Q monitored by SDS-PAGE. a) and c) Commasie blue stained gel; b) and d) UV picture of B_4F binding activity of protein.

It was noticed that the streptavidin WT was stable at 75°C even after long exposure (30 min). The protein was also stable at 85°C. Besides, the mutant S112Q was stable at 75°C and 85°C but the protein denatured more rapidly compared to WT. Furthermore, mutant S112A turned out to be the most sensitive of the variants investigated to the exposure of elevated temperatures. S112A could tolerate heating for 10 min to 75 °C but denaturation was observed for longer heating times or higher temperatures. The observations made by SDS page were reflected in catalysis results (table 10). A long exposure to elevated temperatures as means for protein purification had a negative effect on the structure of the protein and its ability to bind biotin. This was reflected in the catalytic results using heat treated proteins (entries 4, 5, 9,10, and 14, table 10).

Table 10. Results of the asymmetric allylic alkylation performed with heated proteins.

Entry	Protein	Condition	Active Site [a]	Conv [c]	ee [d]
1		Standard	3.6	87 [b]	65
2		75 °C, 5 min	3.6	86 [b]	65
3	**WT**	75 °C, 10 min	3.6	85 [b]	64
4		75 °C, 30 min	3.6	77 [b]	30
5		85 °C, 5 min	3.6	77 [b]	34
6		Standard	4	90 [b]	92
7		75 °C, 5 min	4	87 [b]	92
8	**S112A**	75 °C, 10 min	2	88 [b]	93
9		75 °C, 30 min	< 1.6	82 [b]	27
10		85 °C, 5 min	< 1.6	80 [b]	37
11		Standard	4	99 [b]	-35
12		75 °C, 5 min	4	98 [b]	-33
13	**S112Q**	75 °C, 10 min	4	99 [b]	-35
14		75 °C, 30 min	2.8	89 [b]	-19
15		85 °C, 5 min	4	99 [b]	-35

[a] active sites were determined by B_4F titration [b] only product and hydrolysis product were observed, [c] conversion in %, [d] ee in %, positive ee values in favor of the (R)-enantiomer, negatives values in favor of the (S)-enantiomer; conversion and ee determined by HPLC; substrate: 1,3-diphenylpropenylacetate, artificial metalloenzyme: ([Pd(Ph$_2$allyl)**Biot-4^{ortho}-1**]$^+$ $\subset$ protein), nucleophile: dimethylmalonate, base: K_2CO_3, surfactant: DMB.

The best condition for fast extract purification found employed heat treatment at 75°C for 10 min.

2.2.5.2. Quick screening of artificial alkylase using thermo-purified cell free extracts.

Cell free extracts are obtained from the expression of the plasmid containing the streptavidin gene in E.Coli (Scheme 23). The cell free extracts were heated at 75°C for 10 min and the supernatant was recovered.
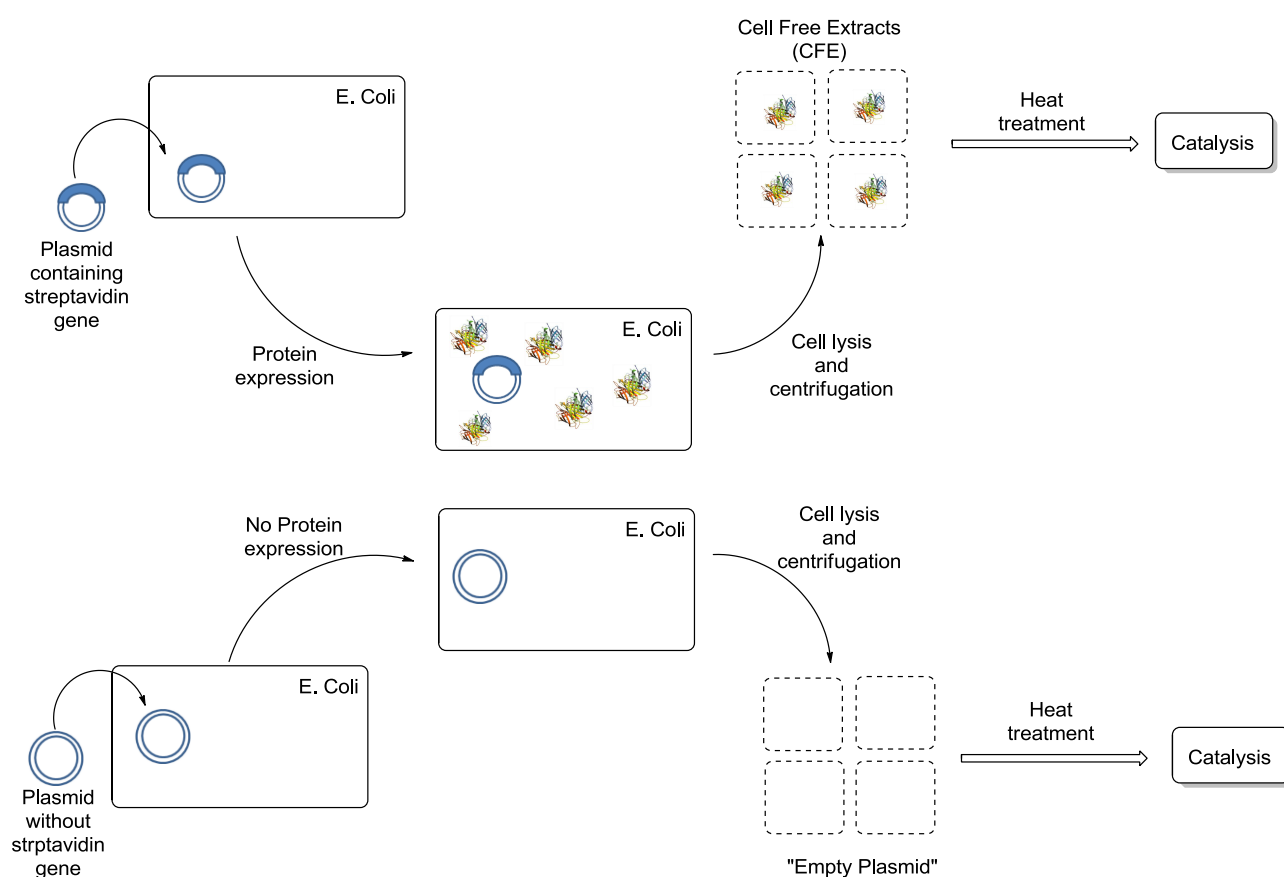
Scheme 23. Cell free extracts production and purification process.

According to SDS-page, a large proportion of components of the cell free extracts other than streptavidin can be removed by this method. The supernatant was then used in catalysis. Pure protein was added to cell free extracts obtained from cells containing an empty plasmid to determine the effects of soluble cell components on catalysis. The results are summarized in table 11.

Table 11. Results of the asymmetric allylic alkylation performed with heated crude cell extracts.

Entry	Protein	Condition	Conv [a][b]	*ee* [c]
1		$[Pd(\eta^3\text{-Ph}_2\text{allyl})$**Biot-4^{ortho}-1**$]^+$	39 [a]	2
2		$[Pd(\eta^3\text{-Ph}_2\text{allyl})$**Biot-4^{ortho}-1**$]^+$ in crude empty plasmid extract	3.1 [a]	57
3	--	$[Pd(\eta^3\text{-Ph}_2\text{allyl})$**Biot-4^{ortho}-1**$]^+$ in heated empty plasmid extract	3.0 [a]	4
4		$[Pd(\eta^3\text{-Ph}_2\text{allyl})$**Biot-4^{ortho}-1**$]^+$ in heated empty plasmid extract dialyzed	25 [a]	3
5		$[Pd(\eta^3\text{-Ph}_2\text{allyl})$**Biot-4^{ortho}-1**$]^+ \subset$ pure WT	59 [a]	79
6		$[Pd(\eta^3\text{-Ph}_2\text{allyl})$**Biot-4^{ortho}-1**$]^+ \subset$ crude extract	0.8 [a]	68
7	WT	$[Pd(\eta^3\text{-Ph}_2\text{allyl})$**Biot-4^{ortho}-1**$]^+ \subset$ heated extract	1.0 [a]	71
8		$[Pd(\eta^3\text{-Ph}_2\text{allyl})$**Biot-4^{ortho}-1**$]^+ \subset$ heated extract dialyzed	3.0 [a]	79
9		$[Pd(\eta^3\text{-Ph}_2\text{allyl})$**Biot-4^{ortho}-1**$]^+ \subset$ Spiking with pure protein dialyzed	5 [a]	78
10		$[Pd(\eta^3\text{-Ph}_2\text{allyl})$**Biot-4^{ortho}-1**$]^+ \subset$ pure S112A	98 [a]	91
11	S112A	$[Pd(\eta^3\text{-Ph}_2\text{allyl})$**Biot-4^{ortho}-1**$]^+ \subset$ Spiking with pure protein dialyzed	9 [a]	89
12		$[Pd(\eta^3\text{-Ph}_2\text{allyl})$**Biot-4^{ortho}-1**$]^+ \subset$ pure S112Q	100 [a]	-31
13	S112Q	$[Pd(\eta^3\text{-Ph}_2\text{allyl})$**Biot-4^{ortho}-1**$]^+ \subset$ Spiking with pure protein dialyzed	22 [a]	34

[a] only product and hydrolysis product was observed, [b] conversion in %, [c] *ee* in %, positive *ee* values in favor of the (*R*)-enatiomer, negatives values in favor of the (*S*)-enatiomer; conversion and *ee* determined by HPLC;

substrate: 1,3-diphenylpropenylacetate, artificial metalloenzyme: ([Pd(η^3-Ph$_2$allyl)**Biot-4$^{\text{ortho}}$-1**]$^+$ ⊂ protein), nucleophile: dimethylmalonate, base: K$_2$CO$_3$, surfactant: DMB.

Catalysis in cell free extract without purification led to dramatic erosion in conversion and *ee* compared to the purified protein (entry 6, table 11). The main product observed was hydrolysis product. This drop of activity was not caused by a change in concentration of the reactants (overall diluton) since catalysis with pure protein under the same condition of dilution provided higher activity (entries 1 and 5, table 11). The complex alone (without addition of purified streptavidin) used in cell free extracts obtained from the empty plasmid culture gave a higher conversion but led surprisingly also to a significant enantiomeric excess that must be attributed to components of the cell free extracts (entry 2, table 11). Although catalysis with purified extracts led to an enantiomeric excess in the expected range, the conversions remained low (entry 7, table 11). These values of *ee* cannot be attributed to impurities since the catalyst alone in heated empty plasmid do not lead to enantiomeric excess. (entry 3, table 11). In addition to the above described heat treatment, a dialysis of the supernatant of the cell free extracts increased the conversion (entries 4 and 8, table 11).

Spiking experiments with the mutant S112A gave the expected results (entry 11, table 11) meaning an increase in *ee*, but the spiking experiment with S112Q gave a surprising result (entry 13, table 11).

These experiments demonstrated that cell free extract may be used to evaluate the potentiality of different mutants. Supplementary steps were added but significantly reduce throughput, which was the initial design idea for the method. Besides, the feasibility of the method is severely questioned by the result obtained with S112Q.

2.3. Artificial tyrosinase based on the biotin-streptavidin technology.

2.3.1. General.

Francis et al. showed a first example of tyrosine modification, on a protein, employing palladium-catalyzed allylic alkylation.

As a model protein, Chymotrypsinogen A, bearing an accessible tyrosine on the protein surface, was selected. The synthesized allylic substrate contained a rhodamine. This fluorophore allowed straightforward detection of the protein modification (Scheme 24).
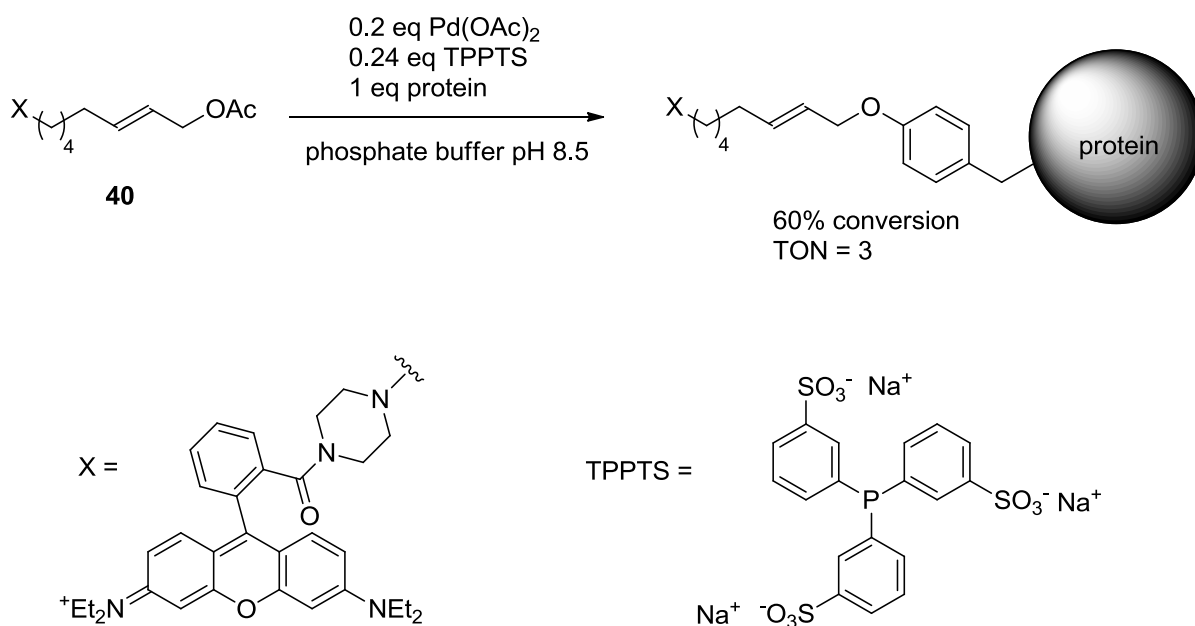
Scheme 24. Allylic tyrosination on proteins developed by Francis *et al*.

The study of Francis et al. is an example of transition metal catalyzed bioconjugation on native proteins, but as for other similar methods, peptide sequence-selectivity was not achieved. In comparison to the lack of selectivity of many metal-catalysts, nature achieves

exquisite selectivity through the use of enzymes. Artificial metalloenzymes might be better suited for the desired molecular recognition than traditional small molecule catalyst.

Based on the experience gathered with artificial metalloenzymes in allylic alkylation, the investigation of allylic tyrosination seemed attractive particularly with a view on potential sequence selectivity that might be achieved.

This work was carried out in collaboration with Ruben Cal during his Master thesis.
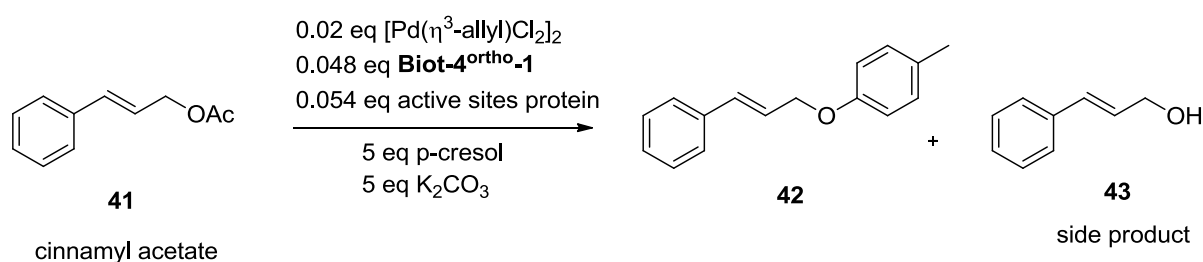
Scheme 25. Allylic phenolation of cinnamyl acetate using an artificial metalloenzyme based on the biotin-streptavidin technology.

In a test reaction, cresol was used as a tyrosine analogue in order to determine the suitable conditions for further applications with proteins (Scheme 25).

2.3.2. Optimization of the reaction of allylic phenolation.

Since the cytotoxicity of DMB presents a problem for an application for the modification of protein, the allylation reaction was performed in the absence of DMB. Preliminary results suggested that the pH has an important influence on the outcome of the reaction (Figure 18). No formation of the product was observed for pH $\leq$ 5 and $\geq$ 11. The best result was obtained with a phosphate buffer at physiological pH (12% conversion, TON= 2).
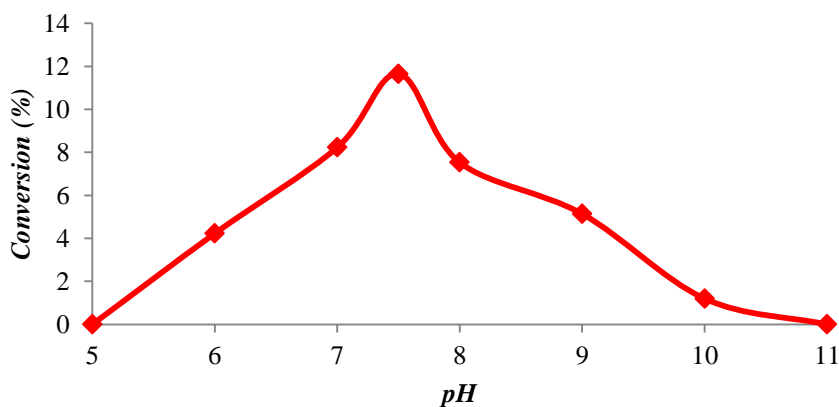
Figure 18. Results of the pH on the palladium catalyzed allylic phenolation. Artificial metalloenzyme: $[Pd(\eta^3\text{-allyl})(\textbf{Biot-4}^{\textbf{ortho}}\textbf{-1})]^+ \subset$ streptavidin WT, substrate: cinnamyl acetate, nucleophile: *p*-cresol, base: K_2CO_3, pH 5-6 0.2 M MOPS buffer, pH 7-8 0.2 M phosphate buffer, pH 9-11 0.2 M carbonate buffer.

Sinou et al. as well as other research groups reported a temperature-influence on the reactivity of the allylic phenolation [273]. Based on these reports and the knowledge of the high thermal stability of streptavidin, the temperature was set to 50°C (Favoring the investigation of the system over the purpose to work under physiological conditions). Heating the reaction to 50°C improved the conversion to 17%.

2.3.3. Genetic optimization of the artificial metalloenzyme.

Previous studies within the group showed that mutation of the protein has an influence on the reactivity, as the second coordination sphere is varied. Mutation at amino acid residue 112, was found to have a significant effect on the catalysis outcome. We thus, tested 18 streptavidin mutants resulting from saturation mutagenesis at position S112 (Figure 19).
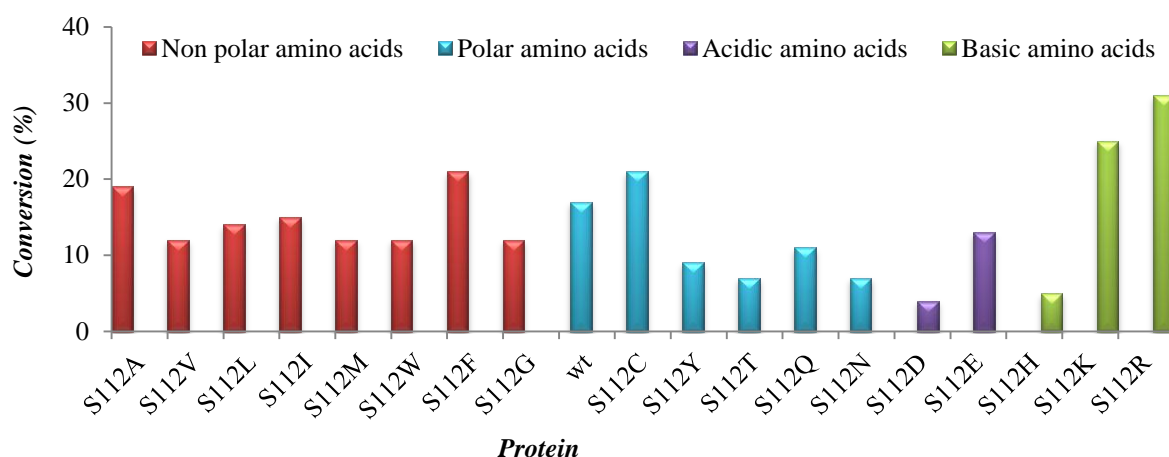
Figure 19. Result of the screening of 18 S112X streptavidin mutants under optimized conditions. Artificial metalloenzyme: [Pd(η^3-allyl)(**Biot-4^{ortho}-1**)]$^+$ $\subset$ protein, substrate: cinnamyl acetate, nucleophile: *p*-cresol, base: K_2CO_3, 0.2 M phosphate buffer (pH 7.5), 50 °C

The best results were obtained with basic amino acids at position S112 (25% for S112K and 32% for S112R). However, the third basic amino acid bearing mutant S112H gave poor results (5%). Possibly the positive charge on lysine and arginine at physiological pH had a positive influence on the catalyst. The repulsion between the positively charged amino acid side chain and the catalyst may relocate the catalyst to a place better accessible for the reactants. Alternatively the proximity of the two positive charges may destabilize the palladium allyl complex and therefore increased the reactivity of the Pd-allyl intermediate. In contrast, histidine has a significantly lower pKa value and could potentially also act as an additional ligand with a deleterious effect.

2.3.4. Influence of temperature and pH on the reaction.

Although the reactivity of the allylic phenolation was improved, the conversion remained low (compared to the results obtained for the allylic alkylation). To address this problem, the best mutant S112R was compared with the protein-free system at various temperatures and pHs (Figure 20).
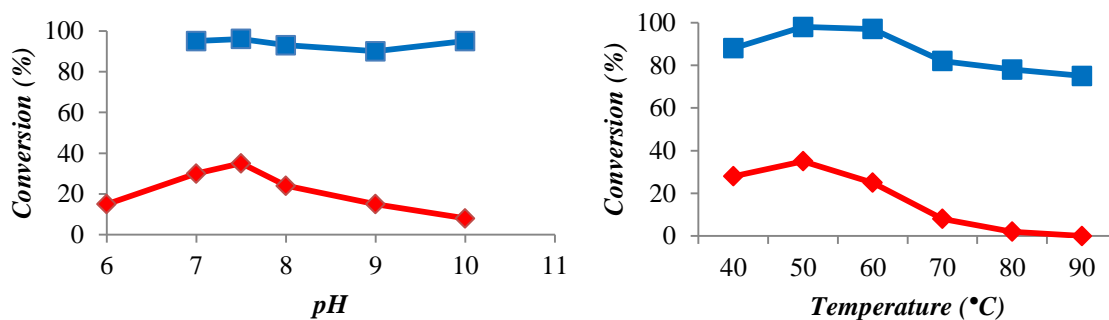
Figure 20. Allylic phenolation at various temperatures (right) and at various pHs (left) in absence of protein (blue) and in presence of S112R (red). Artificial metalloenzyme: [Pd(η^3-allyl)(**Biot-4^{ortho}-1**)]$^+$ $\subset$ protein, substrate: cinnamyl acetate, nucleophile: *p*-cresol, base: K_2CO_3. The pH screen was performed at 50 °C and the temperature screen at pH 7.5.

The protein-free system is more active than the catalyst incorporated into streptavidin. The conversions for the protein-free system increased with temperature and reached total conversion at 50°C.

Further heating (70°C-90°C) led to less product formation. The same profile was reflected when the streptavidin incorporated catalyst was tested at various temperatures, albeit with much lower conversions.

The protein-free allylic phenolation was not influenced by the variation of pH, whereas an influence of the pH was observed for the catalysis in the presence of streptavidin. A possible explanation for this observation is that variation in the pH influences the charge of the protein which may cause changes in the conformation of the protein. Since the rate of hydrolysis of the starting material can be pH dependent, it can be envisaged that the side reaction can compete more efficiently in presence of the protein since the catalyzed reaction is lowered.

2.3.5. Influence of the concentration of the substrate on the reaction.

It was supposed that a higher substrate concentration may increase the activity of the catalyst, in presence of the protein. Therefore, the mutant screening was repeated using 7.8 mM of the allylic substrate.
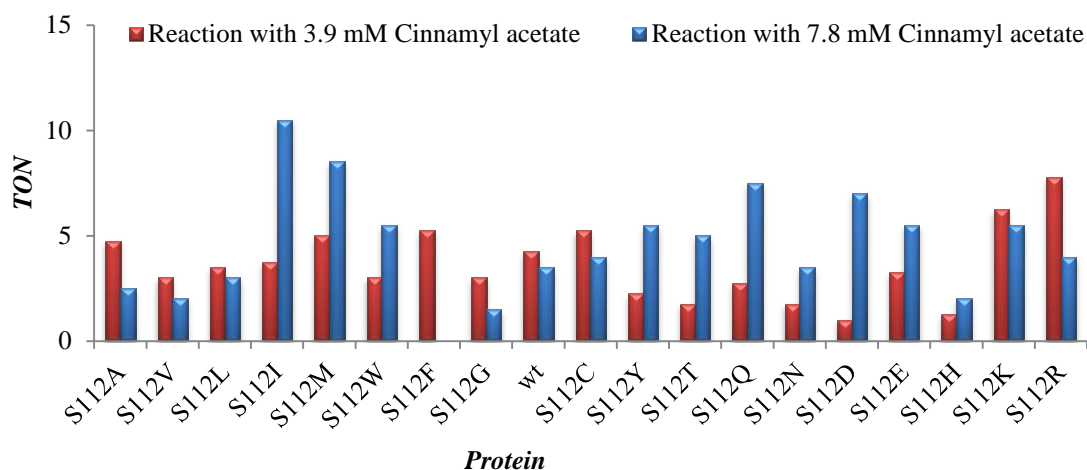
Figure 21. TON of the reactions with 18 streptavidin S112X mutants using 3.9 mM (red) and 7.8 mM (blue) of substrate. Artificial metalloenzyme: $[\text{Pd}(\eta^3\text{-allyl})(\textbf{Biot-4}^{\textbf{ortho}}\textbf{-1})]^+ \subset$ protein, substrate: cinnamyl acetate, nucleophile: *p*-cresol, base: K_2CO_3, 0.2 M phosphate buffer (pH 7.5), 50 °C.

The results were compared with the results from previous experiments, where a concentration of 3.9 mM of allyl acetate was used (Figure 21). To compare the outcome of these reactions, the TON was calculated. Some mutants (e.g. S112I, S112D) revealed higher turnover numbers when 7.8 mM of allyl acetate were used. In contrast, in some cases a decrease of activity was observed (e.g. S112A, S112R). These results suggest that no simple relationship between concentration and activity exists.

Until now, we tried to use the artificial metalloenzyme catalysis system developed for allylic alkylation for the phenolation of a *p*-cresol under physiological conditions. However, the TON in the reaction was low, despite our efforts to improve the system. A more detailed analysis of the reaction may give more clues to improve the system.

2.3.6. Detailled analysis of the reaction of allylic phenolation.

During the allylic phenolation, the formation of a white suspension was observed in the presence of the host protein (Figure 22). The analysis of the precipitate is described in the following paragraphs.

Reaction time in minutes

| 10 | 20 | 30 | 40 | 50 | 60 | 80 | 100 | 120 | 180 | 240 | 360 | 480 | 960 |
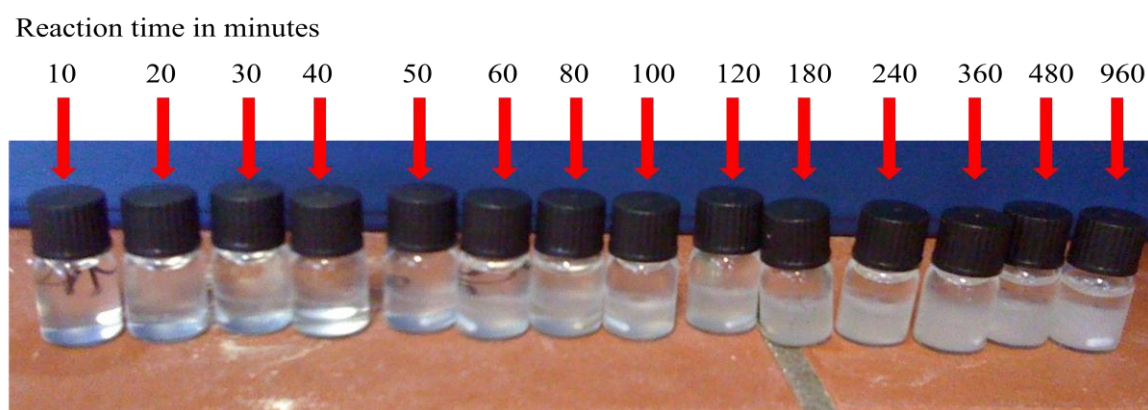
Figure 22. Catalytic samples after different reaction times

The formation of the white solid is gradual and no suspension appeared in the absence of protein. The solid was analyzed using SDS-Page (Figure 23). After centrifugation of a reaction sample, the resulting supernatant was removed and the remaining pellet was washed with H_2O and diethylether prior to analysis.
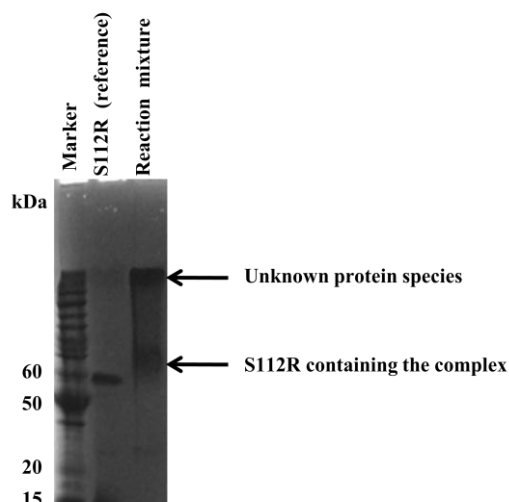
Figure 23. Post catalytic SDS-Page analysis of the white solid and the pure S112R as reference.

The result of the electrophoresis is shown in Figure 23. In the post catalysis sample, two protein species are present, the tetrameric streptavidin containing the complex (pure protein), as well as a species which did not migrate into the loading gel (this slow migrating protein species will be referred to SMPS). Therefore, the SMPS can be either a highly charged or a very large species.

2.3.6.1. Investigation on the formation of the SMPS.

Different experiments were performed to determine the nature of the SMPS and to determine under which conditions it is formed. Control reactions were performed where individual components of the reaction mixture were omitted to identify the cause of the protein aggregation. The samples were analyzed by SDS-Page (Figure 24).
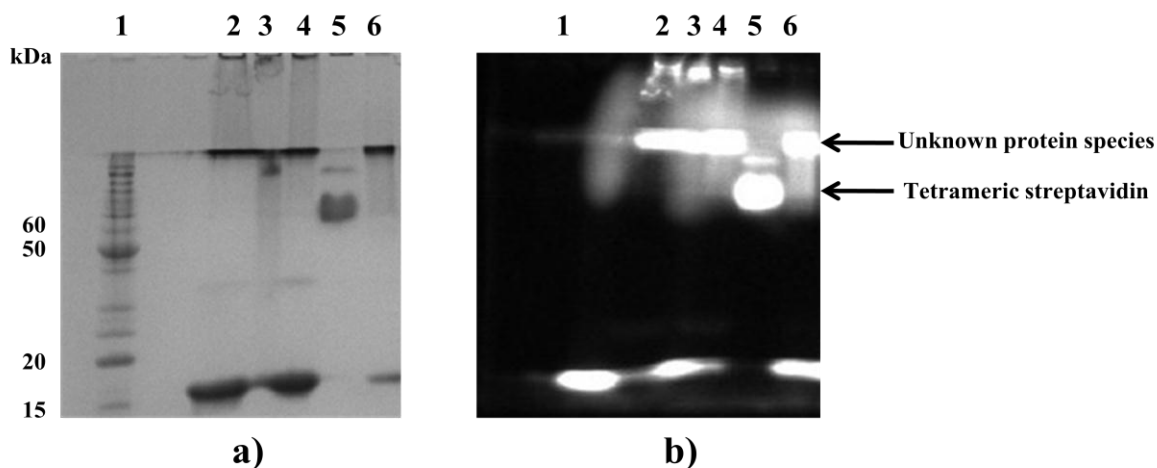
Figure 24. Post catalytic SDS-Page analysis of the reactions in absence of one reactant.
a) Commasie blue stained gel; b) UV picture of B$_4$F binding activity of protein.**.** **1**) Marker. In absence of: **2**) buffer, **3**) base, **4**) *p*-cresol, **5**) allylic substrate. **6**) Sample of the reaction with all reactants.

The SMPS is present in every sample except for the one where the allylic substrate is missing. It was concluded that the allylic moieties may interact with the protein. All reactions that formed a precipitate also showed the formation of SMPS by SDS-page.

As it can be seen in Figure 24, SMPS is able to bind biotin. In conjunction with the low mobility, this suggests that SMPS is aggregated streptavidin which is, at least in part, correctly folded.

Having identified the link between cinnamyl acetate and the formation of SMPS, possible interactions were investigated. To address this problem, the reaction was performed in the absence of biotinylated Pd-catalyst. As no formation of the precipitate was observed, it was concluded that the catalyst was involved in the formation of the SMPS.

It can be speculated that the streptavidin can be allylated under the reaction conditions and that the allylated protein is the SMPS observed on SDS-Page. This hypothesis is in agreement with Bayer's observation that acetylation of (Strept)avidin strongly affects the migration on SDS-Page [274]. Streptavidin monomer contains six tyrosines and four of these tyrosines are located at the surface of the protein and are therefore potential nucleophiles. In addition, Lysines at the surface of the protein can act as a nucleophile as well.

As shown earlier, the conversion is temperature dependent. We thus investigated whether the formation of SMPS is also affected by reaction temperature (Figure 25).
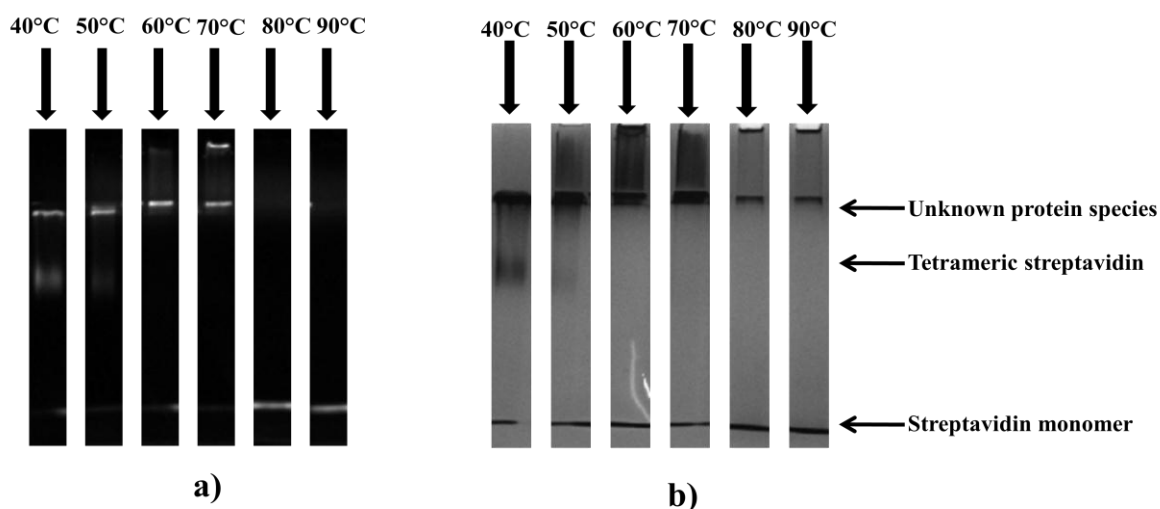
Figure 25. SDS-Page analysis of S112R Sav after the reaction at different temperatures.
a) UV picture of BF4 binding activity of protein; b) Commasie blue stained gel

At 40°C reaction temperature, both the complex containing streptavidin tetramer and the SMPS are present. At 50°C, almost no streptavidin was observed. At 60°C and 70°C, only the SMPS were present. At 80°C and 90°C only traces of the SMPS and no tetrameric streptavidin were observed in favor of monomeric Sav (denaturated streptavidin).

2.3.6.3. Catalytic activity of the SMPS.

To investigate whether the solid protein is active or not, the protein was pre-allylated under the reaction conditions in absence of the nucleophile. The formed SMPS was separated by centrifugation and employed in the allylic phenolation.

The results obtained from the phenolation reaction with the isolated solids are summarized in table 12. The SMPS formed using S112R showed a similar activity compared to the reaction with untreated pure protein under normal conditions (3.9 mM of cinnamyl acetate) (entries 1and 2, table 12). In contrast the SMPS formed using S112I and S112E had a higher activity compared to the reaction under normal conditions (entries 3, 5, 6 and 8, table 12). For these two mutants, the TON obtained with the pre-allylated protein was similar to the TON observed with pure protein when a higher amount of cinnamyl acetate (7.8 mM) was used (entries 4, 5, 7 and 8, table 12).

Table 12. Allylic phenolation with pre-allylated streptavidin mutants compared to the reactions with unmodified protein.

Entry	Protein	Protein state	Substrate concentration [a]	TON
1	**S112R**	Pure	3.9	9
2	**S112R**	Pre-allylated	3.9	9
3	**S112I**	Pure	3.9	4
4	**S112I**	Pure	7.8	11
5	**S112I**	Pre-allylated	3.9	9
6	**S112E**	Pure	3.9	3
7	**S112E**	Pure	7.8	6
8	**S112E**	Pre-allylated	3.9	6

[a] in mM. Artificial metalloenzyme: $[Pd(\eta^3\text{-allyl})(\textbf{Biot-4}^{\textbf{ortho}}\textbf{-1})]^+ \subset$ protein, substrate: cinnamyl acetate, nucleophile: *p*-cresol, base: K_2CO_3, 0.2 M phosphate buffer (pH 7.5), 50°C.

Based on the results obtained with mutants S112I and S112E (higher activity of the pretreated protein compared to untreated), we speculate that the allylation of the protein could contribute to the low conversion observed with untreated protein since some of the electrophile might get consumed for the allylation of the protein.

2.3.6.4. LC/MS analysis of the SMPS.

Until now no firm proof for the protein modification had been obtained. To determine whether the protein is indeed allylated, a tryptic digest of the SMPS (using mutant S112R) was performed prior to a LC/MS/MS measurements. This experiment should also allow us to idenify possible modifications of the protein. In the LC/MS/MS experiment, the m/z in the range of 400-1600 was recorded.

```
                1          12                                                    53       59

Seq S112R      MASMTGGQQMGR/DQAGITGTWYNQLGSTFIVTAGADGALTGTYESAVGNAESR/YVLTGR/

Ref S112R      ------------/-----------------------------------------/------/

SMPS S112R     ------------/-----------------------------------------/------/

                          80     84                      103        112        121

Seq S112R      YDSAPATDGSGTALGWTVAWK/NNYR/NAHSATTWSGQYVGGAEAR/INTQWLLTR/GTTEANAWK/

Ref S112R      YDSAPATDGSGTALGWTVAWK/----/NAHSATTWSGQYVGGAEAR/INTQWLLTR/GTTEANAWK/

SMPS S112R     --------------------/----/NAHSATTWSGQYVGGAEAR/INTQWLLTR/---------/

                     132          144              160

Seq S112R      STLVGHDTFTK/VKPSAASIDAAK/K/AGVNNGNPLDAVQQ/

Ref S112R      STLVGHDTFTK/VKPSAASIDAAK/K/AGVNNGNPLDAVQQ/

SMPS S112R     STLVGHDTFTK/VKPSAASIDAAK/K/AGVNNGNPLDAVQQ/
```

Figure 26. Streptavidin S112R peptide fragments detected by LC/MS/MS. Seq S112R represents the complete sequence, Ref S112R corresponds to the pure S112R Sav, SMPS corresponds to protein isolated after catalysis. Fragments of the streptavidin S112R peptide sequence are separated with a slash. Possible allylated tyrosines are highlighted in yellow and possible allylated lysines are highlighted in green.

During tryptic digest, peptide chains are cleaved at the carboxyl side of the amino acids of arginines (R) or lysines (K) residues. The results of LC/MS/MS analysis of these fragments are illustrated in Figure 26. In addition to the detected fragments of the S112R SMPS (SMPS S112R), the complete sequence (Seq S112R) is represented as well as the detected fragments for the pure S112R streptavidin (Ref S112R). As it can be seen, not all expected peptide fragments from the reference sample were detected. The reason for this is probably that some peptides were not ionized and therefore, these peptides were not detected. However, when comparing the detected sequences from the reference to corresponding sequences from the SMPS sample, it can be seen that two additional peptide sequences (60-80 and 113-121) were not detected in the SMPS sample. The absence of these sequences, in the mass spectrum of the post catalysis sample, suggests that these peptides were modified during catalysis.

Only the peptide sequence from amino acid 60-80 bears a tyrosine (Y60) that is accessible in the folded protein. The sequence from amino acids 113-121 possesses an accessible lysine, which is located near the site where the metal center of the catalyst. Lysines are well known to act as nucleophiles in diverse reactions. It can therefore be speculated that next to tyrosines, lysines may have been modified. Additionally to the tyrosine at position 60, the peptide sequence from amino acids 60-80 bears an accessible lysine at position 80, which could have been allylated as well.

Apart from the two fragments that were detected in the reference but not in the SMPS sample, the other sequences which were not detected are potentially allylated sequences, as all of them bear an accessible tyrosine (except from sequence 1-12).

The artificial alkylase based on the biotin-streptavidin technology is a potential candidate for proteins modifications. However, various problems would need to be adressed. Acetates as

substrate are not ideal due to their low hydrolytic stability. Although it could be demonstrated that enantioselectivity can be achieved, the selectivity problems encountered when proteins need to be modified sequence-specifically by allylic substitution are potentially far more complex with various surface accessible nucleophiles present. A reaction which uses less sensitive substrates and makes use of rare residues for modification might offer straightforward approach. Metathesis was therefore investigated.

2.4. Artificial ligase based on the biotin-(strept)avidin technology.

2.4.1. General.

Davis and coworkers recently demonstrated the modification of proteins by selectively converting the cystein's thiol group to an allylthioether . The terminal olefin was further modified by subsequent metathesis using Hoveyda-Grubbs catalyst (Scheme 26) [203-207].
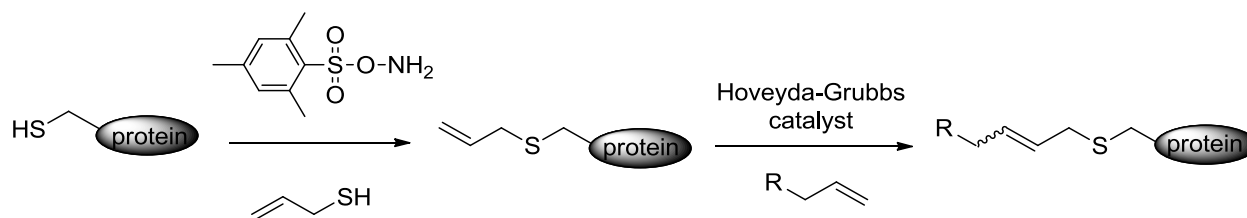
Scheme 26. Selective modification of protein developed by Davis.

In contrast to a small-molecule catalyst, an embedded catalyst within a protein host macromolecule, such as streptavidin, offers the potential for molecular recognition of substrate

by interactions between the second coordination sphere amino acids and others functionalities present on the substrate. For instance, it is imaginable that a peptide sequence can be recognized in the case of a protein target.
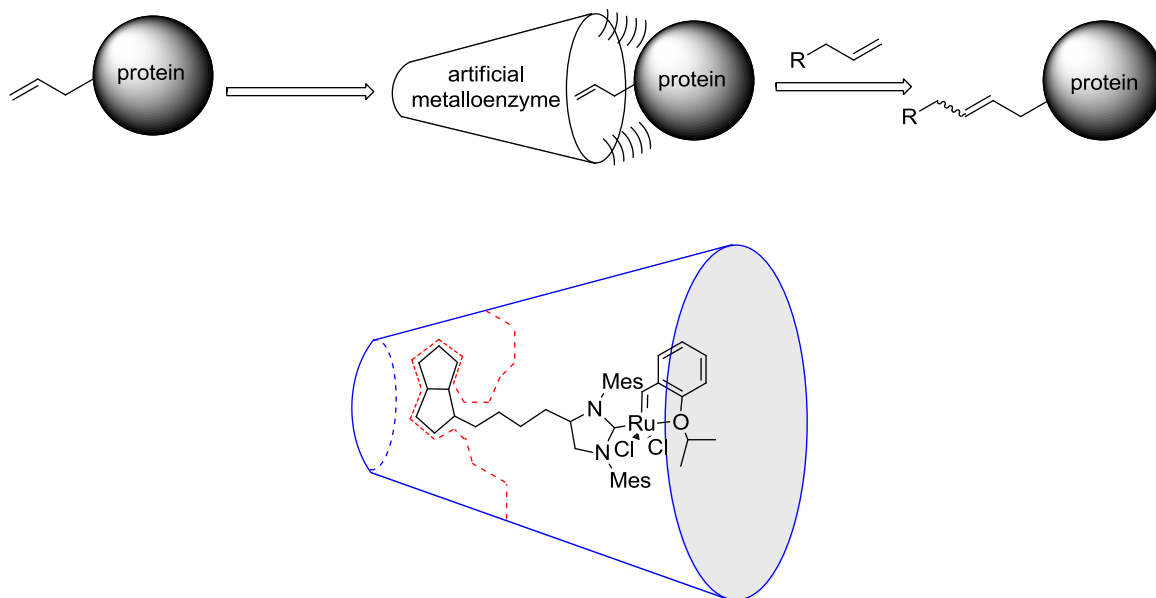
Scheme 27. Principle of selective modification of proteins using artificial metalloenzyme based on biotin-(strept)avidin technology.

For this purpose, artificial metalloenzymes, which can be easily modified through the introduction of single point mutations might be successfully employed in order to achieve this molecular recognition (Scheme 27). With the ambitious goal of selective protein modification, the creation of an artificial ligase based on the biotin-(strept)avidin technology was investigated.

*2.4.2. Synthesis of the biotinylated Hoveyda-Grubbs catalyst (**Biot-HG**).*

The synthesis of N-heterocyclic carbenes and their corresponding organometallic complexes is extensively reviewed in the literature [275-277]. One of the favored routes for generating NHCs is the use of potassium *tert*-butoxide in conjunction with an imidazolium salt. Potassium *tert*-butoxide generates an ether that eliminates *tert*-butanol upon heating and generates the corresponding N-heterocyclic carbene (Scheme 28).

imidazolinium chloride salt   adduct   carbene

Mes =
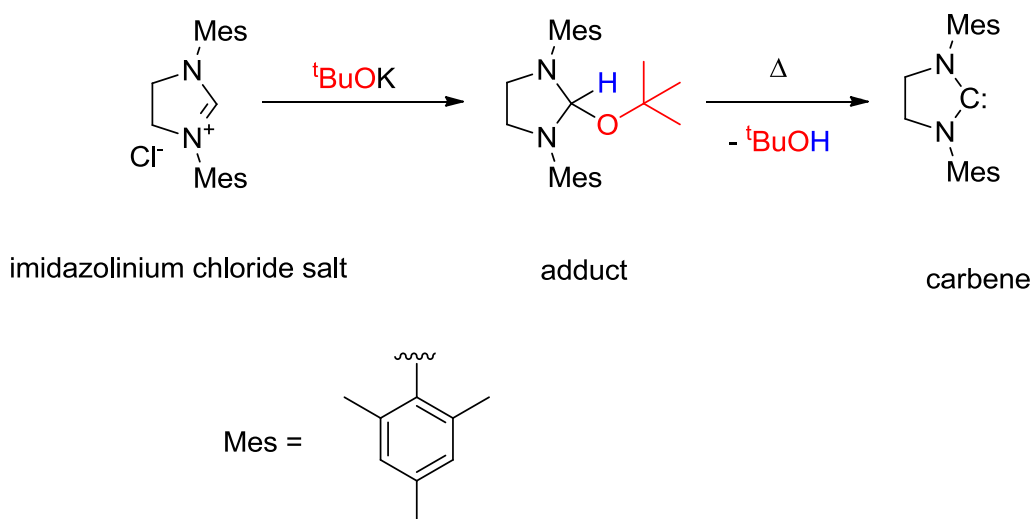
Scheme 28. Formation of the N-heterocyclic carbene from the imidazolinium salt in presence of [t]BuOK.

The synthesis of biotinylated NHC could not be achieved by the deprotonation of a biotin imidazolinium salt with [t]BuOK. It appeared that the presence of biotin prevents the reaction possibly by consumption of [t]BuOK caused by the presence of the two acidic urea protons. Alternative synthetic pathways were examined for accessing carbene precursors. Hedrick and coworkers described an approach that uses a diamine and pentafluorobenzaldehyde which forms the NHC though a Schiff base reaction followed by a nucleophilic attack on the resulting imine to form the intermediate (Scheme 29) [278].
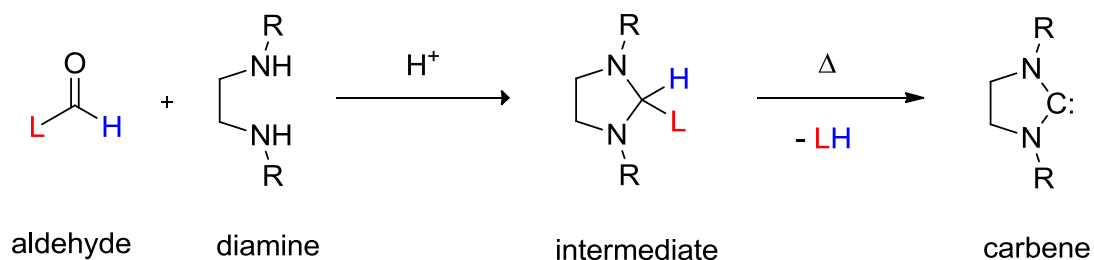
Scheme 29. Formation of the NHC using the method developed by Hedrick.

Hedrick investigated on the nature of the aldehyde. The nature of the leaving group in the intermediate is important. Depending on its nature, the leaving group can be easily liberated upon heating. The pentafluorophenyl leaving group resulted in the formation of pentaflorobenzene which has a low boiling that facilitates purification. Grubbs utilized this method to synthesize Hoveyda-Grubbs second generation catalyst [279].

The method developed by Hedrick was adapted to the synthesis of biotinylated N-heterocyclic carbenes. The adduct was synthesized from the biotin diamine and pentafluorobenzaldehyde under acidic conditions (Scheme 30).
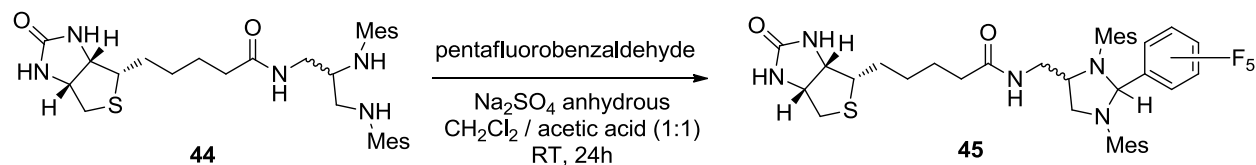
Scheme 30. Synthesis of the biotinylated precursor for the formation of NHC.

The product **45** proved difficult to purify. It was reacted thus further with the Grubbs 1st generation catalyst. These reactions were conveniently monitored by heteronuclear NMR. As outlined in Scheme 31, the formation of the carbene was followed in ^{19}F NMR via the peaks corresponding to the formation of pentafluorobenzene. The reaction of the carbene generated in

situ with Grubbs 1st generation catalyst was monitored by ^{31}P NMR. The signal for Grubbs first generation (37.5 ppm) was shifted and split into two signals (29.4 and 28.5 ppm) (the splitting is due to the slow rotation of the NHC that leads to formation of two diastereoisomers) accompanied by the appearance of free tricyclohexylphosphine (11.2 ppm) or tricyclohexylphosphine oxide (50.7 ppm).
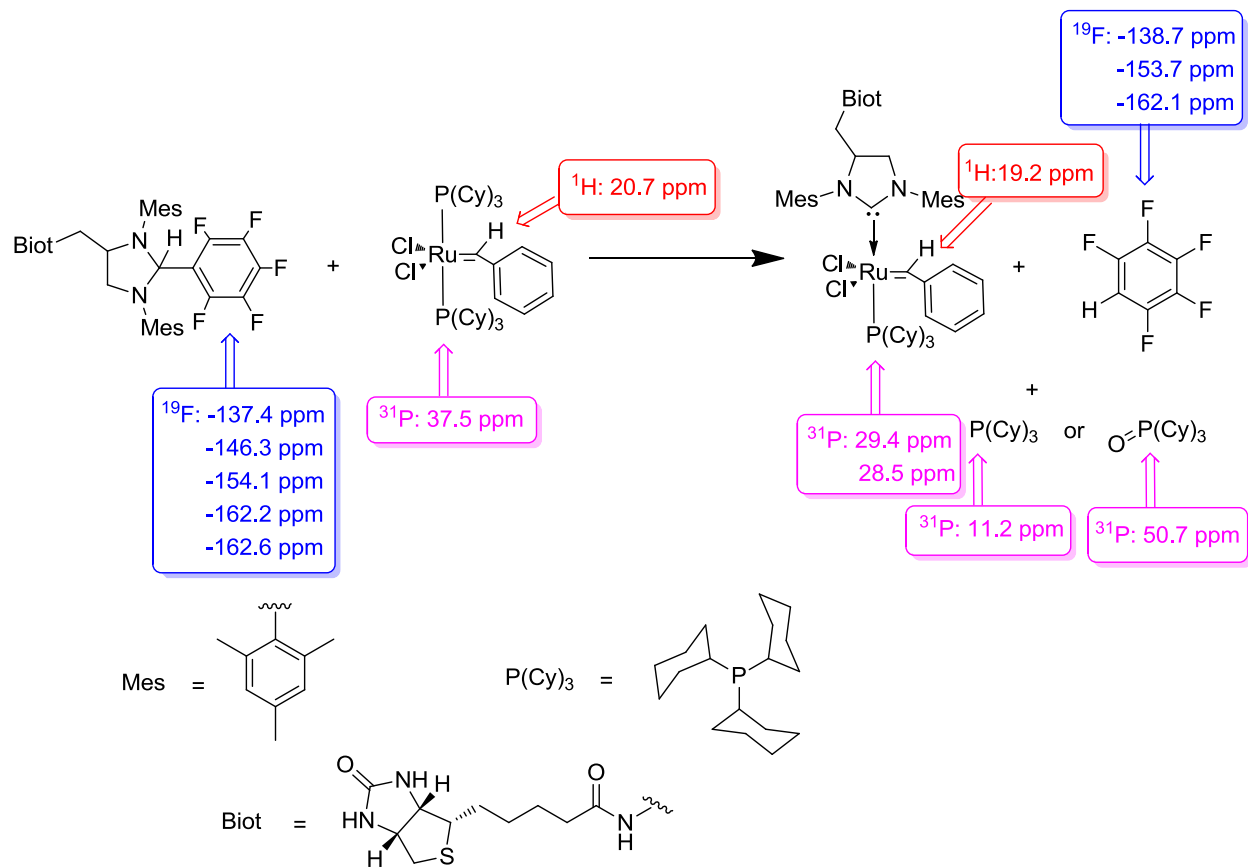
Scheme 31. Synthesis of Biot-HG monitored by NMR.

Unfortunately, experimental results demonstrated that the synthesis of **Biot-HG** failed using this method. When fluorinated precursor was heated in presence of the complex, peaks corresponding to pentafluorobenzene were observed in ^{19}F NMR suggesting that the NHC was generated. At the

same time, in ^{31}P NMR, peaks were observed at 50.7 ppm and at 11.2 ppm but the peak at 37.5 ppm as well as the peak at 20.7 ppm on ^{1}H NMR disappeared.

Another synthetic route was selected whereby the biotin moiety was introduced in the last step (Scheme 32).
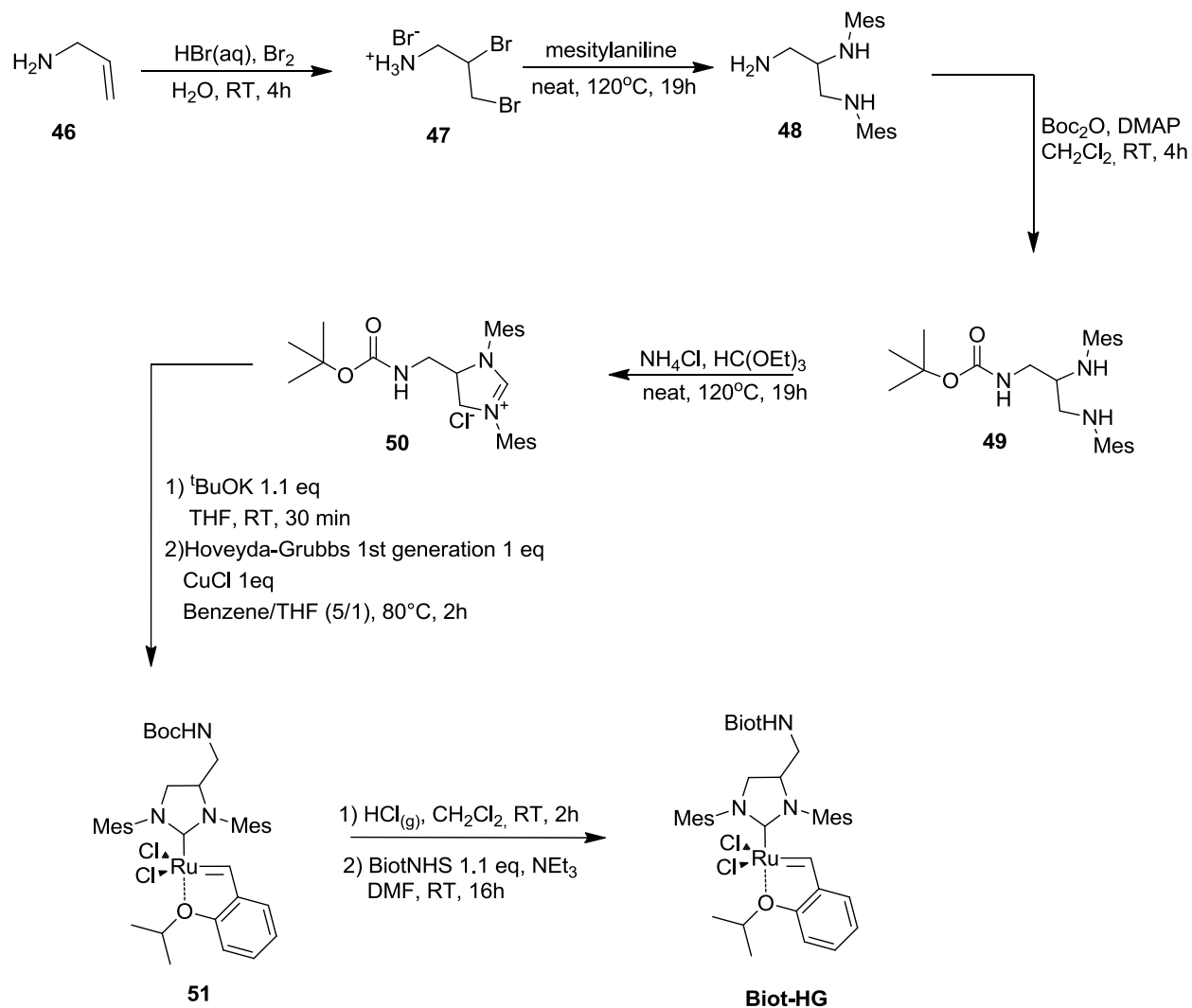
Scheme 32. Synthesis of the biotinylated Hoveyda-Grubbs catalyst (Biot-HG).

The boc-HG **51** was synthetized from the protected imidazolinium salt based on the method described by Grela. The biotin moiety was introduced in the last step of the synthesis of **Biot-HG**

where the deprotected boc-HG was coupled to the activated biotin. A significant amount of **Biot-HG** was lost during purification on silica gel. Therefore, the complex was purified on Sephadex LH 20. This resin was used to purify **Biot-HG** with dichloromethane as eluant.

2.4.3. Incorporation of the **Biot-HG** catalyst into the protein.

In order to probe the localization of **Biot-HG** with Sav, the protein was saturated with 2-(4-hydroxyazobenzene) benzoic acid (HABA), which has a known binding constant to streptavidin [280-282]. It was subsequently displaced by addition of **Biot-HG**. The titration of Sav-HABA with **Biot-HG** was monitored by UV-vis spectroscopy at 506 nm. HABA absorbs at this wavelength only when it is incorporated inside the protein. Depending on the affinity of the biotinylated complex (e. g. **Biot-HG**) for the protein, the equivalence point is exected around 4 equivalents **Biot-HG** to streptavidin.

Aliquots of **Biot-HG** (0.25 equivalents) were added to HABA $\subset$ streptavidin (Figure 27). The value of absorbance decreased until 4.25 equivalents and the absorption increased slowly thereafter as **Biot-HG** absorbs at this wavelength. **Biot-HG** has strong affinity for Sav (much stronger than HABA) and up to four equivalents can be accommodated within tetrameric Sav.
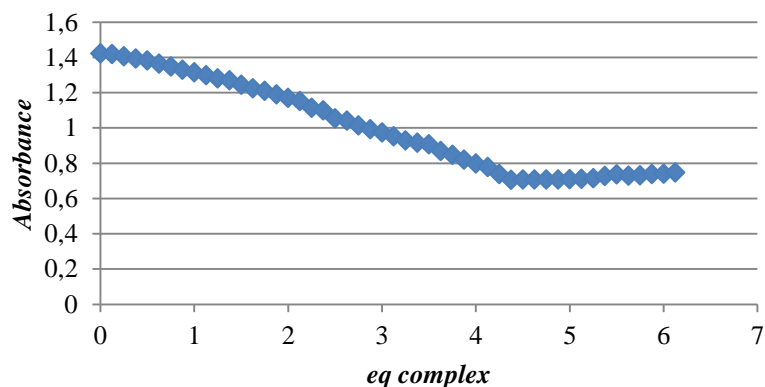
Figure 27. HABA displacement titration curve by Biot-HG in Sav.
15μM streptavidin WT in 20 mM phosphate buffer (pH 7), 15 eq HABA, aliquots of 5μL (0.125 eq) of a 0.87mM solution of the complex was added. Absorbance was measured at 506 nm. 25°C.

Additional information was gathered by CD spectroscopy (Figure 28). Upon incorporation of **Biot-HG** either within WT Sav or K121G, induced CD signals appeared at 390 and 365 nm respectively. Importantly, the CD spectrum **Biot-HG** ⊂ Sav was unaffected upon lowering the pH to 2, thus highlighting the remarkable stability of the system. The shift in induced CD signal upon mutation further illustrates the interaction between the host protein and the biotinylated guest.
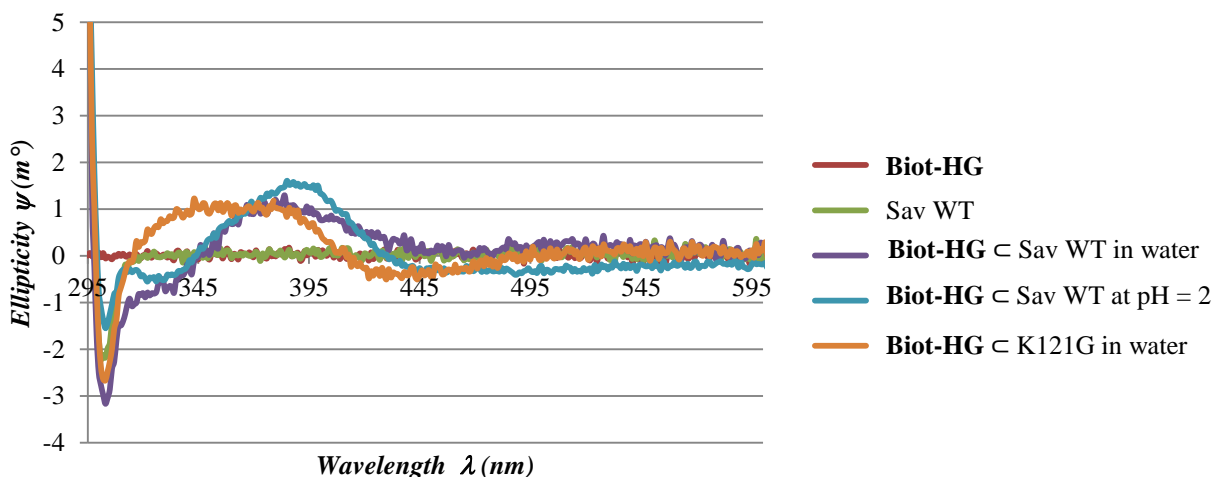
Figure 28. Circular dichroïsm of the biot-HG incorporated in the protein. 30μL artificial metalloenzyme, wavelength: 290-600 nm, step size: 1nm, time per point: 1s, temperature: 40°C.

2.4.4. Catalytic activity of the artificial lyase.

The activity of the artificial metalloenzyme **Biot-HG** ⊂ Sav was evaluated for prototypical ring closing metathesis (RCM) of the diallyltosylamine in aqueous media (Scheme 33). The commercial Hoveyda Grubbs 2nd generation catalyst (HG) was used as reference.
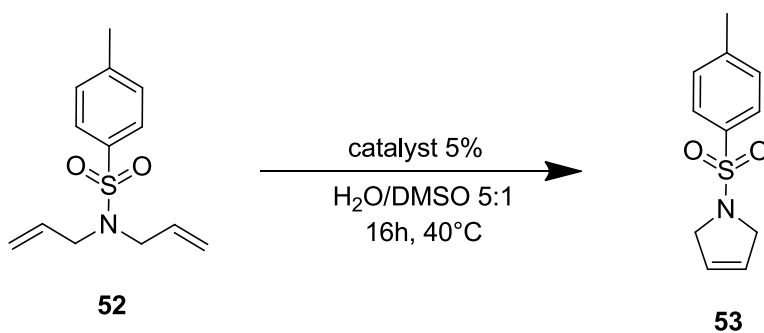
Scheme 33. Ring closing metathesis of diallyltosylamine.

Table 13. Ring closing metathesis of diallyltosylamine with different catalysts.

Entry	Protein	Complex	Conv [a]	TON
1	--	HG	97	19
2	--	**Biot-HG**	74	15
3	Streptavidin WT	**Biot-HG**	4	1
4	Avidin	**Biot-HG**	40	8

[a] conversion in % determined by HPLC reverse phase ; conditions : 0.25 mM protein, 0.73 mM complex, 15.21 mM substrate, total volume: 120 μL, H$_2$O/DMSO = 5/1, 16h, 40°C.

The commercial HG was active (97% conversion, entry 1, table 13) and **Biot-HG** was also active (74% conversion, entry 2, table 13) but when **Biot-HG** was incorporated in the streptavidin WT, the activity decreased dramatically (4% conversion, entry 3, table 13). The activity increased slightly when **Biot-HG** was incorporated in avidin (40% conversion, entry 4, table 13).

The activity of the artificial metalloenzyme toward RCM was tested at various pH (Figure 29).
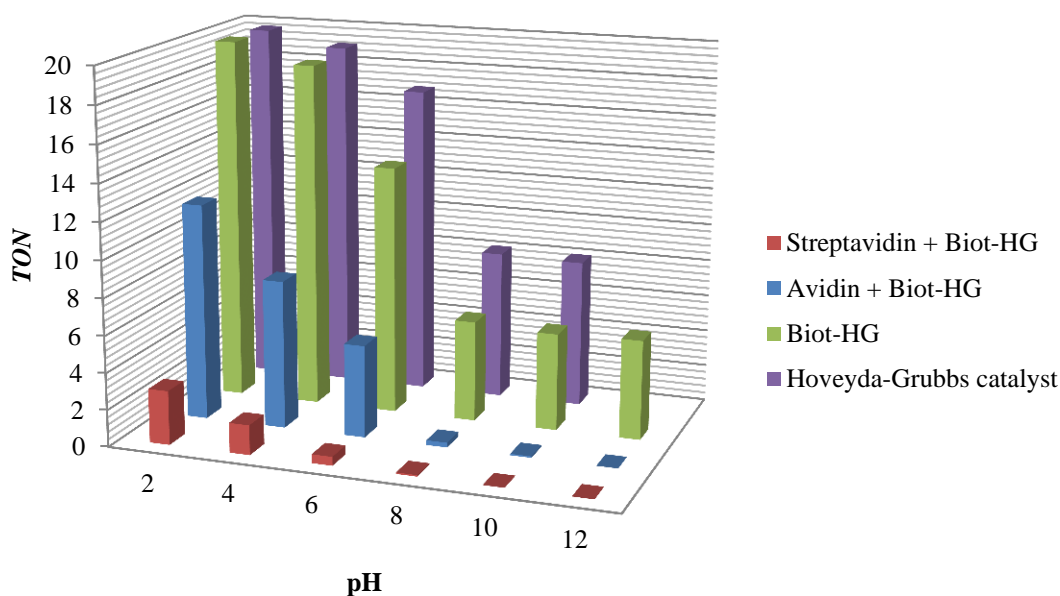
Figure 29. Influence of the pH on the activity of artificial metalloenzyme. conditions : 0.25 mM protein, 0.73 mM complex, 15.21 mM substrate, total volume: 120 μL, H_2O/DMSO = 5/1, 16h, 40°C. pH 10-12: carbonate buffer, pH 6-8: 0.1M phosphate buffer, pH 4: 0.1M acetate buffer, pH 2: 0.2M KCl/HCl buffer.

For the commercial Hoveyda-Grubbs catalyst, the activity was increased when the pH was lowered. The conversion went from 36% at pH 12 to 100% at pH 2. The activity of the **Biot-HG** also was increased when the pH was lowered. The conversion went from 27% at pH 12 to 100% at pH 2. The behavior was the same when **Biot-HG** was incorporated in the (strept)avidin. The activity was low in basic conditions (< 5%) and increased from pH 6 to pH 2 to reach 15% conversion for streptavidin and 59% for avidin.

Two hypotheses were developed in order to rationalize the difference in reactivity of avidin versus streptavidin.

- Lysines on the surface of streptavidin coordinate to **Biot-HG** and thus prevent the olefin from binding thereby inhibiting the catalysis. Avidin is a glycosylated protein. The sugars may act as a protective shield that made lysines at the surface of the protein less

accessible. The lysine at position 121 is near the active site. This amino acid may coordinate and deactivate **Biot-HG**. In these acidic conditions, theses lysines are protonated and thus cannot coordinate to the ruthenium complex.

- Streptavidin and avidin have different isoelectronic points (pI=6 for streptavidin and pI=10 for Avidin). The overall charge of the protein becomes positive when the pH lies below their respective pI. Thus, at pH 7, avidin bears an overall positive charge whereas streptavidin is negatively charged.

Table 14. Result of the RCM of diallyltosylamine using modified proteins.

Entry	Protein	Condition	Conv [a]	TON
1	AcStrept WT	H_2O	1.7	<1
2	K121G	H_2O	1.2	<1
3	rec-Avi	H_2O	20	4
4	rec-Avi	pH= 4	44	9
5	rec-Avi	pH= 2	49	10

[a] conversion in % determined by HPLC reverse phase ; conditions : 0.25 mM protein, 0.73 mM complex, 15.21 mM substrate, total volume: 120 μL, H_2O/DMSO = 5/1, 16h, 40°C. pH 4: 0.1M acetate buffer, pH 2: 0.2M KCl/HCl buffer.

RCM experiments with different proteins were performed to verify these hypotheses. The results are collected in table 14. Neither acetylation (AcSav) nor the removal of the close lying K121 residue (K121G) had a significant impact on the performance of the corresponding hybrid catalyst (entries 1 and 2, table 14). This suggests that the lysine residues of Sav have little influence on catalysis.

Rec-Avi, a recombinant form of avidin with a lowered pI=5.5 and limited glycosylation, was tested as well (entries 3,4 and 5, table 14) [283]. The activity with the modified protein increased in acidic conditions but was comparable to the results obtained with Avidin in the same conditions.

We thus conclude that the activity of the artificial metalloenzyme is not critically dependent on the pI of the protein.

2.4.5. Chemical optimization of the artificial ligase.

Based on qualitative docking simulations performed by M. Schmid, a 3-aminobenzoic acid spacer was selected (Figure 30). Indeed, the corresponding **Biot-*m*ABA-HG** was significantly more accessible in the docked structures **Biot-*m*ABA-HG** ⊂ Sav. No significant differences in the docked structures for both pseudoenantiomers of either **Biot-HG** ⊂ Sav or **Biot-*m*ABA-HG** ⊂ Sav were apparent.
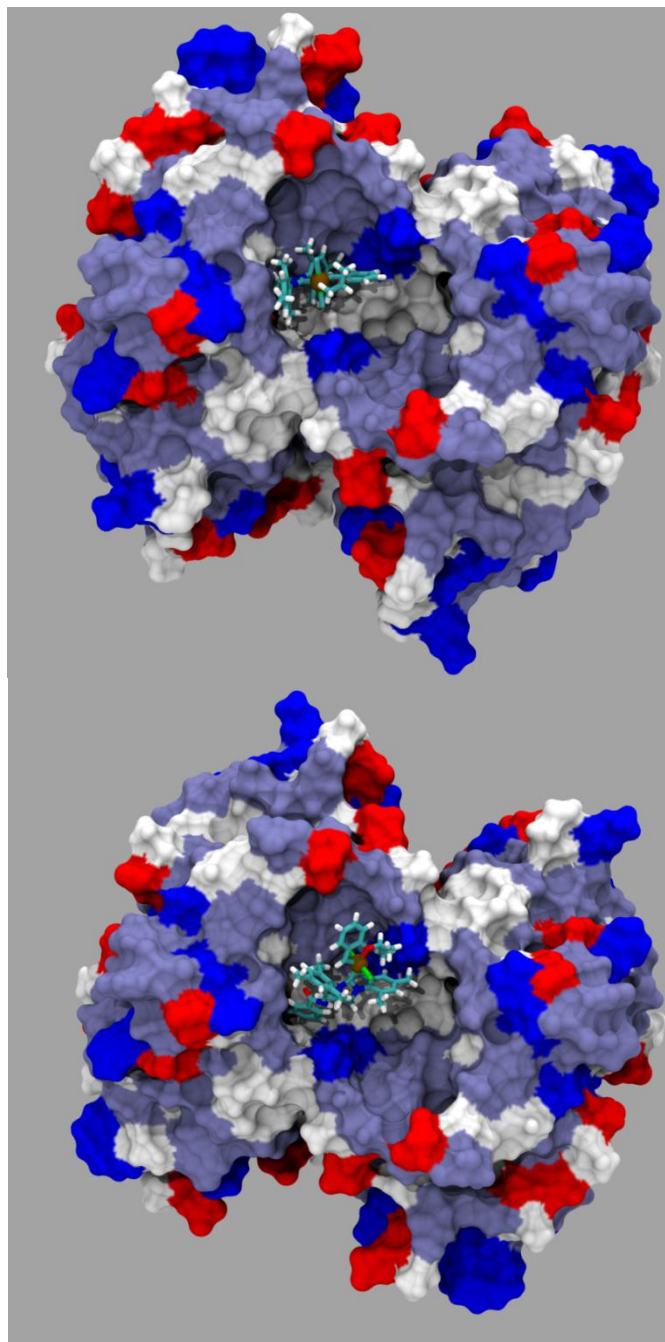
Figure 30. Docking of the complex Biot-HG (up) and Biot-*m*ABA-HG (down) in streptavidin.

The synthesis of the biotinylated complex **Biot-*m*ABA-HG** was performed similarly to **Biot-HG** (Scheme 34). 3-aminobenzoic acid was coupled to activated biotin. The benzoic acid function was then activated with pentaflorophenol and coupled with the deprotected Hoveyda-Gubbs catalyst.
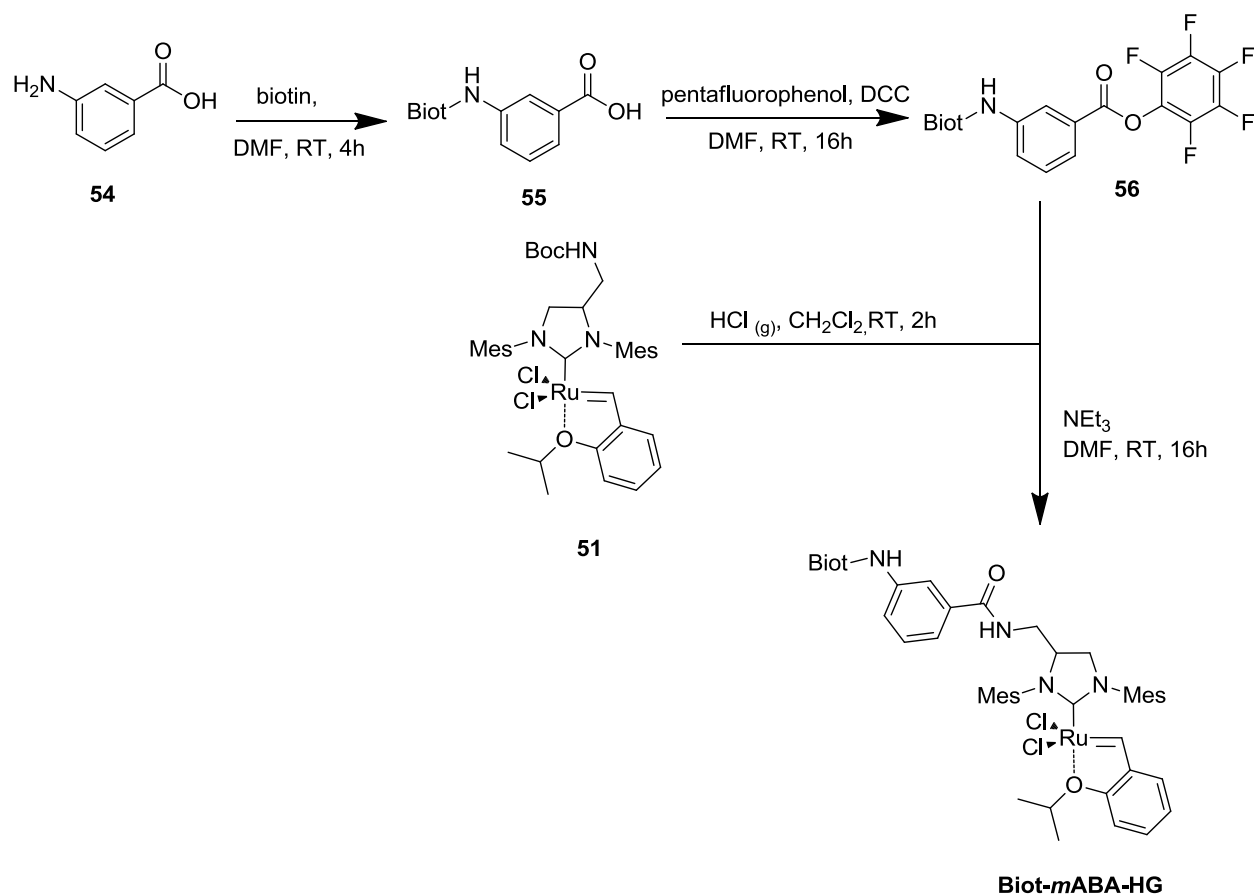
Scheme 34. Synthesis of the Biot-*m*ABA-HG.

The activity of the resulting artificial metalloenzymes towards RCM was tested in acidic conditions (table 15).

As it can be appreciated, the introduction of the aromatic spacer improved the activity of the artificial ligase. As it was observed with **Biot-HG**, the highest activity was obtained with avidin at pH 2 (entry 4, table 15).

Table 15. Experiments performed with the Biot-*m*ABA-HG in acidic conditions.

Entry	Protein	Condition	Conv [a]	TON
1		pH= 4	30	6
	Sav			
2		pH= 2	42	8
3		pH= 4	59	12
	Avi			
4		pH= 2	73	15

[a] conversion in % determined by HPLC reverse phase ; conditions : 0.25 mM protein, 0.73 mM complex, 15.21 mM substrate, total volume: 120 μL, H_2O/DMSO = 5/1, 16h, 40°C. pH 4: 0.1M acetate buffer, pH 2: 0.2 M KCl/HCl buffer.

2.4.6. Influence of the presence of metal salt on the reaction.

The activity of the artificial enzyme was greatest at pH 2 with a HCl / KCl 0.2M buffer. Hypothesizing that the presence of salt may have a large influence on the activity of the artificial metalloenzyme, reactions were performed in presence of various solutions of metal chloride salts (Figure 31).

96
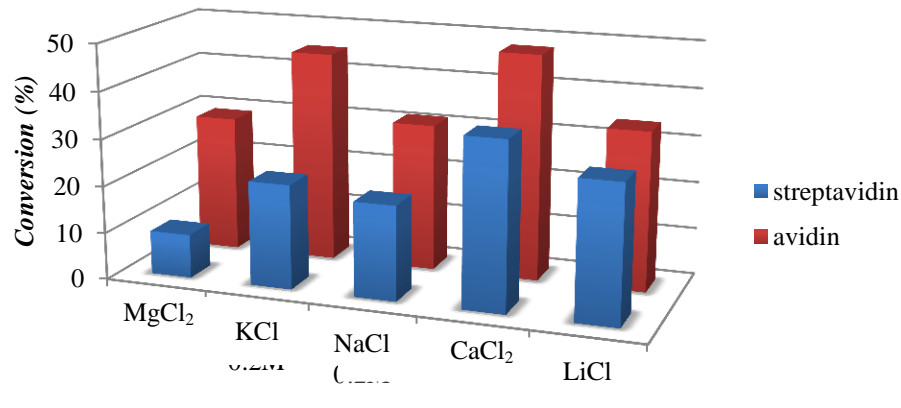
Figure 31. Influence of the different metal chloride salts on the activity of Biot-HG ⊂ protein for RCM. Conditions : 0.25 mM protein, 0.73 mM complex, 15.21 mM substrate, 0.2 M metal chloride, total volume: 120 μL, H$_2$O/DMSO = 5/1, 16h, 40°C.

Based on these results, it was observed that metal chloride may influence the activity of the artificial metalloenzyme. The highest activity was obtained in presence of CaCl$_2$.

It can be speculated that metal ions can block lewis basic sites on the protein as would be achieved by decreasing the pH. The carboxylic functions on the protein are possible binding positions for metals. Streptavidin and avidin have many aspartic acid and glutamic acid in their structures (52 residues for streptavidin and 48 for avidin).

RCM reactions with **Biot-HG** and **Biot-*m*ABA-HG** were performed in presence of metal able to block anionic sites on the protein (Figure 32).

As it can be appreciate, the activity was increased in the activity of the artificial metalloenzyme:

- The activity of **Biot-HG** ⊂ protein is highest in presence of Ni^{2+}.

- The activity of **Biot-*m*ABA-HG** ⊂ protein is highest in presence of Ca^{2+} and Ni^{2+}.
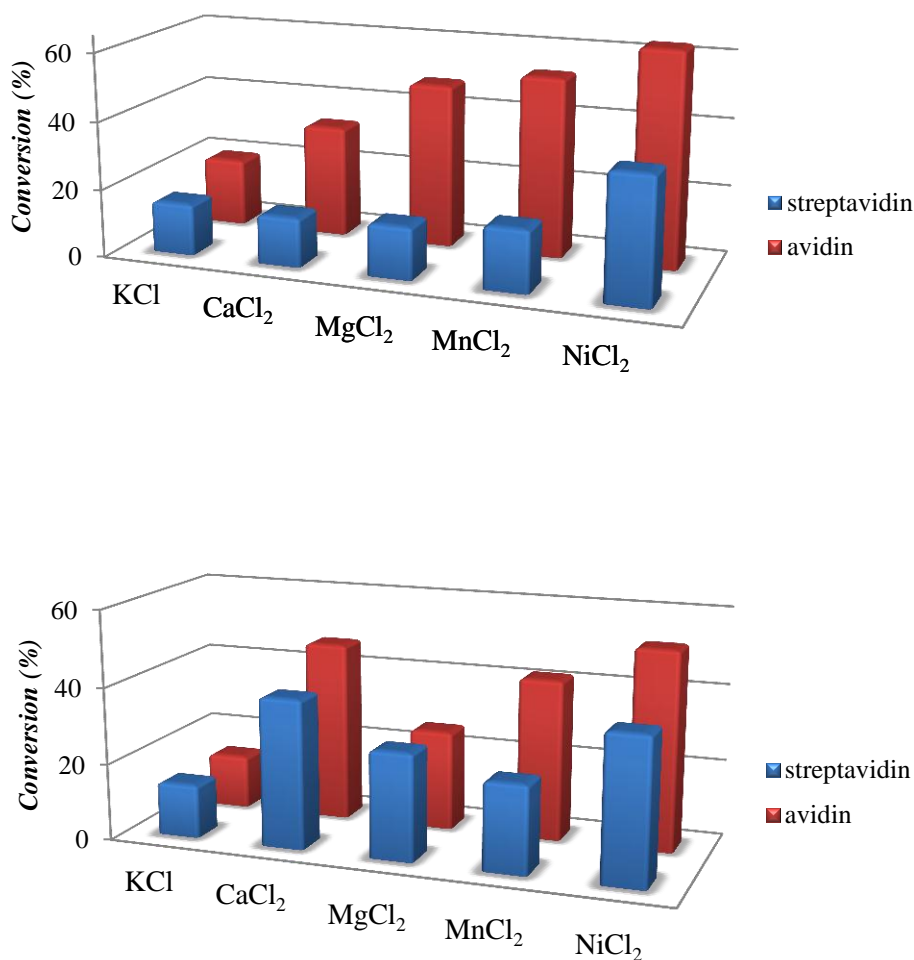
Figure 32. Influence of different metal chloride salts on the activity of Biot-HG ⊂ protein (up) and Biot-*m*ABA-HG ⊂ protein (down). Conditions : 0.25 mM protein, 0.73 mM complex, 15.21 mM substrate, 0.5 M metal chloride, total volume: 120 µL, H$_2$O/DMSO = 5/1, 16h, 40°C

A new artificial ligase was created by the incorporation of a biotinylated Hoveyda-Grubbs in (strept)avidin. The activity of this artificial metalloenzyme was improved in acidic conditions and also in presence of metal chloride salt. It was speculated that the metal was able to block lewis basic position on the protein. Mutations of residues bearing carboxylic acid side chain on the host protein would be an alternative to improve the activity of the artificial ligase.

III. Conclusion

Three different artificial metalloenzymes were investigated in this thesis:

- With practical applications in mind, artificial hydrogenases were screened for their performance towards :

  ➢ Increased substrate concentration (up to 60 mM)

  ➢ Decreased catalyst loading (down to 0.1%)

  ➢ Recyclability/immobilization (two catalytic runs with little erosion of the activity and selectivity).

  ➢ Storage of "ready to use" lyophilized artificial hydrogenase (no loss of activity).

  Although not truly practical at this point, these results pave the way towards widespread use of artificial metalloenzymes.

- The second part present artificial metalloenzymes for allylic substitution reactions. In this context, we have shown that such systems tolerate large portins of organic solvent (up to 50% DMSO) and increased temperatures (up to 40°C, with little loss in activity and selectivity).

  To increase throughput, thermal treatment of crude cell extracts was tested as an alternative to the lengthy dialysis/chromatography protocol. Despite the stability of Sav, a yet uncharacterized cell debris severely limits the usefulness of this approach.

  With chemical biology application in mind and as an extension of the alkylation, phenolation (selected as a tyrosine model) was investigated. This study revealed that the host Sav undergoes self-allylation. Tryptic digest after a catalytic run suggests allylation at lysine residues.

  To circumvent this challenge, a bio-orthogonal reaction was investigated: the olefin metathesis.

- With the aim of creating artificial metalloenzymes for olefin metathesis, two biotinylated Hoveyda-Grubbs catalysts were synthesized. These were for RCM of diallyltosylamine in the presence of Sav or Avi. The catalytic performance could be improved either in acidic condition or by addition of divalent metal salts. Under optimized conditions, these systems rivaled with the "protein-free" Hoveyda-Grubbs catalyst.

Through this work, it was demonstrated that artificial metalloenzymes based on the biotin-(strept)avidin should not be limited to asymmetric reactions. The potential of such a system should be applied for *in vivo* applications as enzyme. Therefore, two points need to be developed. First, it would be worth to combine the outstanding properties of (strept)avidin to compatible aqueous reactions able to mimic or provide new functionalities to cellular media. The other important point that should be investigated is molecular recognition. This aspect was not intensively developed but selected mutations of the host protein should offer a better control of interactions between macromolecules. This point would also be a solution for an "efficient loading" in protein modification since the lack of selectivity of most of reactions involved imposed a high loading of the active species.

IV. Experimental part

4.1 Reagents and solvents

Substance	Origin	Purity
(+)-Biotin	Chanzhou Huaren	≥ 99%
[Pd(Ph$_2$allyl)Cl]$_2$	Own synthesis	
2,3,4,5,6-pentafluorobenzaldehyde	Aldrich	98%
2,3,4,5,6-pentafluorophenol	Aldrich	≥ 99%
2,4,6-Trimethylaniline	Aldrich	98%
3-aminobenzoic acid	Fluka	purum, ≥97.0%
Ac$_2$O	Aldrich	ACS reagent, ≥98.0%
Acetonitrile	Romil	SpS (super purity solvent) UV/gradient quality
Acrylamide	AppliChem	30 % solution
Allylamine	Aldrich	≥99%
Allylpalladium(II) chloride dimer	Aldrich	98%
Ammonium persulfate	Aldrich	≥ 98%
Anisole	Fluka	≥ 99%
Avidin	Belovo	
B$_4$F	AnaSpec, Switzerland	
Boc$_2$O	Aldrich	reagent grade, 97%
Br$_2$	Aldrich	reagent grade
CDMT	Aldrich	≥ 97%
Coomassie brilliant blue R250	Fluka	analytical grade
CuCl	Fluka	purum, ≥97.0%
Cynnamyl acetate	Aldrich	99%
D-biotin-sepharose CL-4B	Affiland, Belgium	suspension in TBS buffer pH 7.4, NaN$_3$ 0.02 % (w/v))
DCC	Fluka	puriss., ≥99.0%
Diallylamine	Aldrich	99%

Didodecyldimethylammonium bromide	Fluka	purum, ≥98.0%
Dimethylmalonate	Aldrich	
DMAP	Aldrich	≥99%
DMF	Aldrich	puriss., absolute, over molecular sieve (H_2O ≤0.005%), ≥99.5% (GC)
DMSO	Aldrich	ACS spectrophotometric grade, ≥99.9%
DNase I	Hoffmann-Roche	
HABA	Aldrich	≥98%
HBr	Aldrich	48 wt. % in H_2O, ≥99.99%
Hoveyda-Grubbs 2nd generation	Aldrich	
Hoveyda-Grubbs 1st generation	Aldrich	
Isobutyl chloroformate	aldrich	98%
N-Acetamidoacrylic acid	Acros	≥ 99%
N-Hydroxysuccinimide	Fluka	purum, ≥97.0%
N-Methylmorpholine	Fluka	≥ 98%
N-*N*-Diisopropylethylamine	Fluka	≥ 98%
Orthoformiate	Aldrich	anhydrous, 98%
p-cresol	Aldrich	puriss. p.a., ≥99.0%
Phenylethanol	Fluka	>99%
Phenylmethylsulfonyl fluoride	AppliChem	≥ 99%
Potassium carbonate	Fluka	purum p.a., anhydrous, ≥99.0%
p-toluenesulfonyl chloride	Aldrich	reagent grade, ≥98%
Pyridine	Fluka	purum, ≥99.0% (GC)
Rh(COD)$_2$BF$_4$	Umicore	
SDS	AppliChem	20 % solution (molecular biology grade)
Streptavidin	Own production	
TEMED	Fluka	≥ 99%
tert-butoxypotassium	Aldrich	reagent grade, 95%
tert-butoxypotassium	Aldrich	sublimed grade, 99.99% trace metals basis

THF	Fluka	puriss. p.a., ACS reagent, ≥99.5% (GC)
trans-1,3-Diphenyl-2-propen-1-ol	Aldrich	≥98.0% (HPLC)
Tributylamine	Aldrich	≥98.5%
Triethylamine	Aldrich	puriss. p.a., ≥99.5% (GC)
Trimethylsulfonium hydroxide	Fluka	0.25M in MeOH
Triphenylphosphine	Fluka	puriss., ≥98.5%
Water	Romil	SpS (super purity solvent)

4.2 Synthesis.

General: ^{1}H, ^{13}C and ^{19}F spectras were obtained on a Bruker 400 MHz, 500 MHz, 600 MHz. Chemical shifts are reported in ppm (parts per million). Signals are quoted as s (singulet), d (doublet), t (triplet), br (broad), and m (multiplet). Electron Spray Ionization Mass Spectra (ESI-MS) were recorded on a Bruker FTMS 4.7T bioAPEX II.

The following compounds were prepared as described in the literature [111, 263, 265]

Biot-1

Biot-(R)-Pro-1

Biot-4°-1

37
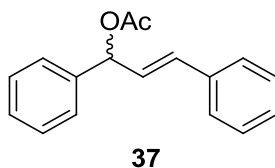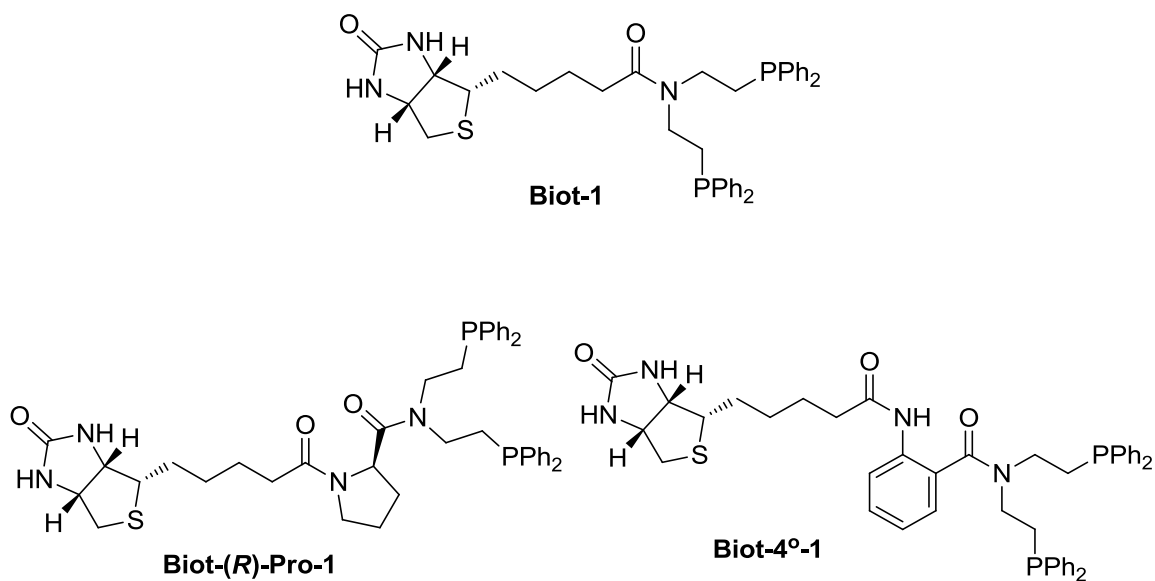
Synthesis of 1,3 diphenylpropenacetate **37** [284].

To a solution of *trans*-1,3-Diphenyl-2-propen-1-ol (2 g, 9.51 mmol) in Ch_2Cl_2 was added anhydride acetic (4.5 ml, 47.56 mmol) and Et_3N (6.6 ml, 47.56 mmol). The reaction mixture was stirred for 16 h. The solvent was removed by vaccum and purified on silica gel using hexane/AcOEt 10:1 as eluent to yield the product **37** as colorless oil. (2,13 g, 88%)

^{1}H NMR (400 MHz, CDCl$_3$) δ: 7.49 – 7.23 (m, 11H), 6.67 (d, *J* = 15.8 Hz, 1H), 6.48 (d, *J* = 6.8 Hz, 1H), 6.38 (dd, *J* = 15.8, 6.8 Hz, 1H), 2.16 (s, 3H). ^{13}C NMR (101 MHz, CDCl$_3$) δ: 170.46, 139.68, 136.60, 133.03, 129.07, 129.02, 128.62, 128.51, 127.94, 127.49, 127.14, 21.79.
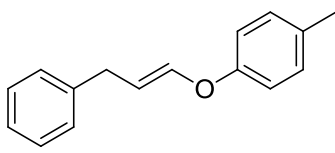
42

Synthesis of allyl ether **42.**

Allylpalladium chloride dimer (13.5 mg, 0.07 mmol) and triphenylphosphine (18 mg, 0.28 mmol) were dissolved in 3 ml dry and degassed THF. *p*-Cresol (183 mg, 1.7 mmol) cynnamyl acetate (250 mg, 1.42 mmol) and pyridine (240 μl, 2.84 mmol) were added. The reaction mixture was stirred at 50 °C for 16h. The solution was extracted three times with NaHCO$_3$. The combined aqueous layers were re-extracted three times with CH$_2$Cl$_2$. The combined organic layers were dried over MgSO$_4$, filtered and evaporated to dryness. The product was purified on silica gel using hexane/ethyl acetate 20/1 as eluant to yield **42** as a white solid. (140 mg, 44%)

^{1}H-NMR: (500 MHz, CDCl$_3$) δ: 7.42 (d, 2H, 7.6 Hz), 7.33 (t, 2H, *J* = 7.6 Hz), 7.26 (d, 1H, *J* = 7.6 Hz), 7.10 (d, 2H, *J* = 8.3 Hz), 6.87 (d, 2H, *J* = 8.3 Hz), 6.73 (d, 1H, *J* = 16.0 Hz), 6.44 (dt, *J* = 5.7, 16.0 Hz, 1H), 4.68 (d, 2H, *J* = 5.7), 2.29 (s, 3H).^{13}C-NMR (50 MHz, CDCl$_3$) δ: 136.93,

133.24, 130.55, 130.34, 128.98, 128.25, 126.98, 125.14, 115.07, 63.16 20.89. MS for $C_{16}H_{16}O$: 222.6 $[M+H]^+$

47
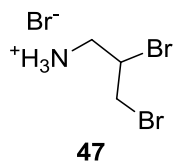
Synthesis of 2,3-dibromopropan-1-amonium bromide **47** [285].

Allylamine (13.16 ml, 175 mmol) was dissolved in 100ml of water and cooled in a ice bath. HBr (19.6 ml, 193 mmol) was added dropwise at 0°C under stirring. A solution of bromine in water (13.5 ml, 263 mmol) was then added. The reaction was stirred at room temperature for 4 hours. The solvent was then removed by vaccum and the solid was recrystallized in MeOH to yield the product **47** as a white solid. (37.4g, 72%)

1H NMR (400 MHz, D_2O) δ: 4.51 (dddd, J = 9.5, 8.9, 4.5, 3.2 Hz, 1H), 3.96 (dd, J = 11.0, 4.6 Hz, 1H), 3.75 (ddd, J = 17.2, 12.6, 6.0 Hz, 2H), 3.37 (dd, J = 14.1, 9.6 Hz, 1H). ^{13}C NMR (101 MHz, D_2O) δ: 46.98, 44.74, 33.74.

48
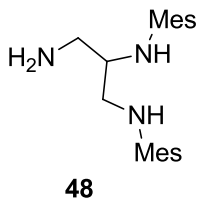
Synthesis of N^1,N^2-dimesitylpropane-1,2,3-triamine **48** [286].

Amine **47** (10g, 33.6 mmol) was dissolved in mesitylamine (61ml, 433.3 mmol). The mixture was heated under nitrogen at 120°C for 19 hours. The excess of mesitylamine was removed by

distillation. The oil was dissolved in a mixture of Et$_2$O/ NaOH 15% (1:1) and was stirred for several minutes. The organic phase was washed with brine and dried over Na$_2$SO$_4$. The solvent was evaporated and the crude was purified on silica gel using CH$_2$Cl$_2$/MeOH/NEt$_3$ 4:1:0.1 as eluent to yield **48** as brown oil. (3.64 g, 33%)

^{1}H NMR (400 MHz, CDCl$_3$) δ: 6.84 (dd, *J* = 7.3, 4.0 Hz, 4H), 3.53 – 3.39 (m, 2H), 3.19 (dd, *J* = 11.8, 5.7 Hz, 1H), 2.92 (dd, *J* = 5.0, 2.7 Hz, 2H), 2.37 – 2.17 (m, 18H). ^{13}C NMR (101 MHz, CDCl$_3$) δ: 144.03, 142.33, 131.78, 131.14, 130.23, 130.20, 129.89, 129.28, 59.13, 51.91, 45.36, 20.98, 20.93, 19.59, 18.75. ESI-MS for C$_{21}$H$_{31}$N$_3$: [M+H]$^+$

44
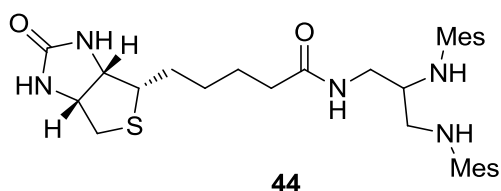
Synthesis of biotinylated diamine **44.**

Biotin NHS (230.7 mg, 0.67 mmol) and **48** (200 mg, 0.61 mmol) was dissolved in DMF. Et$_3$N (0.25 ml, 1.83 mmol) was added and the reaction mixture was stirred 16 hours at room temperature. The solvent was removed and the crude product was purified on silica gel using CH$_2$Cl$_2$/MeOH 8:1 as eluant to yield **44** as a pale solid. (211 mg, 62%)

^{1}H NMR (400 MHz, CDCl$_3$) δ 6.77 (dd, *J* = 15.3, 5.2 Hz, 4H), 5.99 (s, 1H), 5.79 (s, 1H), 4.42 – 4.27 (m, 1H), 4.28 – 4.17 (m, 1H), 3.62 (m, 1H), 3.55 – 3.37 (m, 2H), 3.09 – 2.91 (m, 2H), 2.83 – 2.66 (m, 2H), 2.41 (dd, *J* = 42.4, 12.8 Hz, 1H), 2.23 – 2.09 (m, 18H), 1.66 (m, 4H), 1.39 (m, 2H). ESI-MS for C$_{31}$H$_{45}$N$_5$O$_2$S: 552.3 [M+H]$^+$
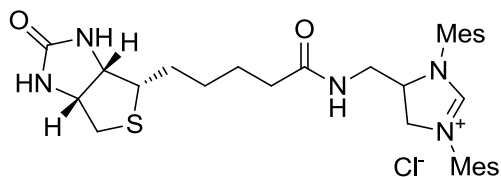
57

Synthesis of biotinylated imidazolinium chloride salt **57.**

To the diamine **44** (100 mg, 0,18 mmol) and NH$_4$Cl (9.69 mg, 0.18 mmol) triethyl orthoformate was added (1 ml, 5.44 mmol). The mixture was stirred overnight at 120°C. The mixture was dissolved in a small amount of CH$_2$Cl$_2$ and poured in Et$_2$O. The solid was washed with Et$_2$O and purified on a small plug of silica gel using CH$_2$Cl$_2$/MeOH 10:1 to yield **57** as a white solid. (45 mg, 41%)

^{1}H NMR (400 MHz, CDCl$_3$) δ: 8.61 (d, J = 11.9 Hz, 1H), 7.03 − 6.77 (m, 4H), 6.64 (m, 1H), 6.12 (m, 1H), 5.37 − 5.11 (m, 1H), 4.65 − 4.33 (m, 2H), 4.24 (s, 1H), 3.99 (m, 1H), 3.87 − 3.60 (m, 1H), 3.37 − 3.15 (m, 1H), 2.70 (m, 1H), 2.64 − 2.46 (m, 1H), 2.22 (m, 20H), 1.66 − 1.34 (m, 4H), 1.35 − 1.12 (m, 2H). ESI-MS for C$_{32}$H$_{44}$ClN$_5$O$_2$S: 562.32 [M-Cl]$^+$
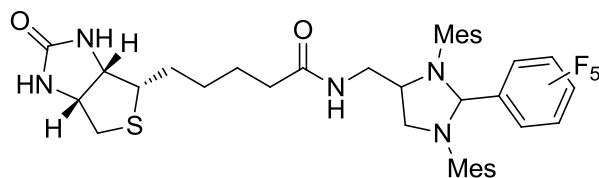
45

Synthesis of biotinylated intermediate **45.**

Diamine **44** (100 mg, 0.18 mmol), pentafluorobenzaldehyde (22 µl, 0.18 mmol) and anhydrous sodium sulfate were dissolved in 5 ml CH$_2$Cl$_2$/acetic acid (1:1). The mixture was stirred overnight at room temperature. The solvent was evaporated and the solid was washed with Et$_2$O. **45** was directly used for the following reaction without further purification.

^{19}F NMR (376 MHz, CDCl$_3$) δ: -137.47 (t, *J* = 19.2 Hz), -146.13 – -146.45 (m), -153.57 – -154.29 (m), -162.18 (td, *J* = 21.7, 10.7 Hz), -162.26 – -162.86 (m).

49
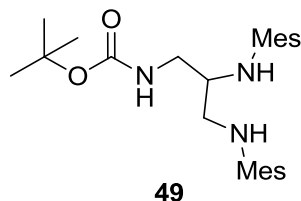
Synthesis of *tert*-butyl (2,3-bis(mesitylamino)propyl)carbamate **49** [286].

Amine **48** (1.8g, 5.53 mmol) and Boc$_2$O (1.2g, 5.53 mmol) were dissolved in dry and degassed CH$_2$Cl$_2$ and cooled to 0°C. DMAP (67mg, 0.56 mmol) was added to the reaction and stirred at 0°C for 30 min and then at room temperature for 4 hours. The reaction mixture was washed twice with water and brine and the organic phase was dried over Na$_2$SO$_4$. The solvent was evaporated and the product was purified on silica gel using Hexane/AcOEt 7:1 as eluent to yield **49** as a white solid.(1.67g, 71%)

^{1}H NMR (400 MHz, CDCl$_3$) δ: 6.80 (d, J = 7.1 Hz, 4H), 3.44 (m, 2H), 3.26 (m, 2H), 3.16 – 3.05 (m, 1H), 2.87 (dd, J = 12.3 Hz, 4.4Hz, 1H), 2.22 (m, 18H), 1.45 (s, 9H). ^{13}C NMR (101 MHz, CDCl$_3$) δ: 156.87, 143.93, 141.73, 131.81, 131.44, 130.40, 130.21, 129.86, 129.28, 79.84, 57.48, 51.22, 43.68, 28.79, 20.92, 20.89, 19.40, 18.72. ESI-MS for C$_{26}$H$_{39}$N$_3$O$_2$: 426.31 [M+H]$^+$

50
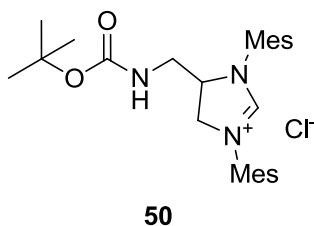
Synthesis of boc-imidazolinium chloride salt **50** [286].

Diamine **49** (2g, 4.7 mmol) and NH$_4$Cl (265mg, 4.7 mmol) were dissolved in 10 ml of neat triethyl orthoformate. The mixture was heated at 120°C under an inert atmosphere for 16 hours. The reaction mixture was allowed to cool down to room temperature and Et$_2$O was added. The precipitate was filtered and washed with a large amount of Et$_2$O to yield the product **50** as a white solid. (1.98g, 89%)

^{1}H NMR (400 MHz, CDCl$_3$) δ: 8.56 (s, 1H), 6.94 (s, 4H), 4.78 (dd, J = 12.3, 8.4 Hz, 1H), 4.46 (t, J = 12.1 Hz, 1H), 3.70 (dd, J = 18.5, 11.2 Hz, 1H), 3.34 (dt, J = 13.0, 4.6 Hz, 1H), 2.33 (dd, J = 43.0, 14.4 Hz, 17H), 1.35 (s, 9H). ESI-MS for C$_{27}$H$_{38}$ClN$_3$O$_2$: 436.29 [M-Cl]$^+$

51
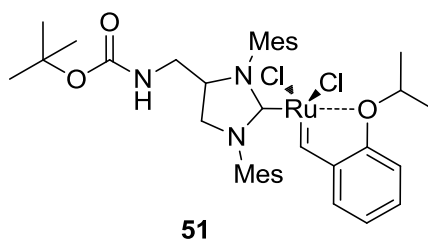
Synthesis of boc-Hoveyda-Grubbs catalyst **51** [287, 288].

A suspension of ^{t}BuOK (112 mg, 0.99 mmol) was added to the imidazolinium salt **50** (471 mg, 0.99 mmol) in dry and degassed THF (15 ml). The mixture was stirred 30 min at room temperature. The yellow solution was transferred into a flask containing Hoveyda-Grubbs 1st generation (500 mg, 0.83 mmol) in benzene (75 ml) and stirred at 80°C. After 1 hour, CuCl (99 mg, 0.99 mmol) was slowly added to the solution and stirred for an additional hour at 80°C. The solvent was evaporated and the product **51** was purified on silica gel using cyclohexane/AcOEt 10:1 as eluent. (277 mg, 44%).

^{1}H NMR (500 MHz, CD$_2$Cl$_2$) δ: 16.32 (s, 1H), 7.48 – 7.43 (m, 1H), 7.05 – 6.95 (m, 4H), 6.88 – 6.74 (m, 3H), 4.82 (m, 1H), 4.43 (m, 1H), 4.18 (t, J = 10.8 Hz, 1H), 3.86 (t, J = 8.9 Hz, 1H), 3.30

(m, 2H), 2.33 (m, 18H), 1.30 (s, 9H), 1.17 (d, J = 5.1 Hz, 6H). ^{13}C-NMR (125 MHz, CD$_2$Cl$_2$) δ: 151.25, 144.28, 138.28, 128.98, 128.96, 121.48, 121.37, 112.96, 78.68, 74.38, 63.89, 55.36, 42.04, 27.23, 20.19, 20.06, 19.99. ESI-MS for C$_{37}$H$_{49}$Cl$_2$N$_3$O$_3$Ru: 720.25 [M-Cl]$^+$
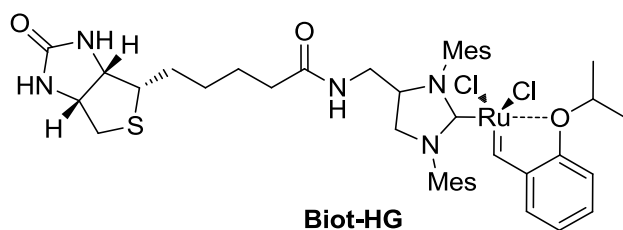
Biot-HG

Synthesis of biotinylated Hoveyda-Grubbs catalyst **Biot-HG**

Complex **51** (40 mg, 0.053 mmol) was dissolved in CH$_2$Cl$_2$ (2 ml) and HCl gas was bubbled through the solution for 1 hour at room temperature. The gaseous HCl was generated by the drop-wise addition of concentrated H$_2$SO$_4$ to NH$_4$Cl. The solution was stirred for 2 hours at room temperature. The deprotection step was monitored by thin-layer chromatography (TLC) (Hexane/AcOEt 2:1). The solvent was evaporated and the resulting green solid was dissolved in DMF (2 ml). Biotin pentafluorophenol (22 mg, 0.053 mmol) and Et$_3$N (0.2 ml, 1.4 mmol) was added to the solution and stirred for 16 h at room temperature. The solvent was removed at reduced pressure. The crude product was purified on sephadex LH 20 eluted with CH$_2$Cl$_2$ to yield **Biot-HG** as a green solid. (27 mg, 58%).

^{1}H-NMR (500 MHz, CD$_2$Cl$_2$) δ: 16.36 (s, 1H), 7.49 (m, 1H), 7.03 – 6.97 (m, 4H), 6.90 – 6.77 (m, 3H), 4.85 (m, 1H), 4.52 (m, 1H), 4.43 (m, 1H), 4.36 (m, 1H), 4.18 (m, 1H), 3.82 (m, 1H), 3.57 (m, 1H), 3.33 (m, 1H), 3.06 (m, 1H), 2.89 (m, 1H), 2.59 (m, 1H), 2.42 – 1.97 (m, 20H), 1.68 – 1.54 (m, 4H), 1.42 – 1.32 (m, 2H), 1.21 (m, 6H). ^{13}C-NMR (125 MHz, CD$_2$Cl$_2$) δ: 174.7, 152.2,

145.0, 143.1 138.7, 136.7, 129.9, 129.8, 122.3, 112.9, 75.2, 63.5, 61.6, 59.9, 55.3, 45.7, 40.5, 35.5, 29.6, 27.9, 26.6, 25.2, 21.2, 20.5, 17.95. ESI-MS for $C_{42}H_{55}Cl_2N_5O_3RuS$: 846.28 $[M-Cl]^+$.

55
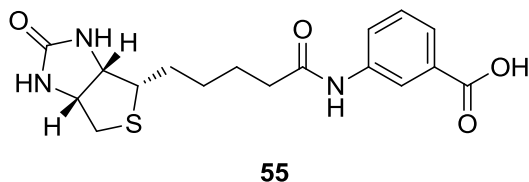
Synthesis of biotinylated benzoic acid **55** [263].

Isobutyl chloroformate (0.32 ml, 2.47 mmol) was added to a solution of biotin (500 mg, 2.05 mmol) in DMF (40 ml) containing tri-*N*-butylamine (0.64 ml, 2.69 mmol). After 10 min at room temperature, the mixture was slowly added to a suspension 3-aminobenzoic acid (4.1 mmol)) in 40 ml DMF at 5°C and was stirred at 5 °C for 2 hrs, the solvent was removed and the crude precipitate was dissolved in warm aqueous ethanol (1:1, 36 ml total). The mixture was acidified with 2.0 N HCl to pH 2 and kept at 0 °C for 12 hours. The resulting precipitate was filtered, washed with water and dried under vacuum to yield **55** as a white solid. (467mg, 63%)

^{1}H NMR (400 MHz, DMSO) δ: 12.91 (s, 1H), 10.04 (s, 1H), 8.21 (t, *J* = 1.7 Hz, 1H), 7.80 (dd, *J* = 8.1, 1.1 Hz, 1H), 7.58 (d, *J* = 7.8 Hz, 1H), 7.39 (t, *J* = 7.9 Hz, 1H), 6.43 (s, 1H), 6.35 (s, 1H), 4.29 (dd, *J* = 7.6, 5.2 Hz, 1H), 4.16 − 4.08 (m, 1H), 3.15 − 3.06 (m, 1H), 2.80 (dd, *J* = 12.4, 5.1 Hz, 1H), 2.56 (d, *J* = 12.4 Hz, 1H), 2.30 (t, *J* = 7.4 Hz, 2H), 1.69 − 1.27 (m, 6H). ^{13}C NMR (101 MHz, DMSO) δ: 172.29, 168.06, 163.58, 140.38, 132.07, 129.79, 124.63, 123.97, 120.63, 61.90, 60.06, 56.25, 40.72, 37.08, 29.08, 28.96, 25.91. ESI-MS for $C_{17}H_{21}N_3O_4S$: 362.12 $[M-H]^-$

56
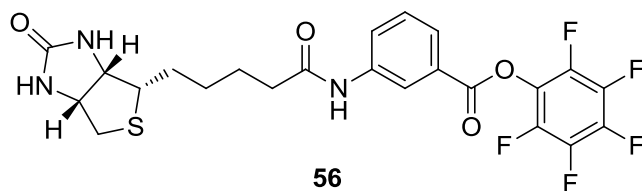
Synthesis of activated biotinylated benzoic acid ester **56.**

The benzoic acid derivative **55** (200mg, 1.37 mmol) and *N,N'*-dicyclohexylcarbodiimide (283 mg, 1.37 mmol) were dissolved in DMF (35 ml). A solution of pentafluorophenol (506 mg, 2.75 mmol) in 10 ml DMF was added and the reaction solution was stirred for 16 hours at room temperature. The reaction mixture was filtered and the solvent was evaporated. The resulting solid was washed with hexane to yield the activated ester **56** as a white solid. (257 mg, 88%).

^{1}H NMR (400 MHz, DMSO) δ: 10.25 (s, 1H), 8.52 (s, 1H), 7.93 (dt, J = 18.0, 9.0 Hz, 1H), 7.82 (d, J = 8.0 Hz, 1H), 7.57 (t, J = 8.0 Hz, 1H), 6.44 (s, 1H), 6.35 (s, 1H), 4.38 – 4.23 (m, 1H), 4.12 (dd, J = 8.9, 3.0 Hz, 1H), 3.18 – 3.04 (m, 1H), 2.90 – 2.68 (m, 1H), 2.56 (d, J = 12.4 Hz, 1H), 2.40 – 2.23 (m, 2H), 1.83 – 1.51 (m, 6H). ^{13}C NMR (101 MHz, DMSO) δ: 172.63, 164.84, 163.58, 142.05, 141.07, 134.54, 130.85, 128.46, 127.14, 126.30, 122.96, 121.08, 61.90, 60.06, 56.25, 40.72, 37.09, 34.21, 30.25, 28.96, 25.82. ^{19}F NMR (376 MHz, DMSO) δ: -153.22, -157.27, -162.00. ESI-MS for $C_{23}H_{20}F_5N_3O_4S$: 552.10 [M+Na]$^+$
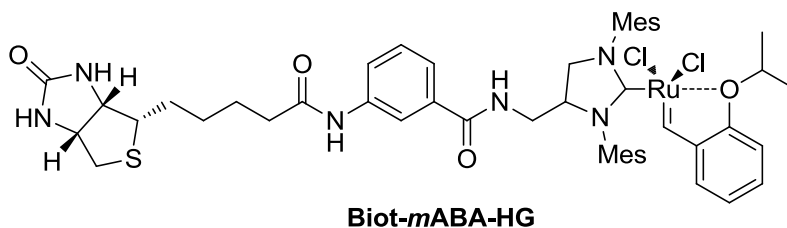
Biot-*m*ABA-HG

Synthesis of biotinylated complex **Biot-*m*ABA-HG**

Complex **Biot-*m*ABA-HG** was synthesized following the procedure for the synthesis of the related complex **Biot-HG** using the boc-protected complex **51** (35 mg, 0.066 mmol) and the activated ester **56** (50 mg, 0.066 mmol). (23 mg, 35%).

^{1}H-NMR (500 MHz, CD$_2$Cl$_2$) δ: 16.38 (s, 1H), δ 7.89 (s, 1H), 7.71 (s, 1H), 7.49 (m, 1H), 7.38 (m, 1H), 7.25 (m, 1H) 7.06 – 6.94 (m, 4H), 6.90 - 6.77 (m, 3H), 4.84 (m, 1H), 4.73 (m, 1H) 4.60 (m, 1H), 4.34 (m, 1H), 4.19 (m, 1H), 3.93 (m, 1H), 3.75 (m, 1H), 3.57 (m, 1H), 3.04 (m, 1H), 2.80 (m, 1H), 2.55 (m, 1H), 2.38 - 2.01 (m, 20H), 1.70 – 1,52 (m, 4H), 1.41 – 1.33 (m, 2H), 1.19 (m, 6H). ^{13}C-NMR (125 MHz, CD$_2$Cl$_2$) δ: 152.3, 151.8, 145.1, 142.8, 138.5, 134.4, 131.1, 130.3, 129.9, 129.5, 122.8, 122.3, 118.2, 113.0, 75.2, 63.6, 61.5, 60.6, 55.6, 42.1, 40.5, 36.5, 29.6, 27.9, 27.6, 25.1, 21.0, 20.7, 17.8. ESI-MS for C$_{49}$H$_{60}$Cl$_2$N$_6$O$_4$RuS: 965.33 [M-Cl]$^+$.

52
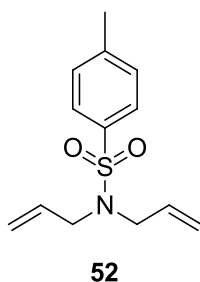
Synthesis of *N*-tosyldiallylamine **52** [289].

Diallylamine (6.33 ml, 51.4 mmol) and toluene-4-sulfonyl chloride (14.7 g, 77.2 mmol) were dissolved in CH$_2$Cl$_2$ (100 ml) and cooled to 0°C. Et$_3$N (20 ml, 154.3 mmol) was added over the course of 10 min and the reaction solution was stirred for 16 hours at room temperature. The reaction solution was placed in a separatory funnel and the organic phase was washed with water and brine, the organic phase was collected and dried over Na$_2$SO$_4$. The solvent was evaporated

and the resulting oil was purified on silica gel using CH_2Cl_2 to yield **52** as colourless oil. (11.8 g, 91%).

^{1}H NMR (400 MHz, CDCl$_3$) δ: 7.73 – 7.67 (m, 2H), 7.30 (d, J = 8.0 Hz, 2H), 5. 61 (ddt, J = 17.3, 9.8, 6.3 Hz, 2H), 5.15(m, 2H), 5.12 (m, 2H), 3.80 (d, J = 6.3 Hz, 4H), 2.42 (s, 3H). ^{13}C NMR (101 MHz, CDCl$_3$) δ: 143.64, 137.78, 133.04, 130.08, 127.54, 119.34, 49.74, 21.89. ESI-MS for $C_{13}H_{17}NO_2S$: 252.1 [M+H]$^+$.
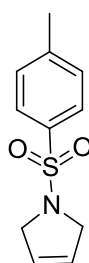
53

Synthesis of metathesis product **53**

Diolefin **52** (100 mg, 0.39 mmol) and Hoveyda-Grubbs 2nd generation catalyst (12 mg, 0.02 mmol) were dissolved in 5ml CH_2Cl_2 and stirred at room temperature for 1 hour. The reaction mixture was filtered on a plug of silica gel using dichloromethane as eluant to yield **53** as a white solid. (79 mg, 88%).

^{1}H NMR (400 MHz, CDCl$_3$) δ: 7.72 (d, J = 8.2 Hz, 2H), 7.31 (d, J = 8.0 Hz, 2H), 5.70 – 5.58 (s, 2H), 4.11 (s, 4H), 2.42 (s, 3H). ^{13}C NMR (101 MHz, CDCl$_3$) δ: 143.82, 134.67, 130.15, 127.81, 125.84, 55.24, 21.93. ESI-MS for $C_{11}H_{13}NO_2S$: 224.12 [M+H]$^+$

4.3 Catalysis procedures.

General Aspects. High purity solvents were used to perform catalysis. All aqueous and organic solvents were flushed with nitrogen during at least *thr*ee *hours*. All manipulations were performed in a nitrogen atmosphere glove box. RCM reactions were performed at air.

Hydrogenation at different concentration of substrate.

[Rh(COD)$_2$]BF$_4$ was dissolved in DMSO (4 mg, 16 ml DMSO), 1.56 µmol of this solution was added to the appropriate aliquoted ligand (2.02 µmol, 1.3 equiv. with respect to the metal source). This solution was stirred for ca. 10 min.

A Pyrex tube (volume ca. 3 ml) was placed in an autoclave and charged with an *N*-acetamidoacrylic acid solution in MES buffer 0.38M. The volume was adjusted with water to 900 µl. The protein solution in water was then added (100 µl of a 0.207 mM solution, 0.0207 µmol of the tetramer). The precatalyst solution in DMSO (100 µl, 0.062 µmol) was added last. The resulting mixture was vortexed, removed from the glove box and hydrogenated at 5 bars H$_2$ for 15h.

Permabond-*L*-Chirasyl-Val (Macheray-Nagel), 25 m x 0.25 mm column: He carrier

gas: 1.2 bar, inlet injector 280°C, oven (85°C; 10 min, 8°C/min; 135°C; 0 min,

10°C/min; 180°C; 35min), FID detector 200 °C; retention time:

Methyl *N*-acetylacetamidoacrylate: 9.3 min

Methyl (*R*)-*N*-acetylacetamidoalanine: 17.2 min

Methyl (*S*)-*N*-acetylacetamidoalanine: 18.5 min

Hydrogenation procedure for recycling of Immobilized Artificial Metalloenzymes.

The commercial suspension of *D*-biotin-sepharose CL-4B (105 µL, which corresponds to 0.031 µmol biotin, capable of immobilizing 0.031 µmol tetrameric (strept)Avidin) (Affiland, Belgium, suspension in TBS buffer pH 7.4, NaN$_3$ 0.02 % (w/v)) was decanted and washed with water to afford wet beads, which were resuspended in water (105 µL total volume). The protein solution (0.062 µmol, 2 equiv. vs. biotin sepharose, 300 µL) was added. After ca. 10 min incubation, the immobilized protein (containing 0.031 µmol of protein, corresponding to 0.093 µmol, free active sites) was washed at least three times with H$_2$O to remove the unbound protein and flushed with nitrogen for three hours. The precatalyst solution in DMSO (100 µl, 0.062 µmol, 0.66 equiv. with respect to the free biotin-binding sites) was added last and was incubated with the immobilized protein for 10 min. The immobilized catalyst was subjected to several cycles of washing and decanting. The catalytic runs were performed according to the standard orbital shaking procedure. After the catalytic run, the immobilized catalyst was decanted, washed and the solution were analyzed. An *N*-acetamidoacrylic acid solution in MES buffer 0.38M (260 µl of a 23.85 mM solution, 6.20 µmol) was added to the immobilized catalyst and subjected to another catalytic run.

Permabond-*L*-Chirasyl-Val (Macheray-Nagel), 25 m x 0.25 mm column: He carrier

gas: 1.2 bar, inlet injector 280°C, oven (85°C; 10 min, 8°C/min; 135°C; 0 min,

10°C/min; 180°C; 35min), FID detector 200 °C; retention time:

Methyl *N*-acetylacetamidoacrylate: 9.3 min,

Methyl (*R*)-*N*-acetylacetamidoalanine: 17.2 min,

Methyl (*S*)-*N*-acetylacetamidoalanine: 18.5 min,

General procedure for allylic alkylation.

The biotinylated ligand (8 mM stock-solution) and the Pd-precursor (6.8 mM stock solution) were dissolved in DMSO and mixed and stirred for 5 min at room temperature. Streptavidin was dissolved in water (275 µL of a 82 µM stock solution, 0.092 µmol, 1.4 eq. active sites vs. metal) and mixed in a vial with the Pd-complex (20 µL of the stock-solution, 0.068 µmol metal) and stirred at room temperature for 30 min. Dimethylmalonate (43 µL, 0.34 mmol) and 1,3-diphenylpropenylacetate (17.1 mg, 0.068 mmol) were dissolved in DMSO (800 µL) and 20 µL of the resulting solution were added to the mixture. Finally, didodecyldimethylammonium bromide (60 µL of 0.056 M a stock solution) and potassium carbonate (60 µL of 0.14 M a stock-solution) were added to the catalysis mixture. The resulting mixture was stirred at desired temperature for 18 h. The reaction was extracted with Et$_2$O (2 x 500 µL), filtered through a short silica gel plug and subjected to HPLC analysis.

Daicel Chiralpak® IA column, 4.6 mm x 250 mm, particle size 5 µm.

Mobile phase: hexane/*i*-propanol 95:5.

Flow rate: 0.8 ml/min

Room temperature

1,3-diphenylpropenylacetate (starting material): 7.3 min

Enantiomer (*S*): 12.4 min

Enantiomer (*R*): 15.0 min

trans-1,3-Diphenyl-2-propen-1-ol (hydrolysis product): 19.4 min

General procedure for allylic alkylation using 25% and 50% DMSO.

The biotinylated ligand (8 mM stock-solution) and the Pd-precursor (6.8 mM stock solution) were dissolved in DMSO and mixed and stirred for 5 min at room temperature. (Strept)Avidin was dissolved in water (270 µL of a 83.5 µM stock solution for 25% DMSO and 160 µL of a 141 µM stock solution for 50% DMSO, 0.092 µmol, 1.4 eq. active sites vs. metal) and mixed in a vial with the Pd-complex (20 µL of the stock-solution, 0.068 µmol metal) and stirred at room temperature for 30 min. the volume was adjusted to 360 µL by adding DMSO. Dimethylmalonate (43 µL, 0.34 mmol) and 1,3-diphenylpropenylacetate (17.1 mg, 0.068 mmol) were dissolved in dimethylsulfoxide (800 µL) and 20 µL of the resulting solution were added to the mixture. Finally, potassium carbonate (60 µL of 0.14 M a stock-solution) was added to the catalysis mixture. The resulting mixture was stirred at room temperature for 18 h. The reaction was extracted with Et$_2$O (2 x 500 µL), filtered through a short silica gel plug and subjected to HPLC analysis.

Daicel Chiralpak® IA column, 4.6 mm x 250 mm, particle size 5 µm.

Mobile phase: hexane/*i*-propanol 95:5.

Flow rate: 0.8 ml/min

Room temperature

1,3-diphenylpropenylacetate (starting material): 7.3 min

Enantiomer (*S)*: 12.4 min

Enantiomer (*R)*: 15.0 min

trans-1,3-Diphenyl-2-propen-1-ol (hydrolysis product): 19.4 min

General procedure for allylic alkylation using Cell Free Extract (CFE).

Catalysis with cell free extracts was performed according to the general procedure of allylic alkylation. Cell free extracts samples were obtained following the procedure described in the thesis of Dr Alessia Sardo. These samples were degassed for 2 h prior to use.

General procedure for allylic substitution of cinnamyl acetate with *p*-cresol.

The ligand (8 mM stock-solution) and the allylpalladium chloride dimer (6.8 mM stock solution) were dissolved in DMSO, mixed and stirred for 5 min at room temperature. (Strept)avidin was dissolved in water (170 µL of a 82 µM stock solution, 0.092 µmol, 1.4 eq. active sites vs. metal) and mixed in a vial with the Pd-complex (20 µL of the stock-solution, 0.068 µmol metal) and stirred at room temperature for 30 min. 170 µL of a 0.5M phosphate buffer and potassium carbonate (60 µL of 0.14 M a stock-solution) were added to the catalysis mixture. *p*-Cresol (11.1 mg, 0.0856 mmol) and cinnamyl acetate (6 mg, 0.0342 mmol) were each dissolved in 100 µl DMSO and 10 µL of the resulting solutions were added to previous mixture.. The vial was sealed and stirred at 50°C for 16h. After adding 10µl of the solution of internal standard (0.25 mg, $3.42*10^{-3}$ mmol, 1eq), the reaction was extracted with Et_2O (2 x 500 µL), filtered through a short silicagel plug and subjected to HPLC analysis.

Daicel Chiralcel® OH-J column,

Mobile phase: hexane/*i*-propanol 96:4.

Flow rate: 0.8 ml/min

Room temperature

Starting material (cinnamyl acetate): 19.6 min

Product: 24.3 min

Hydrolysis product: 27.7 min

4-phenyl-2-butanone (standart): 17.3 min

General procedure for ring closing reaction of 7.

A 9 mM stock solution of the complex **3** was prepared by adding DMSO (50 µl) to an aliquot of the complex (0.4 mg, 0.46 µmol). A 183 mM stock solution of the substrate **7** was prepared by adding of *N*-diallyltosylamine (27 µl, 91.6 µmol) to DMSO (500 µl). In a pyrex tube was added 0.30 mM stock solution of protein in buffer or water (100 µl), followed by the addition of the stock solution of the catalyst (10 µl) and the mixture was stirred for one minute. The substrate was added (10 µl) and the reaction flask was placed in an orbital shaker for 16 hours at 40°C.

A 15 mM solution of phenylethanol (120 µl) was added to the reaction vial followed by MeOH (800 µl). The reaction solution was transferred to an eppendorf and centrifuged at 14000 rpm for 2 minutes. The supernatant (800 µl) was added to an HPLC vial and water (800 µl) was added. The sample was injected in HPLC reverse phase to determine the conversion.

Column: XDB 150 x 4.6 x 5 µl with guard column, 3 µl.

Method: volume injected: 6 µl, at 5 min 10% CH_3CN, at 15 min 90% CH_3CN, at 20 min 90% CH_3CN, at 25 min 10% CH_3CN, at 30 min 10% CH_3CN.

phenylethanol: 10.690 min

Product: 13.534 min

N-tosyldiallylamine: 15.339 min

4.4 Measurements and analysis protocols.

CD and UV-vis titration.

Avidin (8 μM initial concentration in Tris-HCl buffer pH = 7, 2.4ml, 19.2 nmol) was charged with a large excess of HABA solution (9.6 mM stock solution in Tris-HCl buffer pH = 7, 50 eq relative to the tetramer). Aliquots of **Biot-HG** solution (0.96 mM in MeOH) were added to HABA ⊂ Avidin in 0.25 eq steps (5 μL per step, up to 7 eq) relative to the tetramer. The UV-vis measurement (200 - 700 nm) and the CD measurement (450 – 600 nm, band width 1 nm, 30 nm/min, 3 accumulations) were recorded after stirring (5 min with a magnetic bar at room temperature).

Streptavidin (8 μM initial concentration in 0.5 M $MgCl_2$ in Tris-HCl buffer pH = 7 , 2.4 ml, 19.2 nmol) were loaded with a large excess of HABA solution (9.6 mM stock solution in 0.5 M $MgCl_2$ in Tris-HCl buffer pH = 7, 150 eq relative to the tetramer) to ensure full saturation of the active site. Aliquots of **Biot-*m*ABA-HG** solution (0.96 mM in MeOH) were added to HABA ⊂ streptavidin in 0.25 eq steps (5 μL per step, up to 7 eq) relative to the tetramer. The UV-vis measurement (200 - 700 nm) and the CD measurement (450 – 600 nm, band width 1 nm, 30 nm/min, 3 accumulations) were recorded after stirring (5 min with a magnetic bar at room temperature).

SDS-Page Electrophoresis

12% Loading gel

To 5 ml nanopure H_2O, 6 ml 30% acrylamide, 3.8 ml of a 1.5M Tris/HCl (pH 6.8) solution, 75 µl of 20% SDS, 100 µl of a 0.66M ammonium persulfate solution and 6 µl of TEMED were added. This solution was gently stirred for 1 min and 7 ml of this mixture were poured into the cassette. To prevent the gel of drying up 2 ml of ethanol were added on the top. The gel was allowed to polymerize for 60 min. The ethanol was removed and the top of the gel was washed with H_2O.

5% Stacking gel

To 3.4 ml nanopure H_2O, 1 ml 30% acrylamide, 0.5 ml of a 1.5M Tris/HCl (pH 6.8) solution, 30 µl of 20% SDS, 40 µl of a 0.66M ammonium persulfate solution and 6 µl of TEMED were added. This solution was gently stirred for 1 min and 3 ml of this mixture were added on top of the loading gel. A comb was carefully placed into the stacking gel. After the gel was allowed to polymerize for 60 min. the comb was removed slowly. The gels were placed into the tank and the inner and outer reservoirs were filled up with 1X SDS Buffer.

Sample preparation

20 µl of the sample, 10 µl of a 3X loading buffer and 1 µl of B_4F were placed in an eppendorf tube. The solution was gently mixed with the pipette. 10 µl of this solution were loaded on the gel. After separation over night at 200V, the B4F signals were detected under a UV lamp. The gel was then stained with a Coomassie blue solution for one day and destained over 8h.

3X Loading Buffer

3 ml of a 1M Tris/HCl (pH 6.8) solution was added to 20 ml nanopure H_2O, followed by 1 mg bromophenol blue, 1.5 ml glycerol and 0.6 g of SDS. The mixture was stirred and stored at -20°C.

SDS Buffer

A 5X SDS buffer solution was obtained by dissolving 15.1 g Tris-Base, 72.0 g glycine, 5.0 g SDS powder in 1 l nanopure H_2O. This solution was stored in the fridge. The solution was diluted 5 times to achieve a 1X SDS Buffer before use.

Staining solution

Coomassie Blue (0.25 g) was dissolved in a mixture of 1 l nanopure H_2O, 500 ml methanol and 75 ml glacial acetic acid. The solution was stored at room temperature and protected from light.

Destaining solution

The destaining solution consists of a mixture of 810 ml nanopure H_2O, 70 ml glacial acetic acid and 120 ml methanol.

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