Metallocarbene Artificial Enzymes:

Extending Transition Metal Selectivity and Protein Activity

Metaalcarbeen Artificiële Enzymen:

Uitbreiden van Overgangsmetaalselectiviteit en Eiwitactiviteit

(met een samenvatting in het Nederlands)

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Metallocarbene Artificial Enzymes

Extending Transition Metal Selectivity and Protein Activity

to my parents and sister

to the memory of Adriana Licona

Basauri Molina, Manuel

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Front cover: coordination spheres – three metals in organic frames. Back cover: substrates to products across a metalloenzyme. Design and digital art by Antonio Molina Vasconcelos, www.digitarte.mx.

The cosmos is also within us,
we are made of star-stuff.
We are a way for the cosmos to know itself.

Carl Sagan

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The longer the island of knowledge,
the longer the shore of wonder.
Ralph. W. Sockman

1

Hybridization strategies towards semi-synthetic metalloprotein hybrids: Metallocarbene artificial enzymes as a robust and versatile approach

Abstract

Synthetic transition metal complexes and enzymes, powerful tools in catalysis and synthesis, present properties that are in many cases not interchangeable, like the wide reactivity scope of the metallic species and the superb selectivity of the biological catalysts. The combination of both tools into protein-metal hybrids is a strategy to foster the best features of each component in catalysis, i.e. to expand the reactivity of enzymes by the inclusion of a new active site, while the protein scaffold provides a 2nd coordination sphere that may induce selectivity in catalysis. The mode of anchoring and the location of the metallic fragment are crucial for the control of the activity and selectivity features. In this chapter an overview of the different hybridization techniques with the most relevant applications in catalysis is presented, followed by an introduction of metallocarbene hybrids as an exploitation of the covalent active site-directed approach for the construction of artificial metalloenzymes.

1. Interest and general features of artificial metalloenzymes

The value of catalyst development is ever-present in synthetic chemistry, not only due to the interest to accelerate reactions and reduce the energetic costs of the synthesis of constantly newly demanded end materials, but also because of the common sources of prime materials and fuels becoming scarce. The latter has driven chemists to look for renewable sources, such as biomass, to form part of the products as well as to constitute the catalysts themselves. Therefore, important efforts are conducted in research to match bio-based tools with existing man-made technologies as a strategic step of this resource transition.[1]

Synthetic and enzymatic catalysts, both being broadly applied in industrial processes, are not interchangeable in many chemical transformations. Nowadays, synthetic transition metal catalysts can conduct a myriad of chemical transformations, where their bio-based counterparts, i.e. enzymes, are not competitive or simply cannot carry them out. Examples include the different carbon-carbon cross couplings by palladium complexes[2] and ruthenium-catalyzed olefin metathesis.[3] However, it is clear that enzymes show advantageous features such as superb selectivity, due to their evolutionary driven tertiary structure leading to enantio-, regio- and chemoselectivity, as well as their water solubility and activity under mild reaction conditions.

Whether new enzymes can be designed from their primary structure to promote different transformations, and homogeneous transition metal catalysts can be furnished with advanced stereo-directing ligands, these are independent efforts from biochemists and organometallic chemists, respectively, and have been addressed in the last couple of decades leading to, for example, (enantio)selective catalysts that owe their selectivity to synthetic (enantio)pure ligands.[4] The field of artificial metalloenzymes aims to combine these efforts by merging active, yet unselective, transition metal catalysts with protein hosts. The field aims to match the best features of both types of catalysts, where a diverse number of reactions could be carried out as a function of the chosen organometallic moiety that promotes catalysis, while its surroundings provided by the protein scaffold results in catalytic selectivity due to the so-called 2nd coordination sphere that is generated around the metal center.[5]

This biotechnology features the protein scaffold, or another bio-macromolecular host, such as DNA, as building blocks of the catalyst, as a bioinspired natural source of chemical hindrance and chirality, and allows chemists to explore the influence of a variety of scaffolds over already known active artificial transition metal catalysts, thereby expanding the reactivity repertoire of enzymes and substituting the need of conventional synthetic chiral ligands (Figure 1). Moreover, some of the physical properties of the bio-macromolecular host, like its solubility in water and separability by dialysis, are dominant over those of the embedded and considerably smaller synthetic catalyst, which generally presents an opposite behavior.

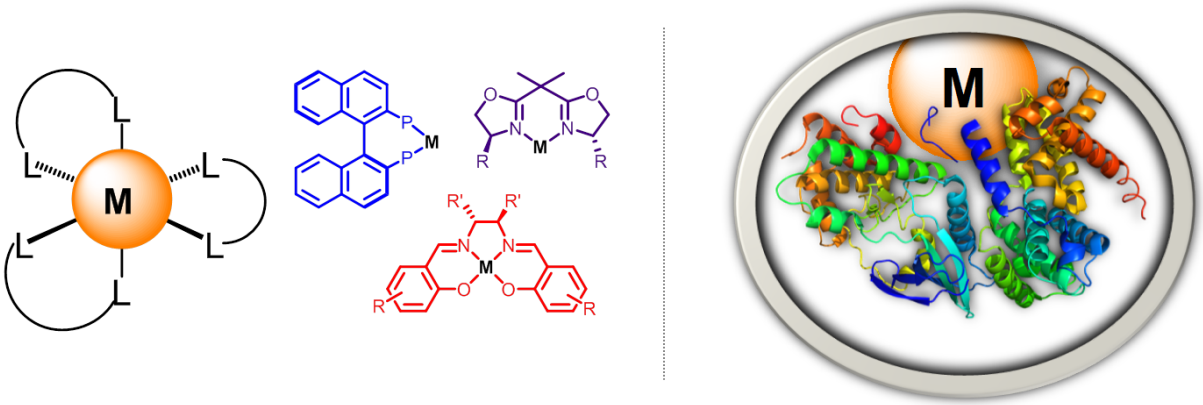

Figure 1. Some common synthetic ligands used as a conventional approach to produce enantioselectivity for a transition metal catalyst (left) and general concept of artificial metalloenzymes (right) using protein scaffolds as a naturally occurring chiral environment.

As anticipated, the resulting effect of the protein on the incorporated metallic moiety has a direct dependence on the position of the active catalyst in the protein host, given that the 2nd coordination sphere will allow or block the attack of substrates or the formation of specific catalytic transition states (Figure 2).[6] The first enantioselective artificial metalloenzyme was reported by Fujii in 1956 who managed the deposition of palladium particles on fibrous proteins from silk, achieving the asymmetric hydrogenation of oximes with low but definite enantiomeric excess (*ee*).[7] However, it was only after more than two decades for the field to meet hybridization techniques with structural control of the protein-

metal conjugation. With milestone works of Kaiser in 1976, who substituted zinc for copper in a carboxypeptidase, incorporating the metal through a coordinative bond,[8] later, in 1978, achieving the alkylation of an aminoacid residue (cysteine) of Papain with a transition metal complex,[9] and of Whitesides, who in the same year positioned a homogeneous catalyst in the pocket of Avidin, making use of its strong supramolecular interaction with biotin functionalized with a transition metal moiety.[10] The field really flourished only fifteen years ago as a result of the required tools for protein structural information, molecular catalyst design and protein mutation.

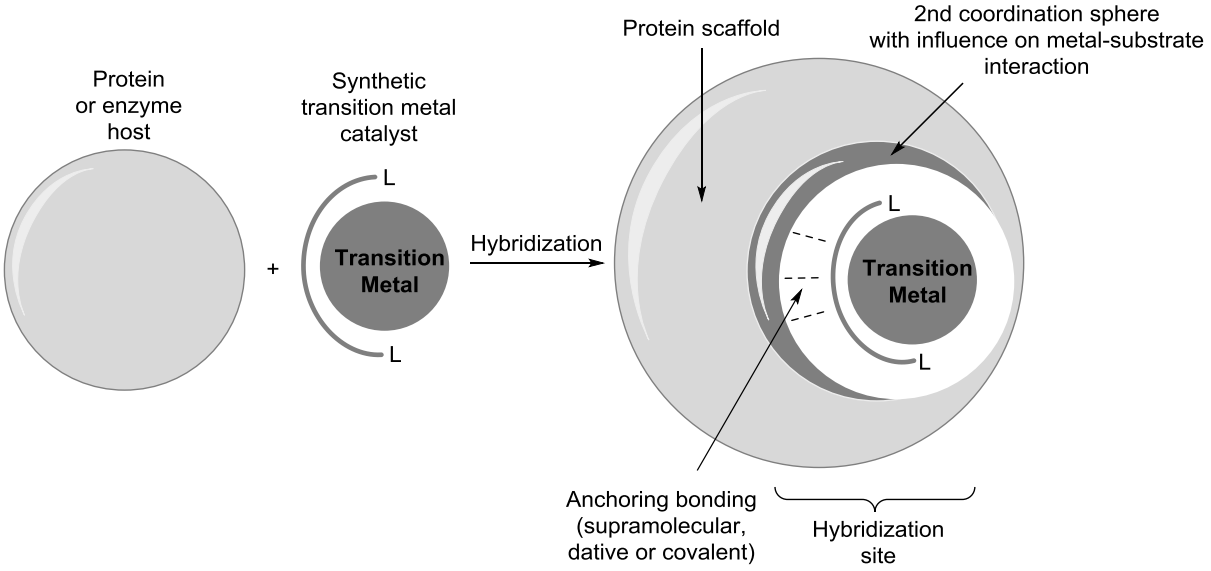

Figure 2. General scheme of a transition metal catalyst embedded in a protein scaffold: an artificial metalloenzyme.

Due to the relevance of the particular hybridization strategy as one of the main features of this thesis, this chapter introduces the diverse metal-protein hybridization methods. Over the years, a handful of strategies have emerged and a number of reviews have provided overviews of these techniques at different stages in the development of the field.[11] Bioinspired catalysts derived from scaffolds such as artificial peptides and DNA strains have been used by the field as well,[12] but these will not be covered here. In this chapter the description and selected applications in catalysis of the different methods towards metal-

protein hybrids are presented, with a discussion of the advantages or disadvantages of each technique. This is followed by our considerations to design and develop metallocarbene aritifical enzymes as products of the use of a covalent active site-directed hybridization approach.

2. Metal-protein hybridization strategies towards artificial enzymes

2.1 Direct coordination of homogeneous metallic species to protein scaffolds

This non-covalent mode of hybridization relies on the rich coordination chemistry of transition metals with the main group moieties that proteins may present accessible to a chosen metallic center, specifically oxygen, nitrogen and sulfur donors present in amino acid residues. Kaiser, as mentioned above, pioneered this strategy by the substitution of zinc in Carboxypeptidase A (CPA) with copper. This very early work was applied many years later by Soumillion and Kazlauzkas using manganese to form active catalysts for the asymmetric epoxidation of styrenes with up to 67% ee.[13] The latter author also achieved substrate *cis/trans* selectivity in the hydrogenation of stilbene and linear/branched selectivity in the hydroformylation of styrene with the use of rhodium as metallic center.[14] These and other examples have proven the applicability of artificial metalloenzymes in selective catalysis following a relatively facile hybridization method. Nevertheless, although coordinating amino acids such as lysines and histidines are mainly employed, the control of allocation of the metal in one single place of the enzyme could be difficult because of metal coordination lability. Moreover, metal de-coordination from the desired pocket is very sensitive to alterations of the protein's tertiary structure and successful (enantio)selectivity depends therefore on the conservation of specific reaction conditions. Interestingly, the site of coordination within the enzyme can be designed using strongly coordinating amino acid residues put closely together in the tertiary structure to promote the binding of the metal center at that position. In this way Reetz reported the modification of tHisF (Imidazole glycerol phosphate synthase) creating an artificial binding site for copper for asymmetric Diels-Alder transformations with up to 46% ee (Figure 3).[15]

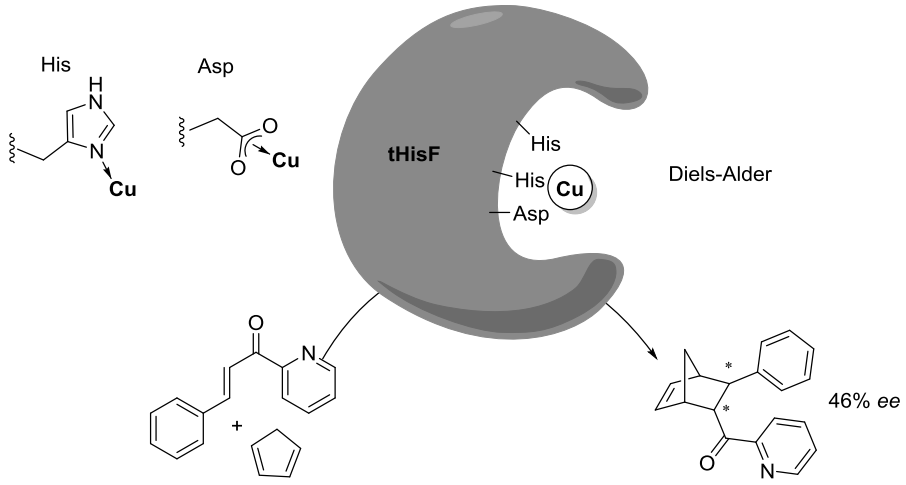

Figure 3. Mutated tHisF with two His and one Asp coordinating Cu for Diels-Alder reactions.

1.2.2 Modified heme cofactor reconstitution and artificial cofactor anchoring

Due to the strong coordination of corroles and porphyrines to metal ions, different transition metals can be incorporated in heme proteins after extraction of the natural cofactor and reconstitution with a modified or artificially designed cofactor (Figure 4). The earliest report comes from Kunitake in 1993 where the iron porphyrine cofactor of Myoglobin was functionalized with a hydrophobic chain and reconstituted in the protein without a change in the tertiary structure, but allowing the incorporated long chain to associate with other lipid species.[16] More recently, the incorporation of other metallic centers has attracted attention to foster new catalytic activity, for example the reconstitution of manganese corroles in human serum Albumin for the oxidation of thioethers leading to ee's up to 74% (Figure 5, top).[17]

Alternatively, these hemeproteins could be reconstituted with synthetic complexes functioning as the heme, i.e. artificial cofactors, expanding the coordination environments around the metallic center for a rationalized control of its activity. For example, in an equivalent catalytic study, Watanabe used chromium and manganese salphen complexes to reconstitute apo-Myoglobin, achieving ee's ranging from 33% to 27% in thioether oxidation for the *R* or *S* enantiomers depending on the catalyst and mutant (Figure 5, bottom).[18]

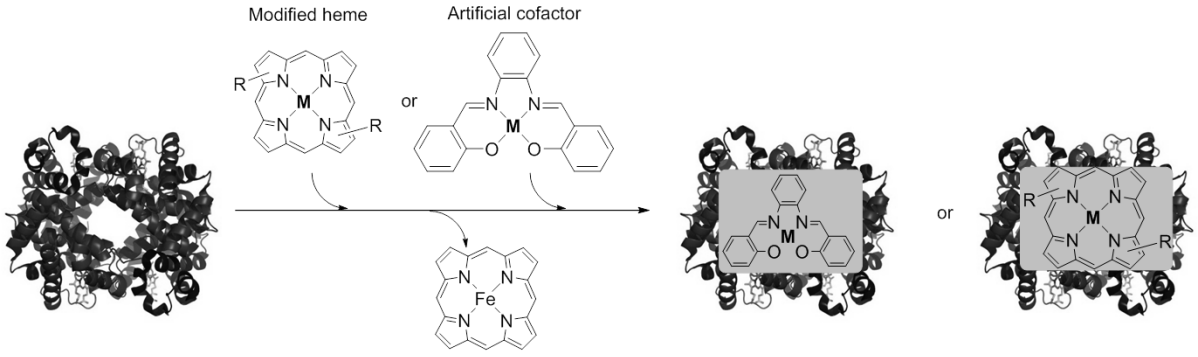

Figure 4. General formation of artificial enzymes with modified or artificial cofactors from hemeproteins.

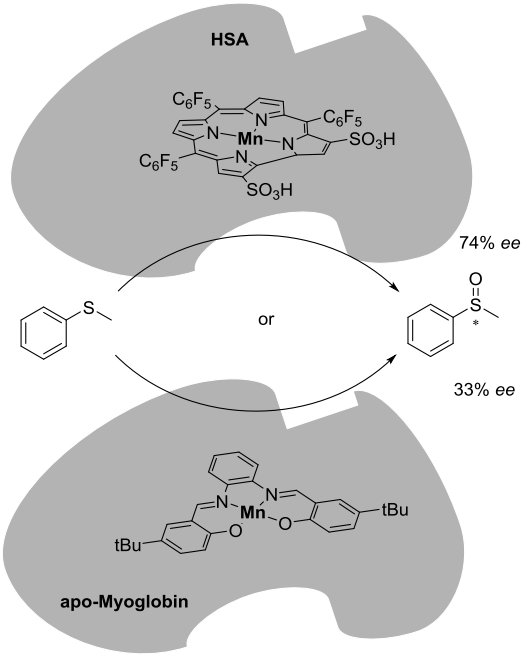

Figure 5. Enantioselective oxidation of thioethers with human serum Albumin coordinated to a modified heme complex (top) and an artificial cofactor (bottom).

Irrespective of the use of modified or artificial cofactors, the technique represents a promising strategy in terms of generation of a 2nd coordination sphere around the metallic center, given that the host protein naturally presents the anchoring site with particular dimensions for heme-like species, in most cases immersed within the protein tertiary

structure. The hybrids are comparatively as robust as the above-mentioned coordinated conjugates but in the case of artificial cofactors, the strength of association is difficult to predict and could be lower. Moreover the 1st coordination sphere around the catalyst is restricted to coordinative square planar aromatic ligands.

2.3 Supramolecular modified biotin-Avidin anchoring

As mentioned above, Whitesides achieved a site-directed hybridization strategy making use of the high supramolecular affinity between the protein Avidin and the molecule biotin (K_a = 10^{15} M^{-1}). The latter is a small bicyclic organic compound that was functionalized with artificial tether chains bearing chelating phosphine ligands for its further coordination with metallic centers, for example using rhodium in the first example for the asymmetric hydrogenation of acetamidoacrylic acid (Figure 6, top). Different modifications in the biotin tether and phosphine ligands of the original biotin-ligand cofactor have later been studied by several groups.[19] As an important advance, Ward discovered that the use of Streptavidin instead of Avidin produced an accentuated 2nd coordination sphere effect, mainly due to a deeper location of the binding site, leading to over 90% ee in the Rh-catalyzed hydrogenation of acetamidoacrcylic acid (precursor of alanine).[20] Further on, the use of spacers in the biotin-ligand compound including enantiopure aminoacid moieties, together with screening of Streptavidin mutants lead to a wide spectrum of catalytic enantioselectivity, with up to 95% ee (S) and a threefold rate increase in this reaction.[21]

This strategy has continued to be extensively studied by Ward, for example by substitution of the chelating phosphines by a N,N ligands to form ruthenium artificial enzymes that perform catalytic transfer hydrogenations of prochiral ketones (Figure 6, bottom).[22] When *p*-cymene was used as the additional arene ligand, the enantioselectivity of the artificial enzymes could be improved by means of protein mutagenesis to lead to 98% ee (*R*) in the transfer hydrogenation of *p*-methylbenzophenone.[23] The complementary use of hydrogen peroxide allowed for the reverse reaction, i.e. the oxidation of alcohols, to be catalyzed by the Ru hybrid (Rh and Ir hybrids were significantly less active).[24] More recently, this group also studied the asymmetric allylic alkylation using palladium artificial enzymes affording over 90% ee (*R*) and 80% ee (*S*) by exploring different (chiral) spacers in the ligand

and mutated protein. Moreover, olefin metathesis has been achieved by functionalizing biotin with tethers bearing an N-heterocyclic carbene, a popular ligand for the coordination of Grubbs-type catalysts.[25]

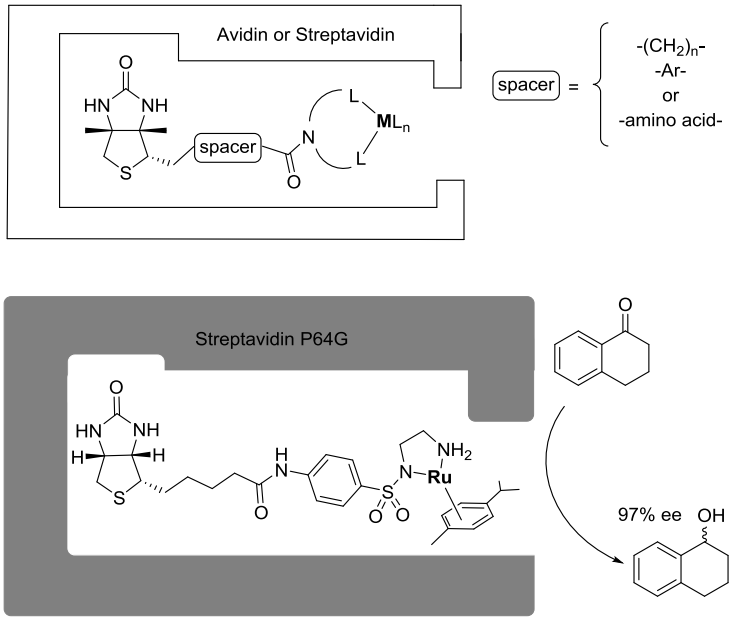

Figure 6. General structure of biotin-(Strept)avidin hybrids (top) and an artificial metalloenzymes for the hydrogenation of ketones (bottom).

The biotin-(Strept)avidin strategy has been the most widely and successfully used hybridization method in the field of artificial enzymes due to the relatively facile functionalization of biotin and the strong interaction with (Strept)avidin, the latter also presenting the anchoring site relatively deep in the tertiary structure and with the amino acids involved in the 2nd coordination sphere being identified for further directed evolution. As a drawback, the system is restricted to two specific, non-abundant proteins, Avidin and Streptavidin, leaving any optimization method to depend on ligand modification, sometimes with enantiopure moieties, and mutation approaches. In addition, many of the catalytic protocols used for these Avidin-biotin hybrids often involve the addition of a metal salt to the ligand-biotin-Avidin construct, therefore requiring sub-stoichiometrical amounts of the metallic source to avoid non-specific metal binding.

2.4 Covalent hybridization through amino acid alkylation

Covalent anchoring of the organometallic fragment to the protein skeleton has several promising advantages in the formation of metalloenzymes. These include a higher reliability of the positioning of the active metal center in the protein scaffold due to a more stable catalyst-protein bond and the decreased change of metal migration during catalysis, which could lead to a different 2nd coordination sphere or even to metal leaching. Moreover, the covalent bond allows for some degree of protein denaturation without the immediate loss of the organometallic fragment, facilitating the screening of reaction media. The treatment and analysis of the hybrids is also simplified due to its higher robustness in comparison with a supramolecular or coordination hybridization strategy.

To address amino acid modification, the organometallic catalyst, or the anchoring ligand must be previously furnished with a reactive functionality that targets the amino acid. This approach makes use of the most nucleophilic groups in proteins, i.e. cysteine, lysine and serine (histidine, although presenting nucleophilicity has been used for the direct coordination of the metal moiety instead, as described above). Thus, nucleophilic substitution of a key functionality in the artificial cofactor to be incorporated leads to irreversible covalent bonding. Cysteine has been most frequently addressed because of being a highly nucleophilic amino acid that is easier to control in terms of single site modification in the protein. In other words, when more than one cysteine residue is present in the protein, these can be selectively mutated to prevent interference in the hybridization reaction. Besides, several hydrolases present a single cysteine residue in their active site. Figure 7 shows the chemical functionalities that have been used for the alkylation of amino acids towards semi-synthetic protein-metal hybrids.

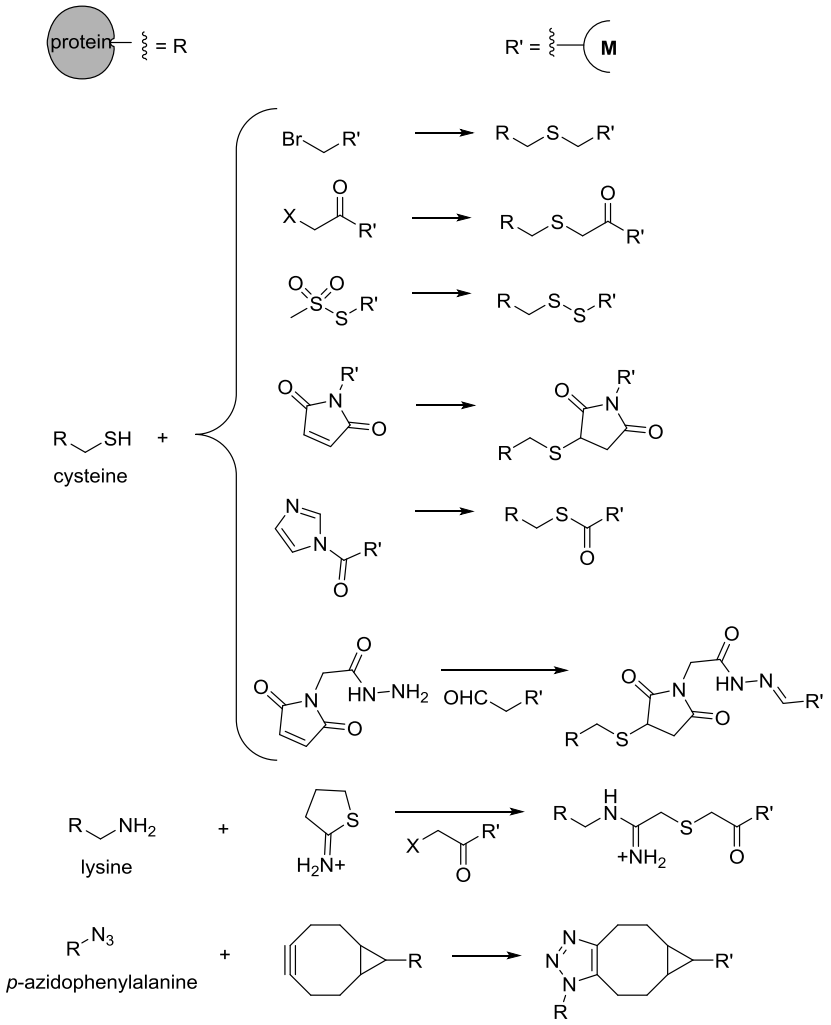

Figure 7. The functionalities that address specific nucleophilic amino acids for their alkylation (an artificial aminoacid, *p*-azidophenylalanine, recently introduced, can undergo click chemistry with alkynes for covalent incorporation of ligands[43]).

Kaiser pioneered the alkylation of cysteine with bromoacetyl compounds bearing flavins,[26] opening the field for more research. Especially with the chosen enzyme host, Papain, catalysis has been carried out later, however since the aminoacid residue modified (Cys_{25}) is part of the catalytic triad of Papain, this will be covered in the next section. Cysteine alkylation has had successful applications, such as the incorporation of a phenantroline ligand in the protein ALBP, which was previously targeted due to the presence of a pocket where an artificial moiety could be included; therein after the introduction of this ligand, its metallation with copper allowed the enantioselective hydrolysis of esters with up to 86% ee of the product.[27] The group of Lu alkylated two cysteine residues with one manganese salen

complex in Myoglobin and compared the enantioselectivity of this catalyst with the mono-ligated counterpart and found that the dual anchoring in the protein led to an ee increment of 21% to 51% (*S*) in the oxidation of anisole.[28] This hybridization method also allowed for the first artificial metalloenzyme for olefin metathesis, reported by Hilvert and Ward by the functionalization of Grubbs catalyst with the haloacetamide group.[29] Recently, high enantioselectivity by a covalently hybridized enzyme was achieved by Roelfes in the Diels-Alder reaction with up to 97% ee by linking haloacetamide-functionalized chelating amines to a dimer enzyme and further metallating with copper salts (Figure 8).[30]

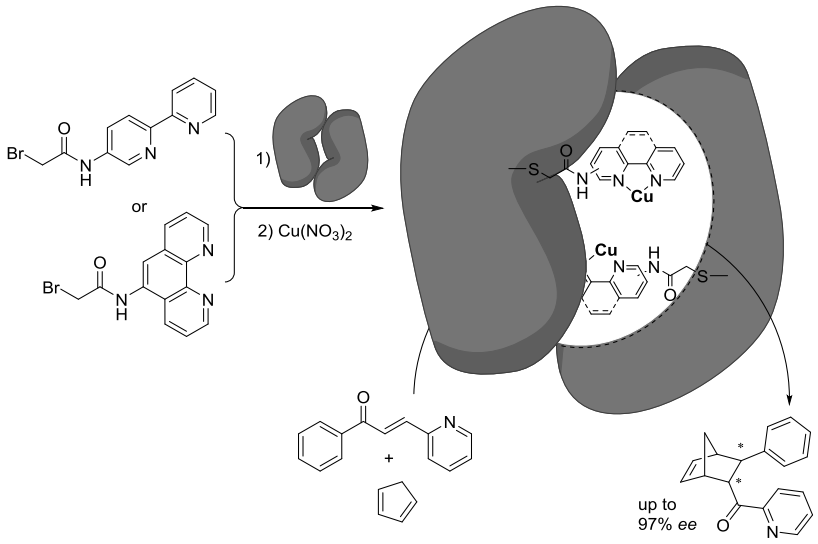

Figure 8. A dimer protein covalently modified with copper catalysts for Diels-Alder reactions.

2.5 Covalent active site-directed hybridization of enzymes

The active site of an enzyme having a naturally selective reaction environment leads to the concept that it is the most promising part of the enzyme to localize a synthetic metal complex in the construction of an artificial catalyst and to develop an effective 2nd coordination sphere around the metal complex. This concept holds the restriction that the enzyme host presents a catalytically active amino acid that is unique in reacting with a chemical functionality bearing the metallic moiety, to avoid competition of other amino acids in the conjugation. Two strategies have been followed, on the one hand the alkylation of Cys_{25} in the active site of Papain, as mentioned before, achieved by Reetz with

haloacetamide functionalities. Salmain performed a similar hybridization but incorporated a η^6-arene ruthenium complex and successfully catalyzed the Diels-Alder reaction of acrolein and cyclopentadiene albeit with no observed ee.[31] De Vries formed a monocoordinated phosphite-rhodium hybrid with Papain active in the hydrogenation of methyl 2-acetamidoacrylate but without enantioselectivity either.[32] The authors have proposed that Papain is not properly suitable for the generation of an enantioselective pocket due to the large conformational flexibility of this enzyme.

Another strategy for the covalent modification in the active site of an enzyme is the use of an inhibitor that selectively reacts with the active site of the enzyme. Specific organic groups are known to perform this after studies on enzyme profiling, generating a library of compounds that, when reacted with an enzyme, are irreversibly bound to the active amino acid in a covalent fashion.[33] Although recently an artificial enzyme with a Grubbs catalyst was bound to α-Chymotrypsin through inhibition of its active site, the anchoring occurred under supramolecular bonding.[34] To our knowledge, covalent constructs have only been addressed using serine hydrolases for the creation of hybrids using a suicide inhibitor. Reetz addressed the active site of a group of lipases covalently anchoring a bis(*p*-nitrophenyl)phosphonate inhibitor bearing also metallic fragment. Since inhibitors are not functional groups that address a particular amino acid anywhere in the protein but that react selectively with the catalytic triad of these lipases, the risk of competition with other residues in the protein can be discarded and this allows to test different lipase hosts without the concern of reactive amino acids on their surface or, in general, out of the active site. However, in the mentioned work, while the modification of the active site blocks the natural activity of the enzyme host, the restitution of this activity was noticed within days because of cleavage of the hybridization bond due to hydrolytic instability of the bis(*p*-nitrophenolate) conjugate.[35] On the other hand, in our group, Van Koten and Klein Gebbink reported the use of mono *p*-nitrophenylphosphonate esters for the inhibition of serine hydrolases, forming hybrids carrying an organometallic moiety with the advantage that these phosphonate ester form a robust linkage with the lipase and that the metal center is part of the inhibitor, avoiding a further metallation step after its reaction with the enzyme, reducing non-specific association of the metal center (Figure 9).[36]

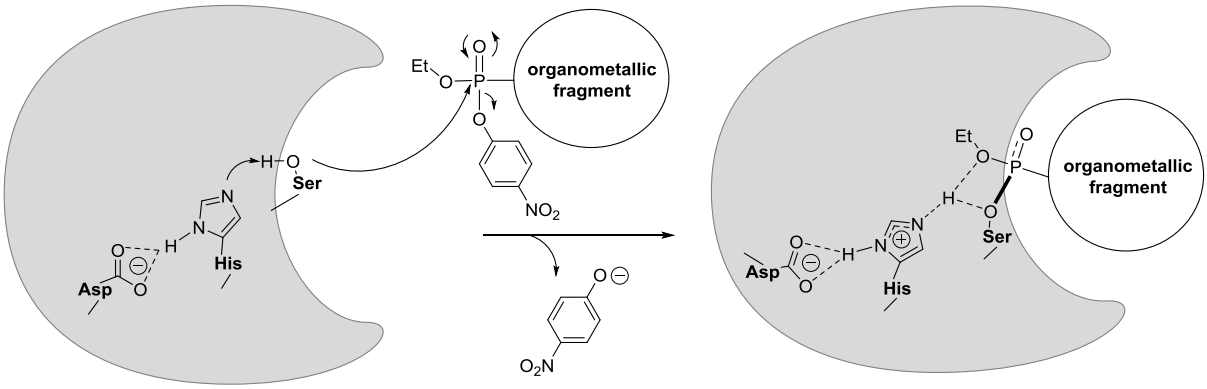

Figure 9. Lipase inhibition mechanism by phosphonate-metal complexes

Exploration of this active site-directed (ASD) approach used Cutinase from *fusarium solani pisi*, a relatively small lipase of 21 kDa with a solvent-accessible active site. The organometallic moieties consisted of ECE-metallopincer complexes bringing to the enzyme pocket palladium and platinum centers bound in a terdentate coordination fashion to the ligands (Figure 10, left). The positioning of the metallic fragment was later proven by X-ray crystallography of the hybrids.[37] The hybridization was further demonstrated by ESI-MS, showing a 1:1 stoichiometry in the final product of the reaction of cutinase with an excess of the inhibitor (the unreacted excess was eliminated by dialysis), complemented by a test for residual activity of the enzyme which showed the inhibition of the latter due to its modification in the active site. Later, the enzyme Candida antartica lipase B (CalB) was functionalized with a ruthenium catalyst for secondary alcohol racemization. Carrying out the conjugation on supported CalB led to only partial inhibition of the enzyme of the support. This supported bi-functional enzyme/artificial enzyme conjugate was used in the dynamic kinetic resolution of secondary alcohols (Figure 10, right).[38] Other examples that make use of this ASD strategy include the labeling of lipases with organic compounds[39] and the generation of luminescent hybrids with platinum complex.[40]

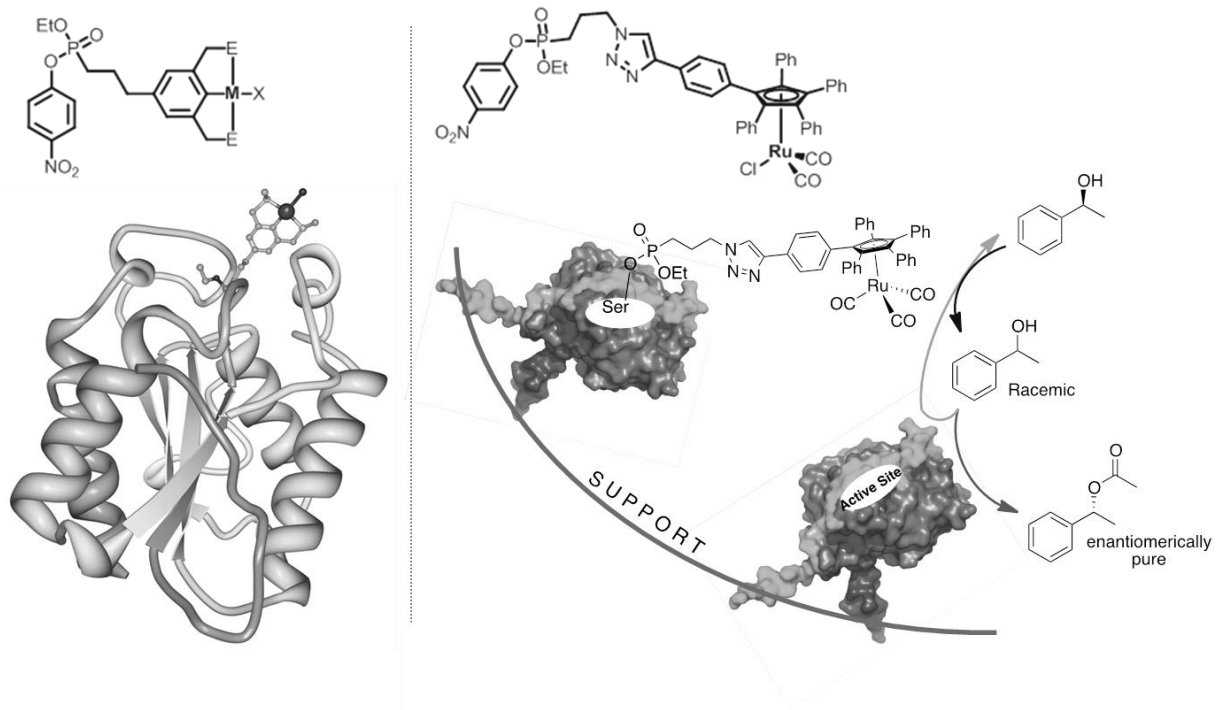

Figure 10. Lipase active site-directed complexes (top) and the resulting metal-protein hybrids. Left: Protein-ECE metallopincer hybrids and right: CalB-Ru hybrid for dynamic kinetic alcohol resolution

In spite of the promising features of the covalent active site-hybridization method, at the beginning of the current thesis there were no observations of catalytic selectivity induced by the protein scaffold in the differently created artificial metalloenzymes. Either because of the large flexibility of Papain (see above), the instability of the hybrids constructed by Reetz, or the formation of active site-directed hybrids without catalytic activity. In the case of the protein-ECE metallopincer hybrids, one obstacle for the reactivity of these hybrids is the inherent stability of terdentate pincer complexes, which derives in the need of relatively harsh reaction conditions, compromising the tertiary structure of the protein host for the activation of the catalytic center, otherwise requiring substantial long reaction times at, for example, room temperature.[41] The latter observations with the pincer-based hybrids encouraged us to look for an alternative ligand system for the work of this thesis as will be discussed below.

3. Aim and scope of the thesis

Due to the interest of our group in the study and development of new catalytic systems, including bioinspired and supported catalysts, we continued our efforts towards the creation of new artificial metalloenzymes. It is our strong believe that the covalent active site-directed approach is a promising strategy for the synthesis of robust hybrids with important interactions with the enzyme resulting in the generation of a 2nd coordination sphere. Our experience in the conjugation of lipases, Cutinase and CalB among others, with phosphonate esters inspired us to further exploit this approach, however, with a different functionalization of the inhibitory functionality to target a ligand system that allows for activity of the organometallic fragment at mild reaction conditions needed to preserve the protein's tertiary structure.

Along this line, we looked for a non-chelating, yet robust and versatile ligand system to circumvent the absent catalytic activity of the former metallopincer systems due to their stability at mild conditions provided by their terdentate structure. N-heterocyclic carbenes (NHC) ligands are well known to take a single coordination site on the metallic center, however presenting one of the most robust carbon-metal bonds and forming organometallic species with a myriad of transition metals. Moreover, opposed to phosphine ligands, the bond in the M(NHC) complexes is oxygen- and water-tolerant, the constitution of the heterocycle is non-toxic and many different species have proven catalytically active in mild and aqueous conditions, where the NHC acts as a spectator ligand with low or no metal leaching,[42] making this a promising system for the conjugation of metal centers to protein scaffolds.

In this work a series of lipase inhibitors were designed based on a phosphonate ester with a tether chain bearing a NHC-metal moiety with different tether lengths and N-substituents. These inhibitors were used for the active site-directed hybridization of lipases in order to produce new artificial metalloenzymes. In this design, the tether chain is integrated in one of the N-substituents to promote an orthogonal interaction of the metal center with the protein backbone (Figure 11). The coordination versatility of NHCs towards transition metals was exploited to form inhibitors of ruthenium, rhodium and palladium. These metals are not present in biological systems, which accordingly allowed us to study

the compatibility between these metals and the chosen lipases, while the nature of the metallic elements from different groups of the periodic table allowed us to study different catalytic applications of the new hybrids.

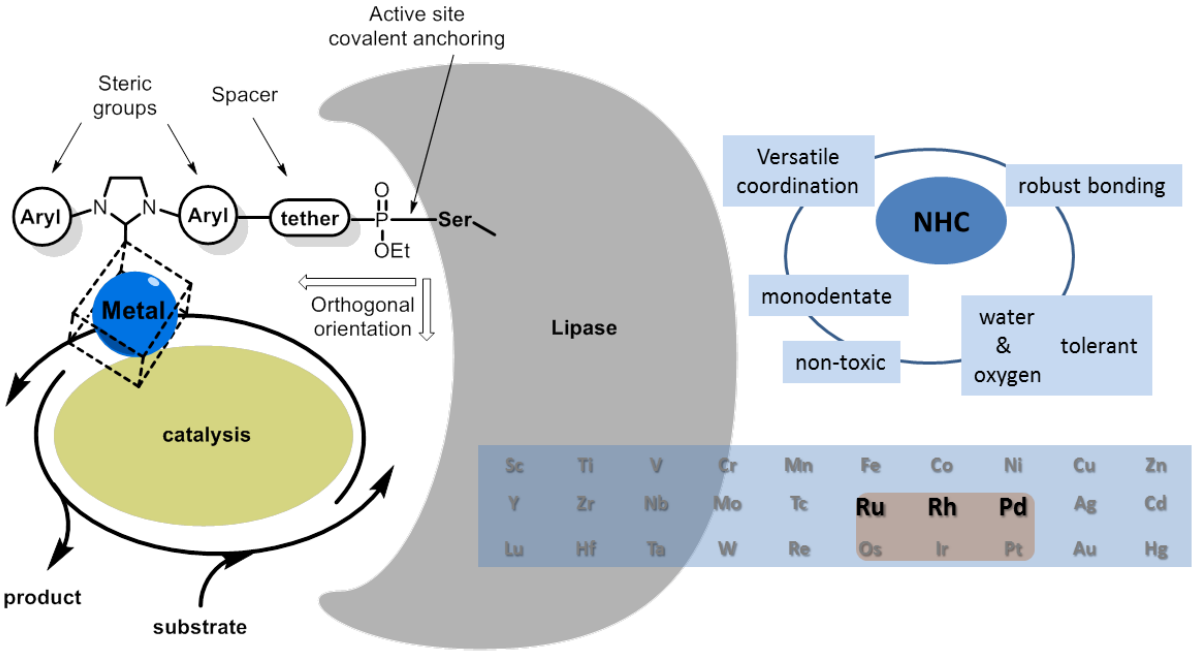

Figure 11. General structure and variations of metallocarbene artificial enzymes developed in this thesis.

After the introductory overview provided by this chapter, Chapter 2 details the initial synthesis of a rhodium(NHC)-phosphonate complex and the anchoring studies of this new type of inhibitor to the lipases Cutinase and CalB. This proved the lipase inhibitory reactivity of a single phosphonate species for the facile screening of different lipases, i.e. different scaffold environments. The new artificial metalloenzymes were used in catalytic ketone and olefin hydrogenation, where the preference of the unsupported catalyst towards the reduction of the olefin was enhanced by its positioning in the lipases. Moreover, with a deeper positioning of the active site, the CalB hybrid promoted the exclusive hydrogenation of the olefin, representing, to the best of our knowledge, the first example of substrate chemoselectivity observed with artificial metalloenzymes.

Next, a series of Grubbs-phosphonate complexes with different NHC N-substituents and different lengths of the NHC-phosphonate tether chain were synthesized, in Chapter 3. The inhibitors were reacted and coupled with cutinase at opposed pH values towards the formation of ruthenium-protein hybrids for olefin metathesis. This showed a subtle sensitivity of the peptide loops protecting the active site of cutinase to block the inhibitory reaction of the enzyme with the bulkier N-substituents-containing inhibitor. In addition, the use of inhibitors bearing a shorter tether resulted in hybrids with a very low catalytic activity in olefin metathesis, while elongation of the tether circumvented the hindering effect of the protein loops, allowing for good yields in ring-closing and cross metathesis.

The development of artificial palladium enzymes for allylic alkylations was studied in Chapter 4 through the use of Pd(NHC)-phosphonate complexes of different tether length. Screening of reaction media and pH and investigation of a phosphine additive were conducted to achieve catalytic activity of the hybrids. The role of the phosphine was revisited to exclude cleavage of the Pd-NHC bond. The hybrids showed low activity towards a symmetric and larger substrate, while an unsymmetrical but smaller substrate was successfully converted. A shift in the linear/branced product ratio for the latter reaction in comparison with the unsupported catalyst was found, constituting the first covalent palladium-protein hybrid with catalytic activity and the first study of regioselectivity with palladium artificial enzymes.

As a conceptual different approach for the study of selectivity, instead of positioning of a catalyst in the relatively exposed active pocket of cutinase, Chapter 5 details the investigation of linking a rhodium catalyst simultaneously to the active site of two units of Cutinase with the aim to develop an encapsulated hybrid catalyst. This study revealed an intrinsic inhibition selectivity issue for the formation of a dimeric hybrid from a racemic difunctional inhibitor and an enantioselective enzyme. The resulting 2:1 enzyme:Rh-NHC hybrid showed quite different physical properties, especially in solubility or aggregation in buffer media, when compared to the monoprotein-metal hybrids developed in previous chapters. Interestingly, although the dimeric rhodium catalyst showed reduced overall activity for the hydrogenation of the same substrates used in Chaper 2, no chemoselectivity was observed inspite of the encapsulated catalyst structure.

Finally, a critical summary of the hybridization method discussed in this thesis is presented, highlighting the advances and providing suggestions for further exploration of the means to confer selectivity in these and related artificial metalloenzymes.

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The important thing in science is not to obtain new facts
As to discover new ways of thinking about them.
William Lawrence Bragg

2

Lipase active site covalent anchoring of Rh(NHC) catalysts: towards chemoselective artificial metalloenzymes

Abstract

A Rh(NHC) phosphonate complex reacts with the lipases Cutinase and Candida antarctica lipase B resulting in the first (soluble) artificial metalloenzymes formed by covalent active site-directed hybridization. When compared to unsupported complexes, these new robust hybrids show enhanced chemoselectivity in the (competitive) hydrogenation of olefins over ketones.

Based on: M. Basauri-Molina, C. F. Riemersma, M. A. Würdemann, H. Kleijn, R. J. M. Klein Gebbink, *Chem. Commun*. **2015**, *51*, 6792.

Introduction

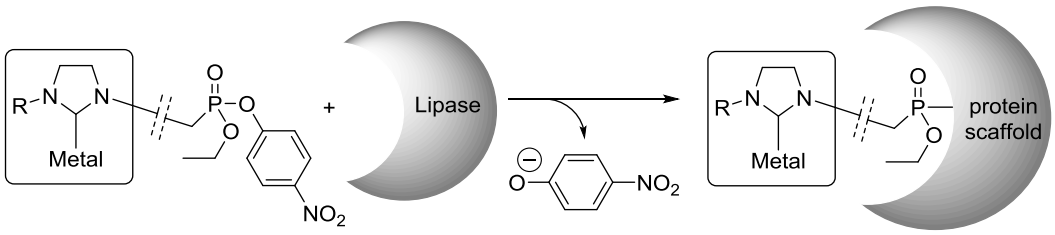
The embedding of synthetic metallocatalysts in protein scaffolds allows for the development of a so-called *2nd coordination sphere* around the metallic center, which can result in catalytic selectivity due to the intrinsically chiral and bulky character of the protein macromolecules, blocking specific sterically demanding transition states in a catalytic reaction.[1] This way, proteins as naturally abundant supports represent an alternative to the development of chiral, bulky or enlarged ligands. Moreover, the solubility of organometallic species, normally restricted to organic media, is expanded by the nature of the protein scaffold, whose macromolecular nature additionally allows for a facilitated separation of the artificial enzymes.

Pioneering work from Whitesides,[2] optimized and extended by Ward,[3] on the supramolecular (Strept)Avidin/biotin system has provided early examples of the development of artificial metalloenzymes. Other hybridization strategies include alkylation of amino acid residues,[4] the use of apo-Myoglobin with metallated artificial cofactors[5] and metal-DNA hybrids.[6] Enantio- and regioselective catalysis can be achieved or further optimized by mutagenic treatment of the proteomic scaffold.[7]

Recently, we have studied the active site-directed (ASD) hybridization strategy using organometallic phosphonate esters that covalently and irreversibly bind to the active serine residue of lipases. In this manner, protein-ECE metallopincer hybrids[8] and supported Ru(Cp)-protein bifunctional catalytic hybrids[9] for lipase labeling and dynamic kinetic resolution, respectively, were developed. ASD hybrids promise robustness of the covalent hybrid for ease of manipulation with reduced leaching, characterization by mass spectrometry, and anchoring of the metallocatalyst in a naturally selective environment (i.e. the lipase active site). Furthermore, phosphonate esters are not restricted to a particular lipase thus potentially allow for the screening of different protein environments.[10]

Our next goal was to further develop the catalytic applications of the ASD method by the use of a versatile ligand (see Scheme 1). To this end, N-heterocyclic carbenes (NHCs) are attractive water- and oxygen-tolerant spectator ligands that present robust σ-donating

monodentate coordination towards a myriad of metallic centers without restriction of the remaining coordination sites for catalytic performance.[11] Functionalization of the N-substituents has allowed for their immobilization[12] and they have been applied recently in the construction of Grubbs-catalyst/protein artificial enzymes,[13] however, to the best of our knowledge, the plausible selective behavior of such hybrids has not yet been documented.



Scheme 1. Covalent inhibition of lipases with *P*-functionalized phosphonate esters towards metal(NHC)-protein hybrids.

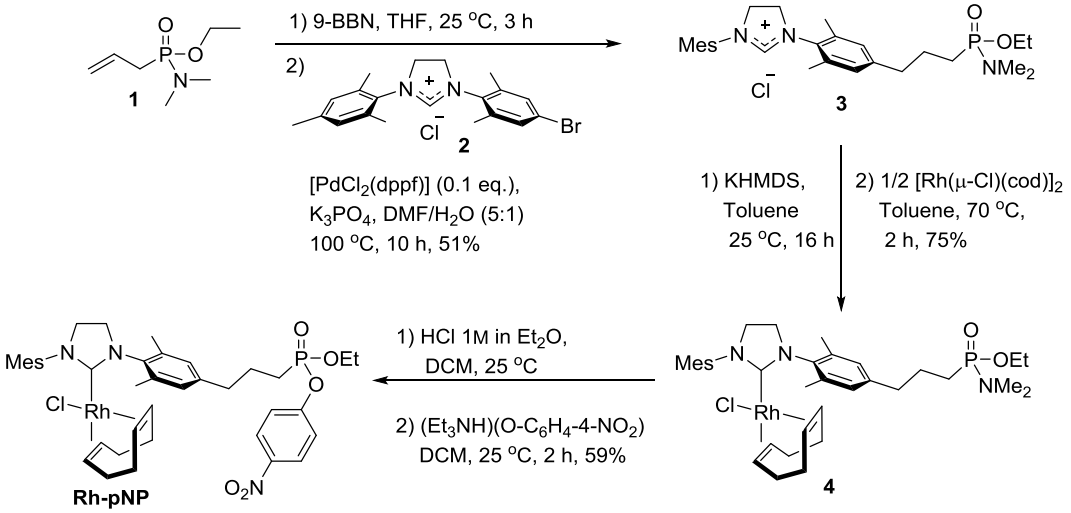
Rhodium species were our first target given their relatively facile synthesis. The absence of this metal in biological systems makes it an attractive moiety to extend enzyme reactivity. The hydrogenation of acetophenone in water with Rh(NHC)-based catalysts has been studied by Hermann and Kuhn and proves the reactivity of such catalytic centers in aqueous media.[14] With other types of ligands, e.g. arenes or bidentate phosphines, the reduction of ketones as well as olefins via transfer hydrogenation with artificial enzymes has been addressed by Ward[15] and Reetz.[16] When monodentate phosphites were addressed by De Vries,[17] bulky protecting groups were needed to avoid its oxidation; yet no selectivity was found in spite of satisfactory catalytic activity. Without the use of protein scaffolds, Noyori's ruthenium catalysts are among the current state-of-the-art catalysts for hydrogenations, which in function of their phosphine ligands achieve excellent enantioselectivities in organic media.[18]

After our successful experience with Cutinase from *fusarium solani pisi*, a lipase that reacts with hydrophobic esters without an initial activation step,[19] as host in the formation of semi-synthetic enzymes,[8,9] we addressed the preparation of Rh(NHC)-protein semi-synthetic hybrids to form (the first) catalytically active Cutinase metallohybrid;[20] and

studied its behavior in hydrogenations to explore the (enantio)selectivity of the active site-embedded Rh(NHC) fragment.

Results

A phosphonate cofactor was designed for an orthogonal metal-NHC-protein orientation. Allylphosphonamidate **1** and brominated imidazolinium salt **2** were synthesized and cross-coupled to form N-tethered NHC ligand **3**. Deprotonation with the non-nucleophilic base potassium hexamethyldisilazide (KHMDS), followed by treatment with $[Rh(\mu\text{–}Cl)(cod)]_2$ (cod = cycloocta-1,5-diene) led to rhodium compound **4** as a mixture of isomers. (Scheme 2, see below). Next, the dimethylamide group was substituted with *p*-nitrophenolate (pNP) towards complex **Rh-pNP** (Scheme 2). The mono *p*-nitrophenyl ethyl *P*-propylphosphonate motif used here has proven an effective inhibitor of Cutinase and CalB,[8,9,21] in contrast to dinitrophenyl phosphonates, which can lead to slow hydrolysis of the phosphorous-serine bond.[16b] Distinctive chemical shifts of the *P*-nucleus in NMR for **1** through **Rh-pNP**, facilitate its characterization in combination with mass spectrometry (for details on synthesis and characterization, see the Experimental Section).



Scheme 2. Synthesis of lipase inhibitor **Rh-pNP.**

Next, cutinase was treated with an excess of **Rh-pNP** for the formation of the hybrid. A dialyzed and denatured aliquot (10% formic acid) was analyzed by ESI-MS, showing complete conversion of Cutinase and formation of the desired **Rh-cut** hybrid (Figure 1, top). Secondary peaks with considerably lower intensity were also observed. These originate from minor impurities in **Rh-pNP**, which due to their smaller size have a high inhibitory competence (see Experimental Section), and do not arise from decomposition of the hybrid. Also, a single hybridization stoichiometry of 1:1 was found, which discarded the association of **Rh-pNP** with the four cysteine (all in cystine form) and six lysine residues at the exterior of the enzyme. In addition, no residual hydrolytic Cutinase activity was found when *p*-nitrophenyl butyrate (pNPB) was treated with **Rh-cut**, leading to a hydrolysis of pNPB only by the buffer, again confirming full inhibition of Cutinase (Figure 1, bottom). These observations demonstrate the covalent character and single site hybridization in forming **Rh-cut**.

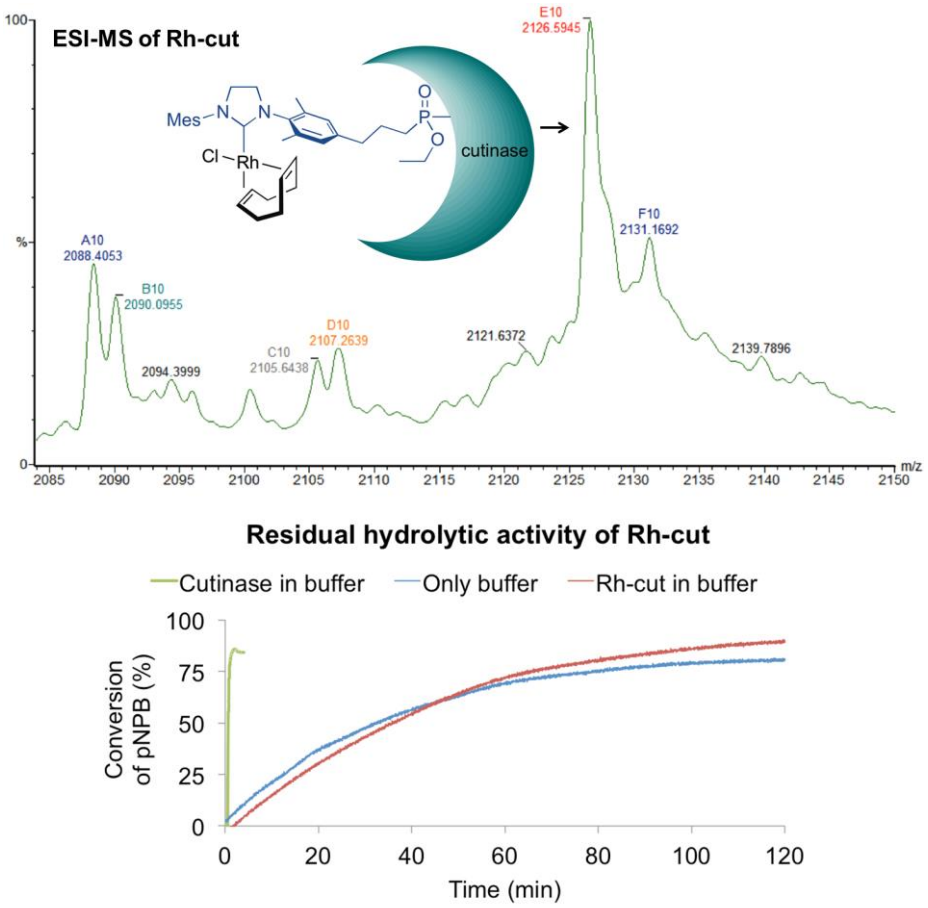


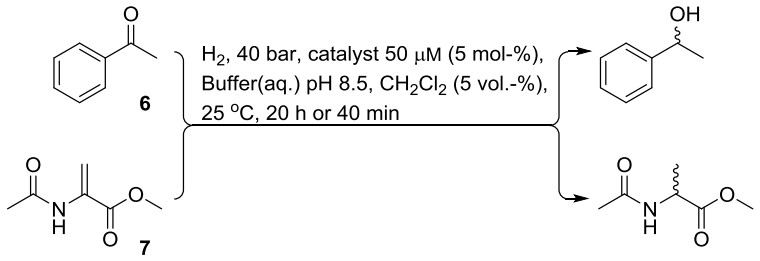
Figure 1. ESI-MS analysis of Rh-cut hybrid (top, $[M]^{10+}$ calc.: 2127.43, found: 2126.59) and the determination of residual hydrolytic activity of cutinase (bottom).

The catalytic performance of **Rh-cut** was evaluated in the hydrogenation of acetophenone, a prochiral ketone, under a H_2 atmosphere (40 bar) at room temperature for 20 h in aqueous biphasic conditions with CH_2Cl_2 (5%, *v/v*) in TrisHCl buffer at pH 8.5. The organic solvent was chosen to promote the interaction of the protein with organic substrates; CH_2Cl_2 also has a low acidity and low coordination behavior with organometallics. At these conditions no product was observed. The same was observed when the non-supported catalyst analog [RhCl(SIMes)(cod)] **5** (where SIMes = 1,3-dimesityl-4,5-dihydroimidazolin-2-ilydene) was used (Table 1, Entries 1 and 2). When the reaction with **5** was not buffered, 1-phenylethanol was produced in 12% yield and this significantly improved to a yield of 90% using a NaH_2PO_4/Na_2HPO_4 buffer at pH 8.5 (Table 1, entries 3 and 4). The difference in conversion using different buffers is attributed to the interference of the high concentration of chlorides of the TrisHCl buffer with the Rh center. Accordingly, using phosphate buffer, **Rh-cut** proved to be an active artificial enzyme for the hydrogenation of acetophenone, albeit at a lower yield of 27% (Table 1, Entry 5).

Next, we investigated the hydrogenation of a prochiral olefin, methyl 2-acetamidoacrylate, using **5** and Rh-cut under equivalent conditions as the previous reaction and found excellent yields with both catalysts towards the desired methyl acetylalaninate product (Table 1, Entries 6 and 7).

Although a clear decrease of activity of the Rh(NHC) motif by its anchoring in the protein was found for the hydrogenation of the ketone, hydrogenation of the olefin did not suffer from a decrease in product formation. This difference in the influence of the protein could originate from a limited approach of acetophenone to the metallic center brought forward by the protein surroundings, or by dative interactions towards the metal center, e.g. from N and O donors reducing the oxophilicity of the catalyst; in these scenarios promoting the formation of the alkane from the olefin but hampering the formation of the alcohol from the ketone.

Table 1. Catalytic hydrogenation of acetophenone **6** and methyl 2-acetamidoacrylate **7** with catalysts **5**, **Rh-cut** and **Rh-calb**.[a]



Entry	Catalyst	Substrate	Buffer	Product formation[b]
1	**Rh-cut**	**6**	TrisHCl	0%
2	**5**	**6**	TrisHCl	0%
3	**5**	**6**	No buffer	12%
4	**5**	**6**	Phosphate	90%
5	**Rh-cut**	**6**	Phosphate	27%
6	**5**	**7**	Phosphate	>99%
7	**Rh-cut**	**7**	Phosphate	>99%
8	**Rh-calb**	**7**	Phosphate	>99%
9	**Rh-calb**	**6**	Phosphate	0%
10	**5**	**6** + **7**	Phosphate	71%, 93%[c]
11	**Rh-cut**	**6** + **7**	Phosphate	15%, 78%[c]
12[d]	**5**	**6** + **7**	Phosphate	15%, 70%[c]
13[d]	**Rh-cut**	**6** + **7**	Phosphate	0%, 63%[c]

[a]Reactions carried out with H_2 (40 bar) at room temperature for 20 h. [b]Determined by chiral GC and GC-MS. [c]Yield for 1-phenylethanol and methyl acetylalaninate respectively. [d]Reaction stopped at 40 min only.

Rhodium catalysts are known to show higher catalytic rates in the hydrogenation of olefins than in the hydrogenation of ketones.[22] Hence, the difference in yield between 1-phenylethanol and methyl acetalaninate by **Rh-cut** does not show by itself a selectivity gain derived from the hybridization, but the comparison of the yields achieved in both transformations between the unsupported catalyst **5** and the **Ru-cut**

hybrid does show a larger influence of the protein over the ketone hydrogenation. In none of the reactions any enantioselectivity in product formation was observed.

The latter observations encouraged us to investigate the anchoring of the rhodium catalyst in a different protein environment. Candida antarctica lipase B (CalB), a lipase with similar exposure of the active site to solvent as Cutinase but at a deeper location,[23] was chosen given our experience on its successful inhibition with metal-functionalized phosphonate esters.[9] A similar methodology as for the previous inhibition was followed resulting in complete inhibition of this enzyme by a ten-fold excess of **Rh-pNP** towards the 1:1 metalloprotein **Rh-calb**. Treatment of pNPB with this hybrid showed no residual hydrolytic activity of CalB according to the release profile of pNP (see the Experimental Section). Successful use of a single inhibitor with different enzymes shows the versatility of the ASD method in protein scope applicability.

Studying **Rh-calb** in catalytic hydrogenations again showed no product enantioselectivity. On the other hand, the difference in reactivity between acetophenone and methyl 2-acetamidoacrylate was larger than with the **Rh-cut** analog. The effect of CalB over the Rh(NHC) motif seemed more pronounced allowing for complete blocking of the ketone in contrast to quantitative hydrogenation of the olefin (Table 1, Entries 8 and 9), suggesting full chemoselectivity by this artificial enzyme.

In order to obtain more insight in the apparent chemoselectivity gained with the hybrids, we performed a series of competition experiments using both substrates in the reaction mixture with **Rh-cut** and **5** (Table 1, Entries 10 and 11). The product yields were slightly lower than in the separate reactions. This decrease is attributed to the distribution of the catalyst's turnover over the two substrates. The olefin was hydrogenated in 93% and 78% by **5** and **Rh-cut**, respectively; in the same manner, the reduction of the ketone yielded 71% and 15%. These results again show an accentuated discrimination of the ketone when the metalloenzyme is used (Figure 2), supporting that the protein backbone increases the difference between the reaction rates of the susbtrates. At shorter reaction times (40 min, Table 1, Entries 12 and 13)

the ketone was reduced in some 15% by **5**, whereas **Rh-cut** afforded no ketone reduction at all (at 63% yield of olefin reduction product), which further supports the observed protein-induced chemoselectivity. The group of Lu has previously reported chemoselectivity when Mn-protein artificial enzymes prevented consecutive overoxydation of thioanisole, as opposed to the unsupported catalyst.[24] Our current study shows a change in reactivity of different chemical functionalities in different substrates.

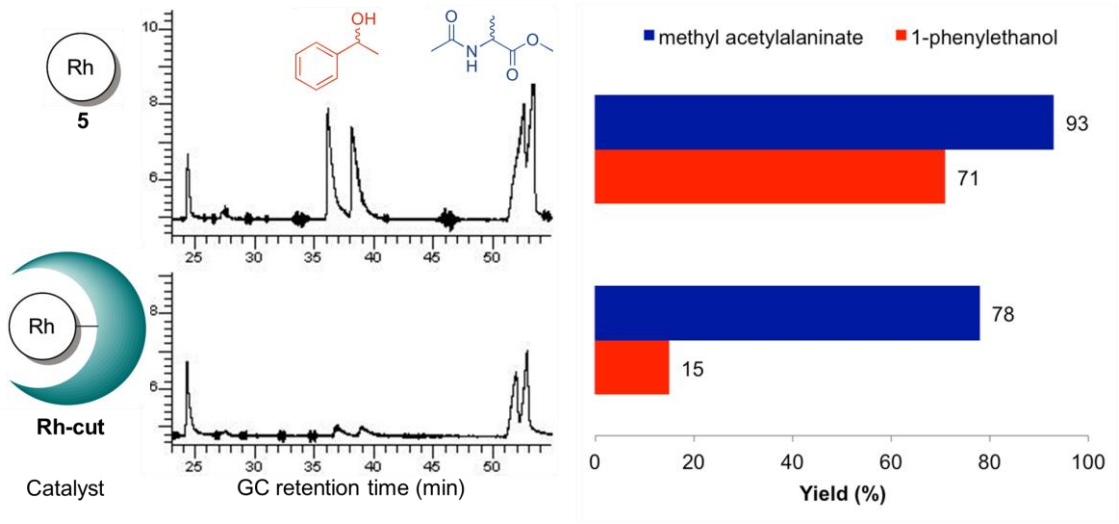


Figure 2. Reaction outcome of the competitive hydrogenation of acetophenone vs. methyl 2-acetamidoacrylate (right) and the corresponding chiral GC chromatograms (center) comparing catalysts **5** [Rh(cod)(SIMes)Cl] and **Rh-cut** (left).

Conclusions

In this work we have demonstrated for the first time catalytic activity for a soluble artificial metallo-enzyme based on the ASD inhibition of lipases. The lipase hybrids reported here catalyze the hydrogenation of the olefin methyl 2-acetamidoacrylate in excellent yields and ambient temperature but show a protein-induced discrimination in the hydrogenation of the ketone acetophenone. The more sterically demanding active site of CalB as anchoring site resulted in exclusive hydrogenation of the olefin

by the corresponding hybrid. This complete chemoselectivity was achieved as well by the cutinase derived hybrid in shorter reaction times.

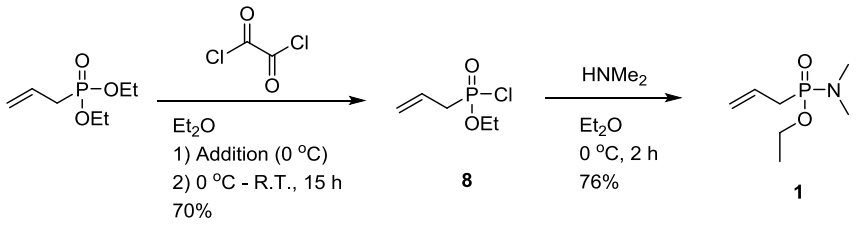
While excellent chemoselective hydrogenation catalysts, preferring either ketone or olefin reduction, have been developed for application in organic synthesis,[18] our current results represent, to the best of our knowledge, the first example of chemoselectivity in reactions catalyzed by artificial metalloenzymes, thereby extending the selectivity repertoire of this specific class of catalysts. These findings may lead to the development of more advanced catalytic tools for the selective conversion of a target substrate in a complex mixture of substrates, which is of interest to the fields of systems catalysis and synthetic biology.

Experimental Section

Materials and methods. Cutinase N172K was obtained from Novonordisk® with a calculated exact mass of 20603.1. Dry acetonitrile, diethyl ether, hexane and toluene were obtained from a MBraun MB SPS-800 solvent purification system; dichloromethane (CH_2Cl_2) and tetrahydrofurane (THF) were dried by distillation from $CaCl_2$ and sodium/benzophenone, respectively and stored over 4 Å molecular sieves. Tris(hydroxymethyl)aminomethane buffer (Tris-HCl) and NaH_2PO_4/Na_2HPO_4 buffer were prepared in degassed Milli-Q water and stored in Schlenk flasks. Other solvents, CalB and reagents were purchased from commercial sources and used without further purification. ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded at 298 K with a Varian AS 400 MHz NMR spectrometer at 400, 100 and 81 MHz, respectively. Chemical shifts are reported in ppm and referenced against the residual solvent signal. UV-Vis spectra were recorded with a Varian Cary50 Scan UV-Vis spectrometer. Chiral GC analyses were performed with a Perkin Elmer Gas Chromatograph AutoSystem XL equipped with an Agilent Cyclodex B 0.25 micron column and flame ionization detector. GC-MS analyses were performed with a Perkin Elmer gas chromatograph Clarus 680 equipped with a acolumn PE Elite 5MS and coupled to a Perkin Elmer mass spectrometer Clarus SQ 8T with EI ionization. Electrospray Ionization (ESI-TOF) mass spectra of chemical products were

recorded with a Waters LCT Premier XE KE317 Micromass Technologies spectrometer; mass spectrometry of protein and hybrid products (calculated as $[M]^{n+}=(M+n)/n$) were performed in positive ion mode using the previous apparatus and a Waters Synapt HD mass spectrometer (ESI-Q-IM-TOF) equipped with a Z-spray nanoelectrospray ionization source. Ultrafiltration dialysis of proteomic samples was performed with Vivaspin 6® tubes, 10,000 M.W. C.O. (PEG membrane). All reactions were carried out under standard Schlenk techniques and under inert conditions with N_2.

Synthesis of ethyl P-allyl-N,N-dimethylphosphonamidate 1

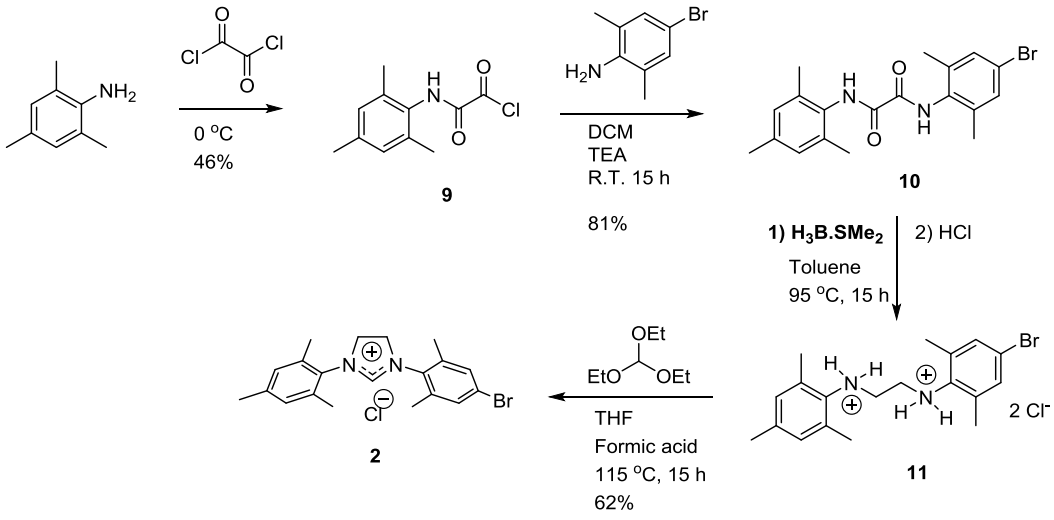


Synthetic procedure for chlorophosphate 8. Following an adapted literature procedure,[8a] diethyl allylphosphonate (10 mL, 58 mmol) was dissolved in 40 mL dry CH_2Cl_2. Slowly oxalyl chloride (15 mL, 176 mmol) was added to the reaction mixture. The reaction was stirred for 21.5 hours. After which it was refluxed for 1 h. All volatiles were removed by vacuum (70% yield). ^{1}H NMR, $CDCl_3$, 400 MHz, δ: 5.75 (m, CH, 1H), 5.28 (m, $=CH_2$, 2H), 4.26 (m, 4H, CH_2O), 2.90 (dd, ^2J(P)=20 Hz, ^3J(CH)=8 Hz, PCH_2, 2H), 1.33 (t, $^3J(CH_2)$ = 8 Hz, CH_3, 3H); ^{13}C NMR, $CDCl_3$, 100 MHz, δ: 125.2 (d, ^2J(P)=13 Hz, CH), 122.1 (d, ^3J(P) = 16 Hz, $=CH_2$), 63.5 (d, ^2J(P) = 9, CH_2O), 38.9 (d, ^1J(P) = 122 Hz, PCH_2), 15.8 (d, ^3J(P) = 7 Hz, CH_3); ^{31}P, $CDCl_3$, 81 MHz, δ: 39.2

Synthetic procedure for phosphonamidate 1.[8a] The residue of the previous reaction was dissolved in diethylether. Dimethylamine gas was condensed in a Schlenk flask submerged in liquid N_2, about 10 mL (355 mmol) was added to the reaction mixture. The resulting reaction produced a large amount of gas and white solid. The reaction mixture was left stirring overnight, after which it was filtered and the residue was washed with dry diethylether (2x20 mL). The filtrate was concentrated on a rotational evaporator and the crude product was obtained as an orange liquid. The product was purified by reduced pressure distillation (50 °C, 0.04 mbar). The product is collected as a colourless oil (76% yield). ^{1}H NMR, $CDCl_3$,

400 MHz, δ: 5.76 (m, =CH, ^{1}H), 5.10 (m, =CH_2, 2H), 3.90 (dm, CH_2-OP, 2H), 2.63 (d, ^{3}J(P) = 8 Hz, PN-$(CH_3)_2$, 6H), 2.5 (m, *p*-CH_2, 1H), 1.24 (t $^{3}J(CH_2)$ = 6.8 Hz, CH_3, 3H); ^{13}C NMR, $CDCl_3$, 100 MHz, δ: 128.2 (d, ^{3}J(P) = 10 Hz, =CH_2), 119.2 (d, ^{2}J(P) = 13 Hz, =CH), 59.4 (d, ^{2}J(P) = 7 Hz, PO-CH_2), 36.4 (d, ^{2}J(P) = 4 Hz, N$(CH_3)_2$) 31.1 (d, ^{1}J(P) = 120 Hz, *p*-CH_2), 16.2 (d, ^{3}J(P) = 7Hz, CH_3); ^{31}P, $CDCl_3$, 81 MHz, δ: 31.66.

Synthesis of 1-mesityl-3-(4-bromo-2,5-dimethylphenyl)-4,5-dihydroimidazolinium chloride 4.



Synthetic procedure for anilide 9. Following a modification of a protocol previously described,[25] oxalyl chloride (15 mL, 176 mmol) was cooled while stirring to 0 °C using an ice bath. 2,4,6-Trimethylaniline (4.9 mL, 35 mmol) was dissolved in dry DCM (30 mL) and was slowly added. The resulting mixture was stirred overnight at room temperature after which the volatiles were removed under vacuum. Diethylether (100 mL) was added and the yellowish suspension filtered. The yellow filtrate was condensed under vacuum and the remaining solid was washed with hexanes. The suspension was filtered and the white solids collected and dried overnight under vacuum (46% yield). ^{1}H NMR, $CDCl_3$, 400 MHz, δ: 7.88 (bs, N-H, 1H), 6.86 (s, Ar-H, 2H), 2,22 (s, Ar-CH_3, 3H), 2.12 (s, Ar-CH_3, 6H); ^{13}C NMR, $CDCl_3$, 100 MHz, δ 167.8 (CClO), 151,4 (CNO), 137.3, 133.6, 128.3, 127.9 (Ar), 19.9, 17.2 (Ar-*C*).

Synthetic procedure for diamide 10. Following the synthesis of Gilbertson and Xu,[26] the product of the previous reaction (2.5 g, 11 mmol) was dissolved in dry DCM (35 mL) and then

cooled to 0 °C while stirring. To this a mixture of 4-bromo- 2,6-dimethylaniline (2.3 g, 11 mmol), triethylamine (1,5 mL, 11 mmol) and dry DCM (10 mL) was slowly added after which the resulting reaction mixture was stirred overnight. Diethylether was added and the solids were collected by filtration. The solids were dissolved in DCM, and the resulting suspension was filtered yielding the first fraction of the product, which was washed with ether. The filtrate was washed with water, then dried over $MgSO_4$. The solvent was removed and the residue was washed with a 10% DCM in diethylether solution. The suspension was filtered and the residue collected as the second fraction of the product. The fractions were combined and dried under vacuum. The product was collected as a white solid (81% yield). ^{1}H NMR, $CDCl_3$, 400 MHz, δ: 8.69, 8.75 (bs, N-H, 1H), 7.21, 6.87 (s, Ar-H, 2H), 2.23 (s, Ar-CH_3, 3H), 2.18, 2.16 (s, Ar-CH_3, 6H); ^{13}C NMR, $CDCl_3$, 100 MHz δ: 158.2, 157.9 (CNO), 137.7, 137.1, 134.0, 131.5, 131.2, 129.5, 121.4 (Ar), 20.9, 18.3 (Ar-CH_3).

Synthetic procedure for diammonium salt 11.[3] The previous product (2.5 g, 6,5 mmol) was suspended in 50 mL dry and degassed toluene. Borane methyl sulphide complex (2.8 mL, 30 mmol) was added. The reaction mixture was stirred overnight at 95 °C, yielding a clear yellow solution. After the reaction mixture cooled down to room temperature a 3M HCl solution was added. The solution started to bubble and solids precipitate. HCl was added until the pH was 2. The mixture was then left stirring for 48 h, after which it was filtered and the residue was first washed with water, then with a 10% acetone in diethylether solution. The product was used directly for the next step. ^{1}H NMR, DMSO, 400 MHz, δ: 7.15, 6.89 (s, Ar-H, 2H) 4.30 (bs CH_2/NH_2^+) 2.43, 2.23, 2.06 (s, Ar-CH_3); ^{13}C NMR, $CDCl_3$, 100 MHz, δ: 143.1, 138.7, 133.1, 132.2, 131.5, 130.9, 115.2 (Ar), 50.1, 44.3, (CH2) 20.7, 18.7, 18.2 (Ar-CH_3).

Synthetic procedure for bromo imidazolinium salt 2. The previous product was dissolved in triethyl orthoformate (20 mL, 152 mmol), 3 drops of concentrated formic acid were added and the suspension was stirred at 125 °C, at which it becomes a clear yellowish brown solution, then the reaction is left to cool. A large amount of hexanes were added (100 - 150 mL) and the mixture was stirred for 3 h. The resulting suspension was filtered and the residue washed with more hexanes. The product was collected as a white solid (62% yield over the last two steps). To our knowledge, there is no previous report of this compound. ^{1}H NMR, DMSO, 400 MHz, δ: 9,29 (s, N_2C-H, 1H), 7.52 (s, Ar-H, 2H), 7.06 (s, Ar-H, 2H), 4.49 (CH_2, 2H), 3.41 (m, CH_2, 2H) 2.39 (s, Ar-CH_3, 6H), 2.34 (s, Ar-CH_3, 6H), 2.27 (s, Ar-CH_3, 3H); ^{13}C NMR,

$CDCl_3$, 100 MHz, δ: 160.7 (N_2CH), 140.0, 139.1, 135.8, 133.3, 131.8, 131.3, 129.9 123.3 (Ar), 56.4, 51.4, (CH_2CH_2), 21.0, 19.0, 17.6 (Ar-CH_3) ESI-MS [M-Cl]: calc. 371.1117, found: 371.1155.

Synthesis of ethyl *P*-(3-(4-(3-mesityl-4,5-dihydroimidazolinium)-3,5-dimethylphenyl)propyl)-*N,N*-dimethylphosphonamidate chloride 3. In the glovebox, [9-BBN]$_2$ (0.15 g, 0.63 mmol of dimer) was weighed, dissolved in THF (15 mL) and added to a solution of the allyl phosphonamidate **1** (0.19 g, 1.05 mmol) also in THF (5 mL) in a Schlenk flask at room temperature and stirred for 20 h. The reaction mixture was concentrated in vacuo, closed and removed from the glovebox. To this hydroboronated phosphonamidate, a solution of the bromo imidazolinium salt **2** (0.39 g, 0.95 mmol) and [Pd(dppf)Cl_2]·CH_2Cl_2 catalyst (54 mg, 67 μmol) in DMF (38 mL) was added, followed by $K_3PO_4.H_2O$ solid (0.22 g, 1.06 mmol). The mixture was stirred under N_2 and 2 mL of degassed water was added. A reflux condenser adapted and the reaction was left stirring at 100 ^oC for 21 h. At the end of this time, the reaction was cooled down to room temperature and concentrated under rotary evaporator and then under high vacuum (10^{-6} mbar at 50 C for 1.5 h). The residue was redissolved in CH_2Cl_2 (15 mL) and the solids filtered off. The product was purified by column chromatography using silica gel with CH_2Cl_2/MeOH (9:1, R_f = 0.5) eluent as a dark yellow very viscous oil (288 mg, 60% yield). ^{1}H NMR, $CDCl_3$, 400 MHz, δ: 8.90 (s, NCHN, 1H), 6.94 (s, ArH, 4H), 4.62 (s, NCH_2CH_2N, 4H), 3.90 (dm, CH_2O, 2H), 2.64 (d ^3J(P) = 8 Hz, NCH_3, 6H), 2.61 (m, $ArCH_2$, 2H), 2.39, 2.38 (s, $ArCH_3$, 12H), 2.27 (s, $ArCH_3$, 3H), 1.84 (m, CH_2P, 2H), 1.63 (m, CH_2CH_2P, 2H), 1.25 (t, ^3J = 7 Hz, CH_2CH_3, 3H); ^{13}C NMR, $CDCl_3$, 100 MHz, δ: 159.2 (NCHN), 144.1, 140.7, 135.2, 134.9, 130.6, 130.1, 130.0, 129.5 (Ar), 59.2 (d ^2J(CH_2) = 6 Hz, CH_2O), 52.3 (NCH_2CH_2N), 36.1 (m, NCH_3), 24.1 (d, ^1J(C-P) = 10 Hz, CH_2P), 23.7, (d, CH_2CH_2P), 23.6 ($ArCH_2$), 21.0, 18.2 ($ArCH_3$), 16.3 (CH_2CH_3); ^{31}P, $CDCl_3$, 81 MHz, δ: 36.22. ESI-MS $[M\text{-}Cl^-]^+$ calc.: 470.2931. Found: 470.2970.

Synthesis of RhCl(cod)(NHC)phosphonamidate 4. The phosphonamidate-SIMes product (**3**) (42.6 mg, 0.084 mmol) was placed in a Schlenk flask and brought into the glovebox. Toluene (10 mL) was added and the mixture stirred, followed by slow addition of KHMDS 0.5 M in toluene (0.18 mL, 0.09 mmol); the heterogeneous mixture was stirred at room temperature for a total of 20 h and to the new dark orange solution, [Rh(μ–Cl)(cod)]$_2$ (17.8 mg, 0.036 mmol) in toluene (5 mL) was slowly added. The mixture was stirred for 0.5 h at room temperature and then for 2 h at 70 °C outside of the glovebox with a reflux condenser under

N_2 atmosphere. After this time, the reaction was cooled down to about 10 °C and filtered via cannula. The solvent was removed with vacuum and the product purified by column chromatography with silica and acetone as the eluent (R_r = 0.65). The product was obtained as a dark yellow to orange viscous oil (38.6 mg, 75%). ^{1}H NMR, CD_2Cl_2, 400 MHz, δ: 7.03, 7.01, 7.00 (s, ArH, 4H), 4.48, 4.36 (s, CH_{cod}, 1H), 4.00 (m, C*H*$_2$CH$_3$, 2H), 3.84, 3.83(3x) (NCH_2CH_2N, 4H) 3.51, 3.45 (d, CH_{cod}, 2H), 2.69 (s, $ArCH_2$, 2H), 2.66, 2.63 (d, NCH_3, 6H), 2.56, 2.57 (d, $ArCH_3$), 2.34 (s, $ArCH_3$), 1.78 (m, CH_2P, 2H), 1.73 (m, C*H*$_2$CH$_2$P, 2H), 1.51, 1.49 ($CH_{2(cod)}$, 2H), 1.27, 1.26 (t, CH_2CH_3, 6H), 1.22 (d, $CH_{2(cod)}$, 2H); ^{13}C NMR, CD_2Cl_2, 100 MHz, δ: 212.4, 212.2 (d, ^1J(C-Rh) = 48 Hz, NCN), 141.3, 141.4, 138.4, 138.3, 138.2, 138.1, 138.0, 137.7, 137.7, 137.0, 136.5, 136.4, 135.6, 135.6, 135.4, 135.4, 129.5, 129.5, 129.1, 129.1, 129.0, 128.5, 128.4, 128.0, 127.9, 127.9, 127.8 (Ar), 96.8, 96.7, 96.6, 96.2 (cod), 68.9, 68.7 (cod), 67.7, 67.6 (cod), 58.9 (d, $POCH_2$), 58.8 (d, $POCH_2$), 51.6, 51.5 (NCH_2CH_2N), 36.2. 35.8 (NCH_3), 32.5 (cod), 28.13 (cod), 25.3, 24.7 (d, CH_2P), 24.0 (*C*H$_2$CH$_2$P), 20.7, 20.5, 20.4, 18.26 ($ArCH_3$), 16.2, 16.1 (CH_2CH_3); ^{31}P, CD_2Cl_2, 81 MHz, δ: 36.95. ESI-MS $[M\text{-}Cl^-]^+$ calc.: 680.2847, found: 680.2859.

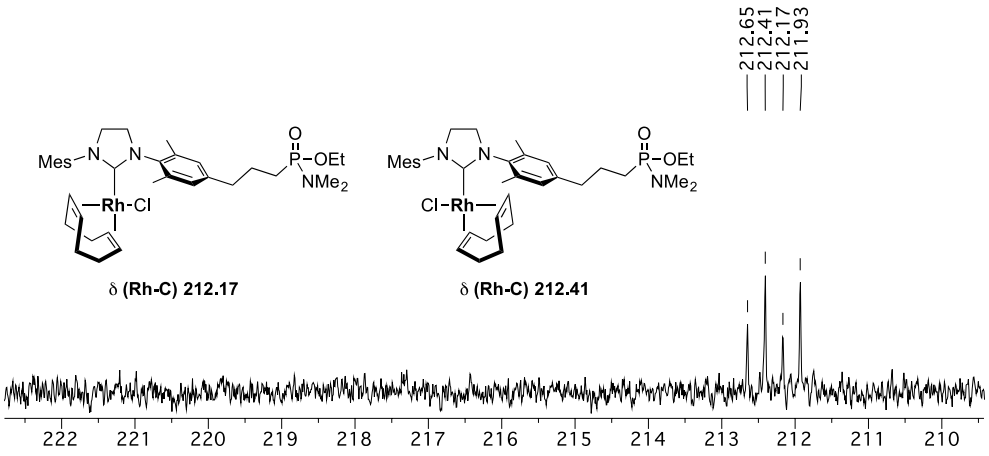
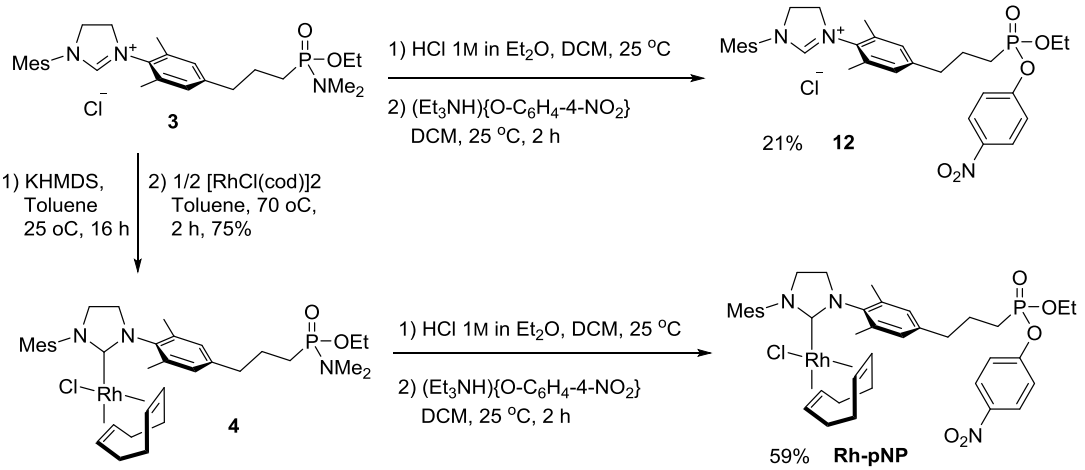


Figure 3. Low field ^{13}C NMR of **4** showing the Rh-C coupling of two isomers.

For both isomers: $J_{C\text{-}Rh}$ = 48 Hz.

Synthesis of lipase inhibitors



Synthesis of non-metallated inhibitor 12. To a solution of the phosphonamidate-SIMes product **3** (92 mg, 0.18 mmol) in CH_2Cl_2 (5 mL), hydrogen chloride (0.9 mL, 1 M solution in diethyl ether, 0.9 mmol) was added and the mixture stirred for 2 h at room temperature. The reaction was concentrated in vacuo (an aliquot of the chloro phosphate product shows a single signal at 43.80 ppm in ^{31}P NMR) and CH_2Cl_2 (5 mL) were added. A pre-stirred solution of *p*-nitrophenol (26 mg, 0.19 mmol) and triethylamine (0.07 mL, 0.5 mmol) in CH_2Cl_2 (2 mL) was added to it and stirred for 3 h at room temperature. Solids were filtered off and the filtrate washed 1 time with aqueous K_2CO_3 1 M and saturated NaCl, collecting the organic fraction and concentrated to give an orange solid, a large portion of the product remained in the aqueous phase (14.7 mg, 21% yield). ^1H NMR, $CDCl_3$, 400 MHz, δ: 9.29 (s, NCHN, 1H), 8.19, 7.37 (d, ^3J=8 Hz, $ArH_{(pNP)}$, 4H), 6.93 (s, $ArH_{(Mes)}$, 4H), 4.54 (s, NCH_2CH_2N, 4H), 4.17 (m, CH_2O, 2H), 2.66 ($ArCH_2$, ^3J=8 Hz, 2H), 2.38, 2.36 ($ArCH_3$, 12H), 2.26 ($ArCH_3$, 3H), 1.95 (m, CH_2P, 2H), 1.91 (b, CH_2CH_2P, 2H), 1.29 (t, ^3J = CH_2CH_3, 3H); ^{13}C NMR, $CDCl_3$, 100 MHz, δ: 160.1 (NCHN), 155.6, 143.2, 140.7, 135.5, 134.9, 130.9, 130.1, 129.4, 125.6, 121.0, 121.0 (Ar) 63.1 (d ^2J(C-P) = 7 Hz, CH_2O), 51.8, 51.8 (NCH_2CH_2N), 29.7 ($ArCH_2$), 26.0 (CH_2CH_2P), 23.6 (d ^2J(C-P)=50 Hz, CH_2CH_2P), 21.0, 18.1, 18.0 ($ArCH_3$), 16.4 (CH_2CH_3); ^{31}P, $CDCl_3$, 81 MHz, δ: 29.47. ESI-MS: Calc. Mass: [M-Cl] 564.26. Found: 564.2628 (100%).

Synthesis of Rh-inhibitor Rh-pNP. RhCl(cod)(NHC)phosphonamide **4** (24.8 mg, 0.035 mmol) was dissolved in CH_2Cl_2 (5 mL), HCl (1 M in diethyl ether, 3.5 mL, 0.35 mmol), was added by syringe through septum and the mixture left stirring for 3 h at room temperature. After this time, the mixture was concentrated in vacuo under N_2, CH_2Cl_2 (5 mL) was added and a pre-

stirred solution of *p*-nitrophenol (4.9 mg, 0.035 mmol) and triethylamine (20 µL, 0.14 mmol) in CH_2Cl_2 (3 mL) was added an stirred for 2 h at room temperature. The reaction was filtered via cannula and concentrated. The product was purified by silica column chromatography using acetone as the eluent (R_f = 0.78) yielding a dark yellow oil (16.8 mg, 59%). ^{1}H NMR, CD_2Cl_2, 400 MHz, δ: 7.00, 6.98 (s, ArH_{Mes}, 4H), 8.09 (^3J=8 Hz, ArH_{pNP}, 4H), 4.35 (b, cod, 2H), 4.19 (m, CH_2CH_3, 2H), 3.86 x2 (s, NCH_2CH_2N, 4H), 3.48, 3.39 (cod, 4H), 2.74 ($ArCH_2$, 2H), 2.55, 2.52 ($ArCH_3$, 6H), 2.34, 2.33, 2.32 ($ArCH_3$, 6H), 2.04 ($ArCH_3$, 3H), 1.73 (m, CH_2P, 2H), 1.50 (m, CH_2CH_2P, 2H), 1.31, 1.30 (m, CH_2CH_3, 3H); ^{13}C NMR, CD_2Cl_2, 100 MHz, δ: 211.61 (d, ^1J(C-Rh) = 48 Hz, NCN), 162.5, 155.5, 155.4, 155.4, 144.7, 141.0, 140.5, 138.6, 138.0, 137.9, 137.2, 136.3, 136.0, 135.4, 129.6, 129.1, 128.6, 128.0, 127.9, 127.9, 125.9, 125.6, 121.0, 121.0, 115.6 (Ar), 97.1, 97.0, 96.9, 96.9 (cod), 68.6, 68.5, 68.2, 68.0 (cod), 63.6, 63.5, 63.4 (CH_2O), 51.6, 51.4 (NCH_2CH_2N), 35.7, 35.5 (cod), 32.7, 32.4 (cod), 28.2, 27.8 (CH_2CH_2P), 25.1 (d, ^1J(C-P)=140 Hz, CH_2P), 23.7 ($ArCH_2$), 20.7 ($ArCH_3$), 19.7, 19.5 ($ArCH_3$), 18.2, 18.1 ($ArCH_3$), 166.2, 16.1 (CH_2CH_3); ^{31}P, CD_2Cl_2, 81 MHz, δ: 30.16. ESI-MS $[M\text{-}Cl^-]^+$ calc.: 774.2538, found: 774.2538.

Synthesis of [Rh(SIMes)(cod)Cl] complex 5. In the glovebox, to a stirred solution of 1,3-dimesityl-4,5-dihydroimidazolinium chloride (93 mg, 0.27 mmol) in toluene (15 mL), KHMDS 0.5 M in toluene (0.54 mL, 0.27 mmol) was slowly added, the flask closed and left stirring at room temperature for 0.5 h. After this time and under stirring, a solution of $[Rh(\mu\text{-}Cl)Cl(cod)]_2$ (66 mg, 0.13 mmol) in toluene (10 mL) was slowly added. The flask was closed and stirred at 70 C for 2 h. After this time, the mixture was cooled down in an ice bath and the solids filtered off. The solvent was evaporated and the product purified by silica gel column chromatography using acetone as the eluent and collecting the dark yellow band. Evaporation of the solvent left an orange-red powder (99 mg, 66% yield). ESI-MS: $[M\text{-}Cl^-]^+$ calc.: 517.2090, found: 517.2090. NMR in agreement with previously reported spectra.[27]

Synthesis of SIMes-cut and SIMes-calb hybrids. A stock solution of the lipase (Cutinase or CalB) prepared from a crystalline sample in Tris-HCl buffer (50 mM, pH 8.5) to give a protein concentration of 30.8 µM was treated with inhibitor **12** (4 mM) at room temperature, so that the initial ratio enzyme/inhibitor was 1:2 for **SIMes-cut** and 1:10 for **SIMes-calb**, final volume 2.5 mL and the buffer concentration 20 mM. The final concentration of protein content was 20 µM. (To determine the initial enzyme concentration, a parallel solution with

the same amount of enzyme powder was dissolved in Milli-Q water and submitted to a nanodrop measurement of absorbance at 280 nm (extinction coefficient calculated from http://web.expasy.org/protparam, protein sequences obtained from uniprot.org database). The mixture was left to stir for additional 24 h at room temperature. Dialysis were conducted in membrane bags or by ultrafast filtration with 10 kDa M.W.C.O. membrane, restoring the volume each time with Mili-Q water and the last time with buffer in Mili-Q water. Hybrids were stored at – 20 ^{o}C.

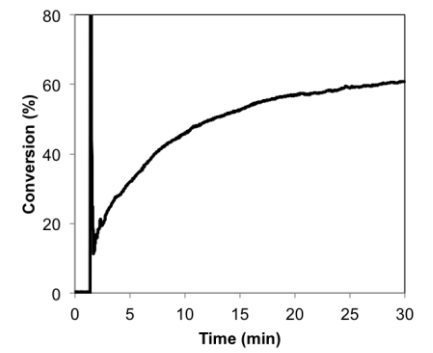
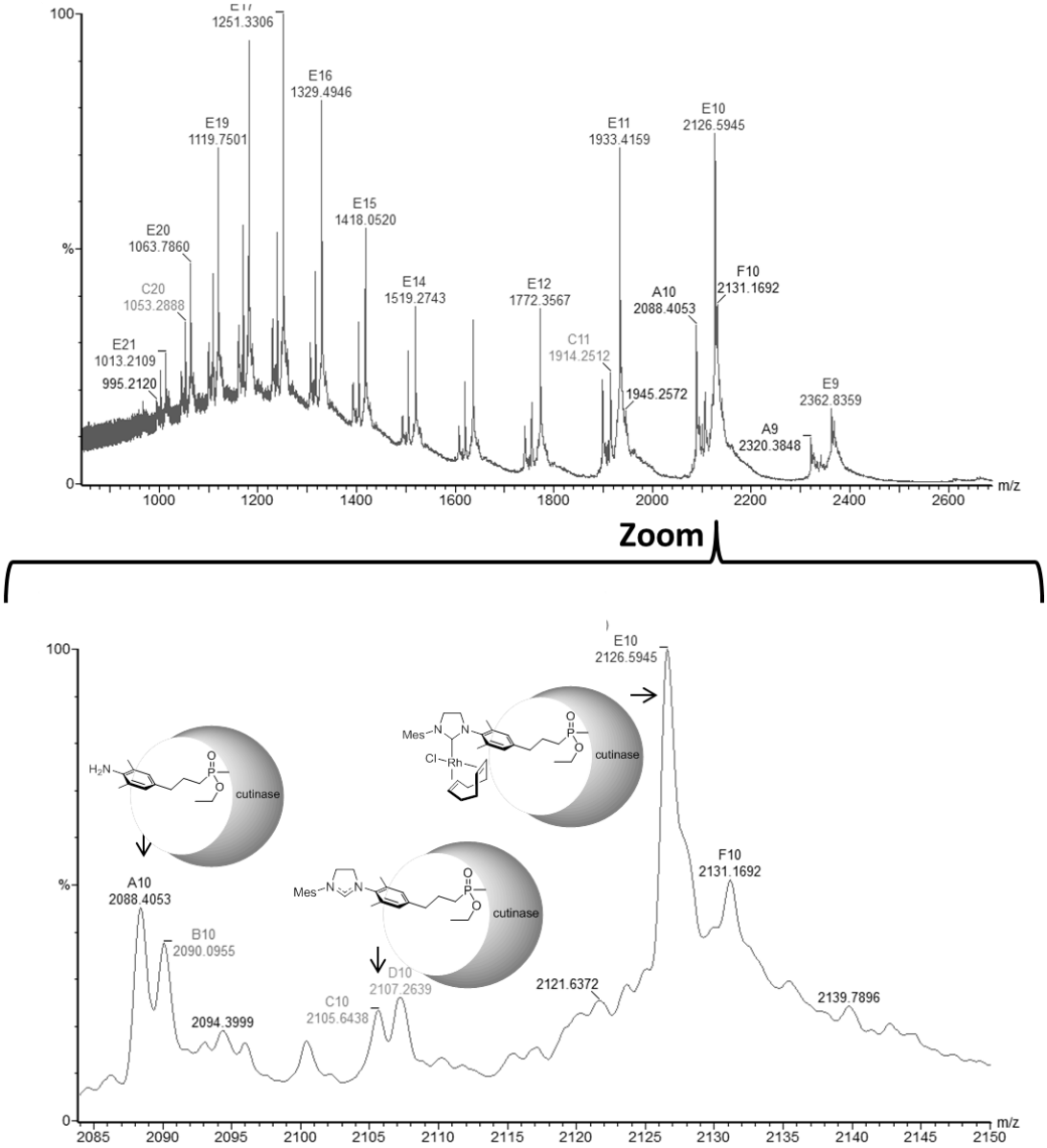


Figure 4. Release of *p*-nitrophenolate (pNP, λ_{max} = 405 nm, ε = 18400 $mol^{-1}cm^{-1}$)[28] during the inhibition of cutinase with **12**.

Synthesis of Rh-cut and Rh-calb hybrids. A similar procedure as for **SIMes-protein** hybrids was applied. To a stirred solution of cutinase or CalB (9.5 mL, 50 μM in degassed Tris-HCl or NaH_2PO_4/Na_2HPO_4 buffer 50 mM, pH 8.5; concentrations determined by similar parallel experiments in Milli-Q water as explained above), inhibitor **Rh-pNP** (3 equivalents with respect to cutinase when buffer = Tris-HCl; or 10 equivalents when phosphate buffer was used and for the synthesis of **Rh-calb**) in degassed dichloromethane (CH_2Cl_2, 0.5 mL, solution was already yellow because of Rh complex) was slowly added via septum with a syringe; the volume of CH_2Cl_2 was 1/19 of the volume of buffer so that it accounted for 5%-vol. in the mixture. The reaction was stirred vigorously at 25 ^{o}C for 24 h. Then the content was transferred to a 250 mL round bottom and placed in rotary evaporator with fast spin at 25 ^{o}C to eliminate the CH_2Cl_2. The solids were filtered off three times and the filtrate was transferred to Vivaspin 6® centrifugation tubes with peg membrane of 10 kDa M.W.C.O. and

centrifuged 3 × 1.5 h at 3500 rpm for dialysis, restoring the volume each time with Milli-Q water and finally to 9.5 mL with buffer 50 mM. The resulting solutions were stored at – 20 °C in degassed vessels. For ESI-MS analyses (Figure 5), an aliquot of 0.5 mL was further dialysed and treated with formic acid for its denaturation before injection.



nH^+	21	20	19	18	17	16	15	14	13	12	11	10	9
Calc.	1013.06	1063.72	1119.70	1181.91	1251.43	1329.64	1418.29	1519.59	1636.49	1772.86	1934.03	2127.43	2363.81
Found	1013.21	1063.79	1119.75	1180	1251.33	1329.50	1418.05	1519.27	1635	1772.36	1933.42	2126.60	2362.84

Figure 5. Extended ESI-MS analysis of **Rh-cut** with the calculated and observed *m/z* values.

Residual activity of SIMes-calb. A cuvette was charged with *para*-nitrophenyl butyrate (pNPB, 1.14 mM, 1.35 mL, 1.5 μmol), the absorbance at 405 nm was set to zero and a measurement of the production of pNP as a function of time was started (see Figure 6). The addition of regents was as follows: At t = 5 min phosphate buffer pH 8.5 (1.35 mL of 100 mM); at t = 15 min **SIMes-calb** (0.3 mL of 50 μM, 0.015 μmol, equivalent to 1 mol-%); at t = 30 min unmodified CalB (0.3 mL of 50 μM, 0.015 μmol, equivalent to 1 mol-%).

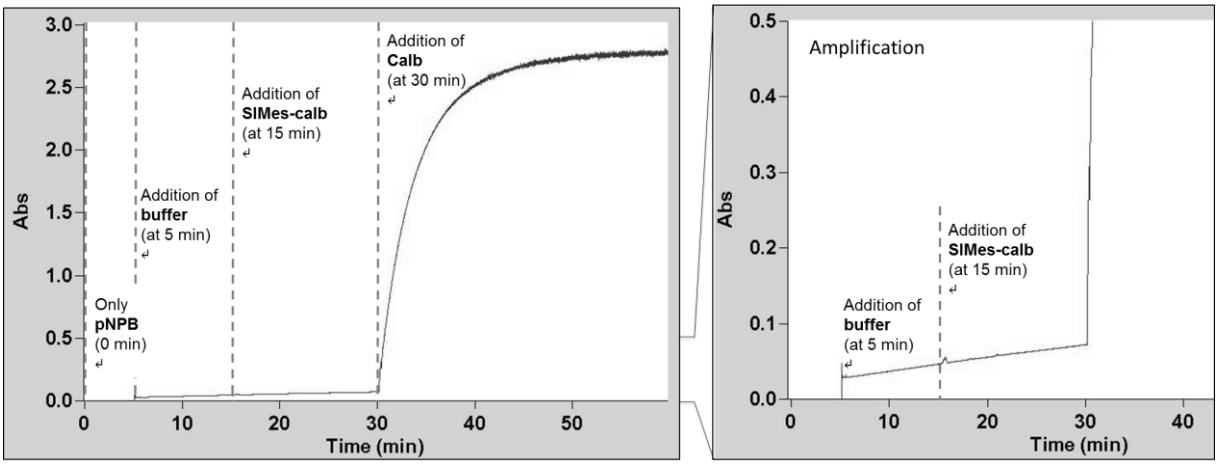


Figure 6. Monitoring of the release of pNP at sequential addition of CalB-containing species. At the addition of **SIMes-calb**, the very slow rate of hydrolysis of pNPB is equal to its hydrolysis only in buffer whereas the rate dramatically increased with the addition of unmodified CalB.

Residual activity of Rh-calb. With the absorbance set to zero the same as the previous procedure, a second cuvette was charged with only **Rh-calb** (0.3 mL of 50 μM, 0.015 μmol, equivalent to 1 mol-%) and phosphate buffer pH 8.5 (1.35 mL of 100 mM). The absorbance at 405 nm started in function of time (see Figure 7). The addition of regents was as follows: At t = 5 min pNPB (1.35 mL of 1.14 mM, 1.5 μmol), at t = 15 min unmodified CalB (Figure 7).

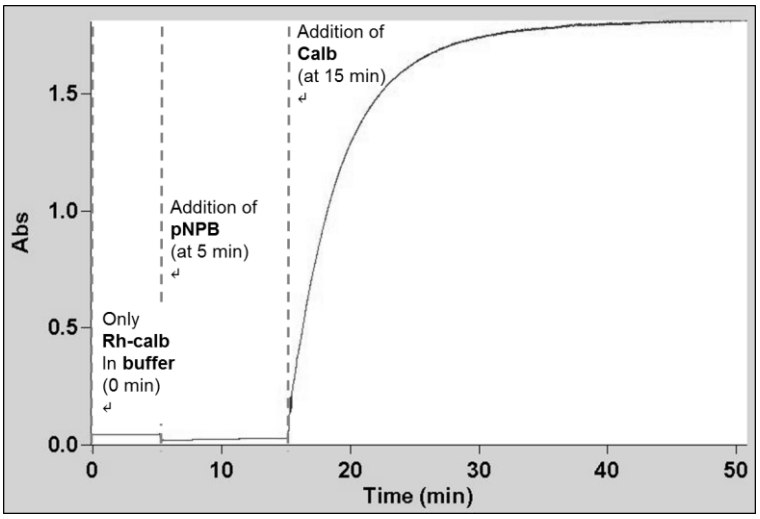


Figure 7. Monitoring of the release of pNP at sequential addition of CalB-containing species. pNPB in the presence of **Rh-calb** and buffer shows a very slow rate of hydrolysis comparable to the hydrolysis in only buffer (see Figure SI-6.1), whereas the rate dramatically increases when CalB is added. **Rh-calb** hybrid absorbs itself at 405 nm. The amount of pNPB is 100 times more than **Rh-calb**; even with higher concentration of the pNPB added, its addition increases the total volume of the system, therefore a decrease of absorbance is observed at 5 min.

Catalytic hydrogenation experiments using catalyst 5. In a typical procedure, a pressurization vessel was charged with substrate (1 mL stock solution of acetophenone and/or methyl 2-acetamidoacetate and 1,2-diphenylethane as internal standard, 4.3 mM, 4.3 μmol each in CH_2Cl_2) and catalyst **5** (1 mL of stock solution 0.21 mM, 0.215 μmol, 5 mol-% in CH_2Cl_2). The solvent was evaporated and the vessel refilled with N_2 and CH_2Cl_2 (0.25 mL). Buffer (4.3 mL, 50 mM) was added to the vessel together with a stirring bar and this was placed in an autoclave chamber. The system was flushed with a gentle stream of H_2 for 1 min, then closed and pressurized under stirring to 40 bar of H_2. After the desired time of reaction, the chamber was opened. To the reaction mixture, CH_2Cl_2 (3 x 5 mL) was added to extract the organic phase and then concentrated to about 0.05 mL and analyzed by chiral-GC and GC-MS, the latter to complement the determination of products identity correlating their ratios (and previous analysis of stock material). Column chromatography and NMR analysis of the reaction mixtures were incompatible due to the low amounts of analytes.

Catalytic hydrogenation experiments using Rh-protein catalysts. In a typical procedure, a pressurization vessel was charged with substrate (1 mL stock solution of acetophenone

and/or methyl 2-acetamidoacetate and 1,2-diphenylethane as internal standard, 4.3 mM, 4.3 μmol each in CH_2Cl_2). The solvent was evaporated and the vessel refilled with N_2, CH_2Cl_2 (0.25 mL). The hybrid (**Rh-cut** or **Rh-calb**, 4.3 mL, 50 μM, 0.215 μmol, equivalent to 5 mol-%) in buffer (50 mM) was added to the vessel together with a stirring bar and this was placed in an autoclave chamber. The system was flushed with a gentle stream of H_2 for 1 minute, then closed and pressurized under stirring to 40 bar of H_2. The resulting mixture had a clear colorless appearance, showing no denaturation of the protein material under the experimental conditions. The reaction and its analyses were equivalent to the proceedings described with catalyst **5**.

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Science is not only a disciple of reason but,
also, of romance and passion.
Stephen Hawking

3

Ring-closing and cross metathesis with artificial metalloenzymes created by covalent active site-directed hybridization of lipases

Abstract

A series of Grubbs-type catalysts bearing lipase-inhibiting functionalities have been synthesized and reacted with the lipase Cutinase leading to the first artificial metalloenzymes for olefin metathesis that present the organometallic fragment bound to the active site of the enzyme host in a covalent fashion. Differences in reactivity as well as accessibility of the active site by the functionalized inhibitor became evident through variations in the anchoring motif and substituents on the N-heterocyclic carbene ligand. Such observations lead to the design of a hybrid active in ring-closing metathesis and the first example of cross metathesis, using *N,N*-diallyl-*p*-toluenesulfonamide and allylbenzene, respectively, in the field of artificial metalloenzymes.

Introduction

Olefin metathesis, the reorganization of alkene moieties into new C-C double bonds, has become an essential tool for the construction of chemicals and polymers from simpler olefinic building blocks. The ring-closing, ring-opening polymerization and cross metathesis reactions promoted by homogeneous transition metal-based catalysts have been largely reviewed.[1] Yet, there are still important efforts to develop methods for the control of selectivity in these reactions.[2] Therein, a great majority of strategies rely on synthetic tools, such as bulky or anionic ligands as well as supports.[3] The option of renewable materials or biological tools for this end represents an attractive alternative strategy due to their abundance and reduced toxicity. Undeniably, biotechnology, with the use or modification of enzymes for the synthesis of demanded commodities as one of its most important goals, has intensely grown in the past decades.[4] Recently, homogeneous olefin metathesis catalysts of the Grubbs type proved to be compatible with protein-based substrates in physiological conditions (in vitro) performing olefin metathesis.[5] This promptly suggested the incorporation of Grubbs-type species, based on ruthenium N-heterocyclic carbenes (NHC), in protein structures to asses the potential promotion of selectivity conferred by the generation of a 2nd coordination sphere,[6] and at the same time to expand the catalytic repertoire of enzymes, forming artificial metalloenzymes for olefin metathesis.[7-10] Along this line, a small number of artificial enzymes for olefin metathesis have been created very recently using different strategies: alkylation of cysteine residues,[7,10] the biotin-(strept)Avidin technology[8] and supramolecular hybridization of an enzyme's active site (Figure 1).[9] Activity in ring-closing metathesis (RCM) and ring-opening metathesis polymerization (ROMP) has been achieved with a range of yields up to that of the parent non-supported catalyst. Modest *E/Z* selectivity in ROMP has also been observed (Figure 1, bottom).[10] These efforts express the interest in the search for new semi-synthetic metalloenzymes for olefin metathesis.

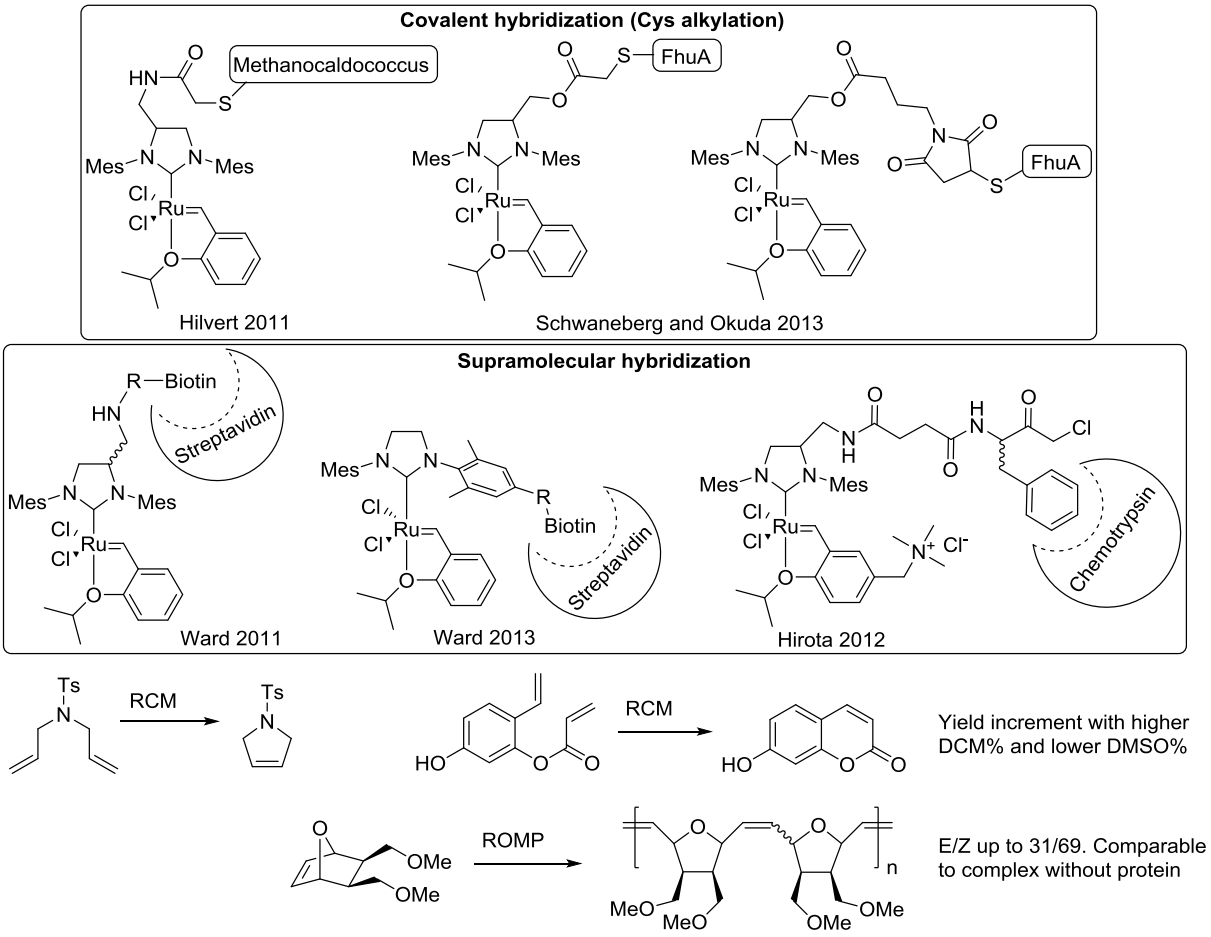


Figure 1. Artificial metalloenzymes for olefin metathesis produced by different strategies (top) and the reactions explored (bottom).

As can be anticipated, the nature of the protein chosen as support for the organometallic catalyst plays a fundamental role in the physical properties of the conjugated construct, i.e. for its solubility in aqueous and other media, its size, aggregation, temperature and pH tolerance, etc. The reactivity and selectivity of the resulting artificial enzyme involves finer interactions between the metallic center and the structural surrounding of the protein residues around it. The latter can allow or hinder the approach of substrates (substrate selectivity), the formation of specific transition states and products (product selectivity), the orientation and configuration of these (e.g. enantioselectivity), or these can hamper the catalyst's activity, having an impact on e.g. its kinetic competence.[11] Because of this, the delicate endeavor of the promotion of catalytic selectivity by the protein without induction of major effects on the metal activity demands subtle tuning of the positioning of the

catalyst in the protein host, while ensuring its robust anchoring for the reproducibility of reactivity.

With a special interest in the development of hybridization strategies, our group has reported a method for lipase active site-directed covalent anchoring of organometallic catalysts in enzymes.[12] A phosphonate ester, functionalized with the organometallic fragment, acts as a suicide inhibitor of the enzymatic family of serine hydrolases and forms a covalent and irreversible bond with the serine residue of the catalytic triad. The phosphonate ligand motif acts as a cofactor, linking the metallic center to the former active site of the enzyme. This method offers a number of interesting features. For instance, the original activity of the enzyme is transformed into that of the incorporated catalyst, although, without loss of the tertiary structure of the protein scaffold; and this catalyst is positioned in the naturally selective pocket of the enzyme, the location with larger potential for the generation of a 2nd coordination sphere. Moreover, covalent and irreversible association between the entities ensures robust anchorage of the catalyst, facilitating the purification, handling and analysis of the hybrid, while diminishing the risk of catalyst leaching. Furthermore, the activity of phosphonate esters is not limited to a single lipase; hence the choice of different lipases is an alternative to the use of mutagenic techniques for the screening of protein scaffolds.

This covalent conjugation with retention of the enzyme's tertiary structure has been documented using X-ray crystallography and mass spectrometry.[13] Monitoring of the loss of residual hydrolytic activity of the lipase in combination with the observation of new activity also proved this mode of hybridization of lipases.[12a,14] More recently, a series of hybrids were produced using a phosphonate-NHC cofactor able to promote the interaction between its metallated derivative and the protein scaffold. These hybrids showed a promotion of chemoselectivity in rhodium-catalyzed hydrogenation, which was exerted in different extents depending on the chosen protein host, i.e. Cutinase or Candida antarctica lipase B.[15]

These results suggested a promising suitability of phosphonate-NHC cofactors in the formation of other selective artificial metalloenzymes, including Grubbs-type protein hybrids for olefin metathesis via the active site-directed approach (Figure 2). This type of constitution has, to the best of our knowledge, not been used in an artificial enzyme for

olefin metathesis, albeit the desirable advantages in terms of ease of manipulation and purification of the constructs that can be met, as well as the promise of important protein-catalyst interactions, which are desirable for the aim of catalytic selectivity. We were also interested if the reaction versatility of Grubbs-type catalysts can be achieved as well with artificial enzymes, studying different types of metathetical reactions and analyzing a possible selectivity effect of the protein scaffold in cross metathesis reactions.

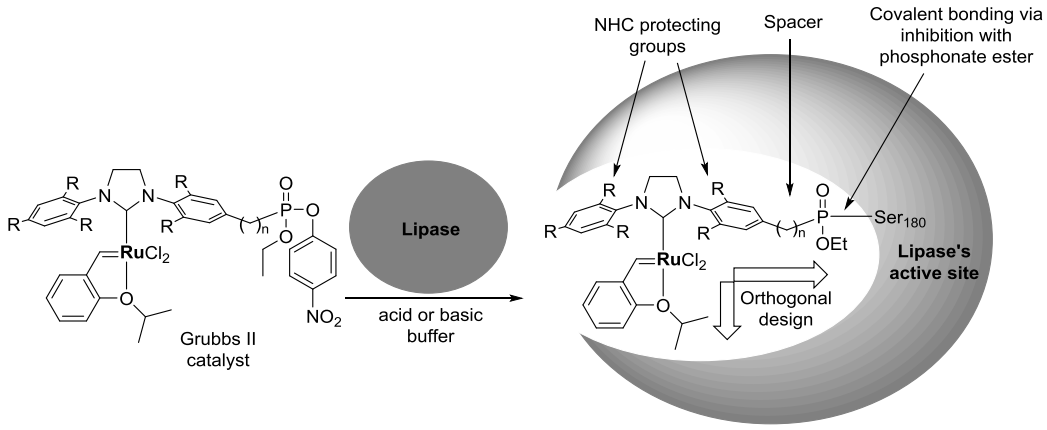


Figure 2. Lipase inhibition and features of the Grubbs-protein hybrids studied in this work.

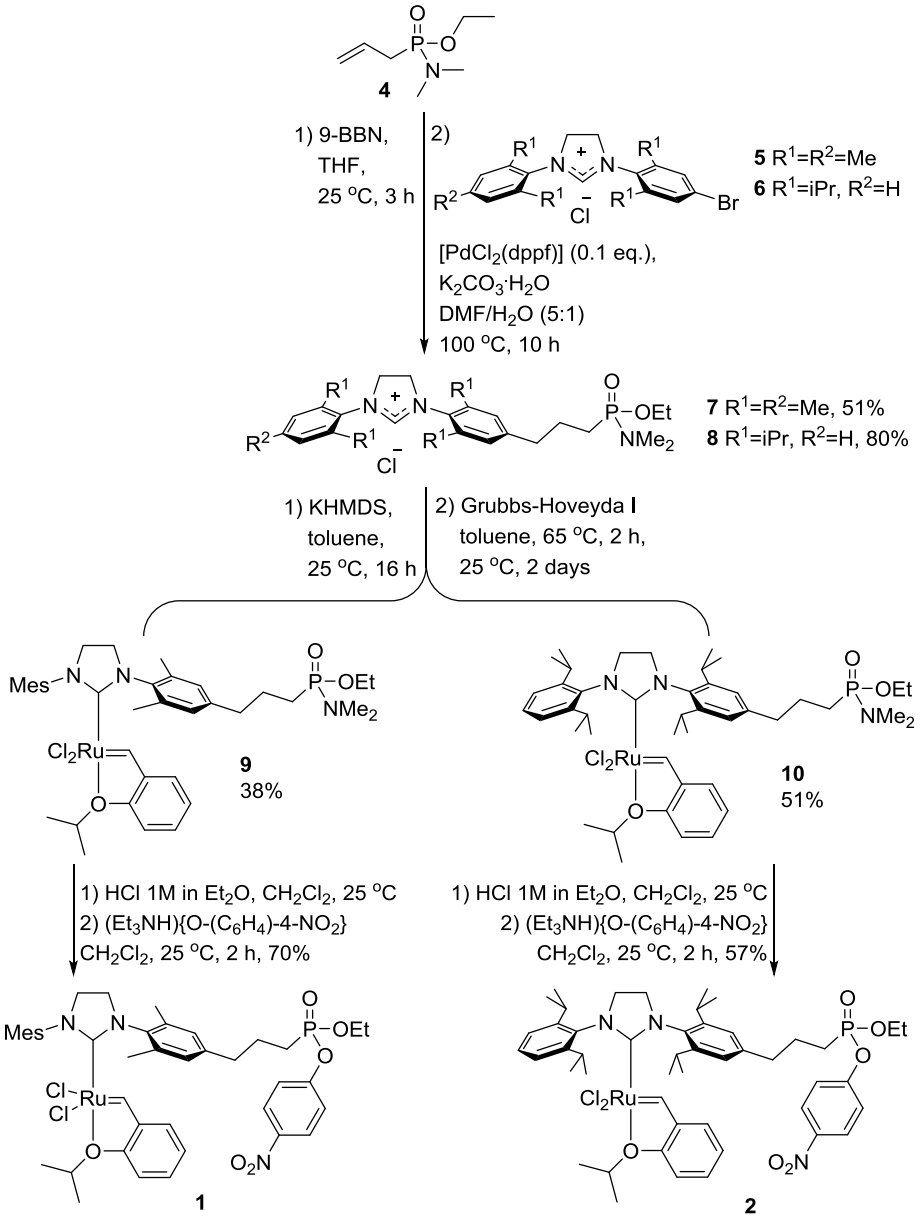
In this work a series of new Ru(NHC)-protein hybrids produced via the active site-directed method using NHC-phosphonate ligands were synthesized and studied. The hybridization and catalytic activities varied depending on the length and sterical demands of the protein-metal tethers. These semi-synthetic metalloenzymes for olefin metathesis proved active in the ring-closing metathesis of *N,N*-diallyl-*p*-toluenesulfonamide and the cross metathesis of allylbenzene, with the latter as a new reaction explored with artificial enzymes.

Results

The general structure of Grubbs II compounds comprises an NHC and a functionalized methylidene ligand. The latter plays an important role in the stabilization of the precatalyst and it has been used previously as an anchoring motif to support Grubbs-type catalysts, for example, to silicon-based dendrimers.[16] However, the catalytic cycle of the metathetical reaction involves the elimination of this methylidene.[17] Some authors claim a boomerang effect of the ligand allowing the preservation of the catalyst on the support, however this is still under discussion.[18] On the other hand, functionalization of the saturated NHC backbone produces a chiral center that may influence the possible selective effect of a protein scaffold. Therefore we chose a functionalization at the N-substituents of the NHC. In this manner, any electronic effect from the modification of the ligand does not have an impact on the metallic center and the ligation of the catalyst to the enzyme occurs via a spectator ligand (the NHC) with an orthogonal orientation (Figure 2).

Accordingly, a short-tethered SIMes-inspired preligand **7** was designed (SIMes = 1,3-dimesityl-4,5-dihydroimidazolin-2-ylidene) for the construction of the lipase inhibitor **1** (Scheme 1). *Ortho*-propoxyphenylmethylylidene, known as the Hoveyda ligand, was chosen as the methylidene ligand because of its wide use in aqueous olefin metathesis.[19] In parallel, we designed the synthesis of a ligand with a slightly more protecting behavior towards the ruthenium center. To this end, we envisioned the substitution of the trimethylphenyl N-substituents for the bulkier diisopropylphenyl substituent groups towards preligand **8** and inhibitor **2** (Scheme 1, right).

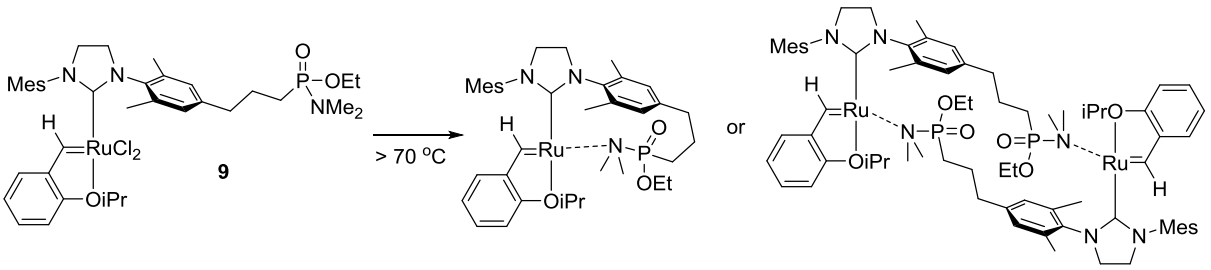
Following the synthesis of preligand **7** from the Suzuki cross-coupling of hydroboronated ethyl *N,N*-dimethyl allylphosphonamidate **4** and 1-mesityl-3-(4-bromo-2,5-dimethylphenyl)-4,5-dihydroimidazolinium chloride **5**,[15] the metallation and further purification of the corresponding ruthenium complex was not trivial. Attempts of transmetallation towards **9** from the silver-NHC derivative in the reaction with Hoveyda-Grubbs I precursor were unsuccesfull even when carried out in the presence of CuCl as phosphine scavenger.



Scheme 1. General synthesis of the short-tethered Grubbs-Hoveyda II lipase-directed inhibitors **1** and **2** with trimethylphenyl (left) and diisopropylphenyl (right) substituents.

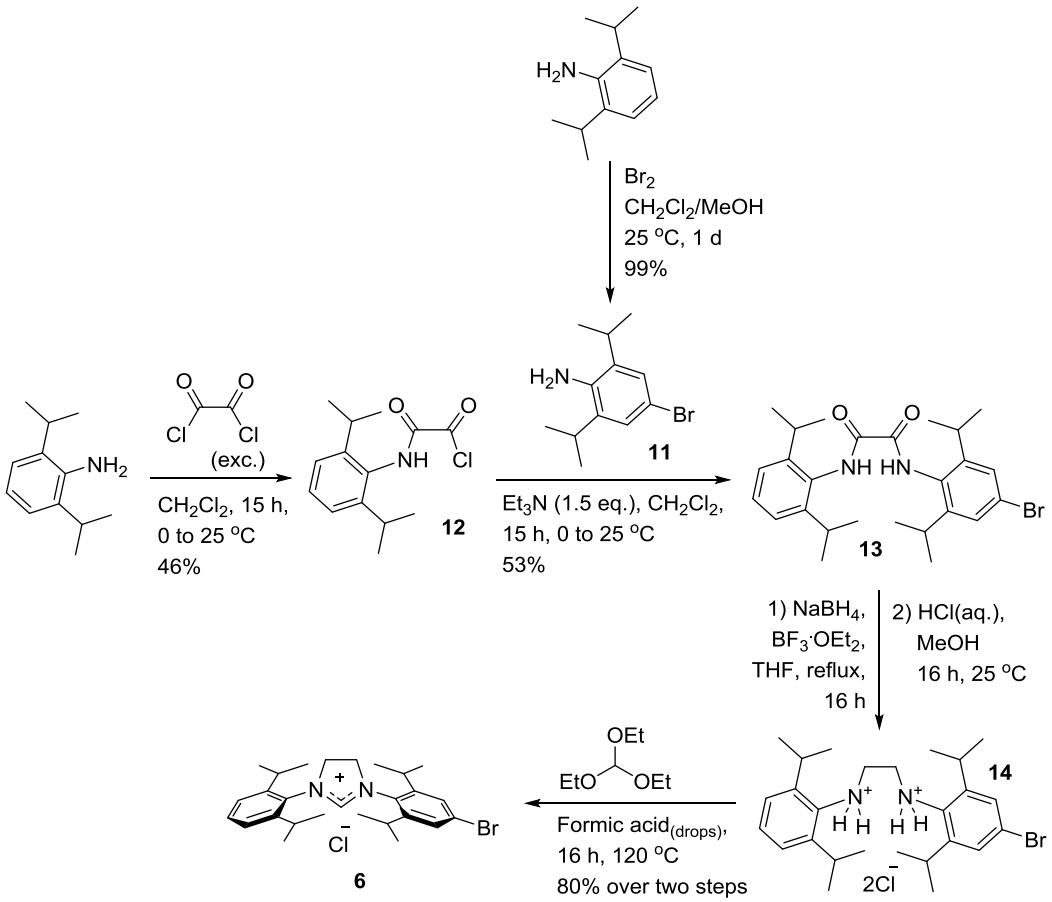
The product **9** was obtained by initial deprotonation of the imidazolinium salt **7** with the non-nucleophillic base KHMDS to avoid nucleophillic attack on the phosphorous center. The resulting free carbene was directly metallated with Grubbs-Hoveyda I precursor at low concentration of reagents, room temperature and long reaction time of 2 days, since at more harsh conditions it was noticed that intra- or intermolecular interactions occurred (Scheme 2). The purified product **9** still shows a limited lifetime of 5 days, resulting in a

change of color from green to brown due to the mentioned interactions, which were observed by a small shift in the ^{1}H NMR resonance of the methylidene moiety. Accordingly, the desired product was used for the next reaction directly after purification. It was at these metallation experiments that we opted for the synthesis of the analog **10**. In earlier reports, the catalyst with isopropyl NHC-substituents has shown increased resistance to aqueous conditions in the presence of an enzyme compared to the catalyst with mesityl groups.[5d]



Scheme 2. Ru-N interactions are found in **9** but not in **10**.

For the latter, the preligand **8** required the preparation of its synthetic precursor, the 1-(2,6-diisopropylphenyl)-3-(4-bromo-2,6-diisopropylphenyl)-4,5-dihydroimidazolinium salt **6**. The salt was formed via bromination of 2,6-diisopropylaniline followed by its coupling with chlorooxallyl anilide **12**. The resulting diamide **13** was reduced using a combination of sodium borohydride and trifluoroborane, and cyclized by protonation followed by reflux with triethyl orthoformate towards the desired bromo imidazolinium chloride **6** (Scheme 3). Afterwards, the synthesis of preligand **8** was conducted in a similar manner as that of **7**. Likewise, the metallation procedure developed for **9** was also successful here (Scheme 1, right) and, to our delight, product **10** showed a better stability since it shows no color or ^{1}H NMR change upon storage.



Scheme 3. Synthesis of brominated imidazolinium chloride **6**.

The final step for the synthesis of inhibitors **1** and **2** involved the elimination of dimethylamine using hydrogen chloride and substitution of the resuling halide with *p*-nitrophenolate (pNP). A small fraction of undesired side product with pNP bound to the ruthenium center was also observed, which was eliminated after chromatographic purification of the target complexes. pNP is a reactive leaving group and it is released from phosphonate esters in the presence of a hydrolase; this anion then acts as a yellow dye due to its absorbance at 405 nm. Interestingly, the metallation of the carbene towards complexes **9** and **10** resulted, for each case, in a mixture of the isomers where the Hoveyda ligand lays in a plane with the benzylidene in the direction of the substituted or the non-substituted mesitylene group. The ratio of these isomers is 72:28 for **9** and 74:26 for **10**; determined by ^{1}H NMR. However, for both inhibitor complexes **1** and **2**, only one isomer was observed.

Next, Cutinase was chosen as the host enzyme because of its capability to react with hydrophobic substrates and its exposed active site,[20,12,13,15] making inhibitors **1** and **2** suitable candidates for hybridization. First, a reaction using cutinase in the conditions previously found with a Rh(NHC) inhibitor[15] in biphasic conditions (Tris-HCl buffer pH 8.5 with CH_2Cl_2, 5% *v/v,* as co-solvent) was carried out with an excess of **1**, resulting in the successful formation of hybrid **Ru1-cut**. The inhibition of Cutinase was determined, in accordance with previous methodologies, by measuring the residual activity in the hydrolysis of *p*-nitrophenylbutyrate. No residual hydrolytic activity was found proving the inhibition of the active site. Excess of inhibitor **1** was eliminated by separation of water-insoluble species and dialysis of the hybrid. The covalent constitution of the hybrid allows for the latter procedure without disintegration. Mass analysis (ESI-MS) of a dialyzed, denatured sample showed the desired conjugate with an incorporation of the Grubbs fragment in a 1:1 stoichiometry (Figure 3, top), lending evidence to the irreversible character of the hybridization at the former active site.

On the other hand, when cutinase was treated with inhibitor **2** the formation of the desired hybrid was not achieved even after several attempts using different reaction times and co-solvents. We propose that the unsuccessful reaction of cutinase with compound **2** originates from the bulkier character of this inhibitor because of the N-substituents, where isopropyl substituents provide a larger steric demand (the van der Waals volumes of the residues $–CH_3$ and $–CH(CH_3)_2$ are 5.873 $Å^3$ and 8.988 $Å^3$, respectively, and if we consider the the furthest distance between hydrogens in mesityl, there is a difference of 6.23 Å with respect with the furthers distance between hydrogens in 2,6-diisopropylbenzene).[21] The difference between the inhibition of Cutinase with **1** and **2** suggests a fine sensitivity of cutinase towards these potential inhibitors and that the steric demands of the organometallic inhibitor lay close to the limit of the motifs that can be incorporated in the active site of cutinase. Furthermore, in one of our attempts to introduce **2**, when the reaction was carried out for several days at room temperature using DMSO as the co-solvent, we observed a fraction of the enzyme incorporating the ligand (hybrid **L2-cut**), but without the ruthenium center (Figure 3, bottom). This may originate from slow decomposition of the Ru-NHC complex in the medium, allowing Cutinase to react with the remaining ligand.

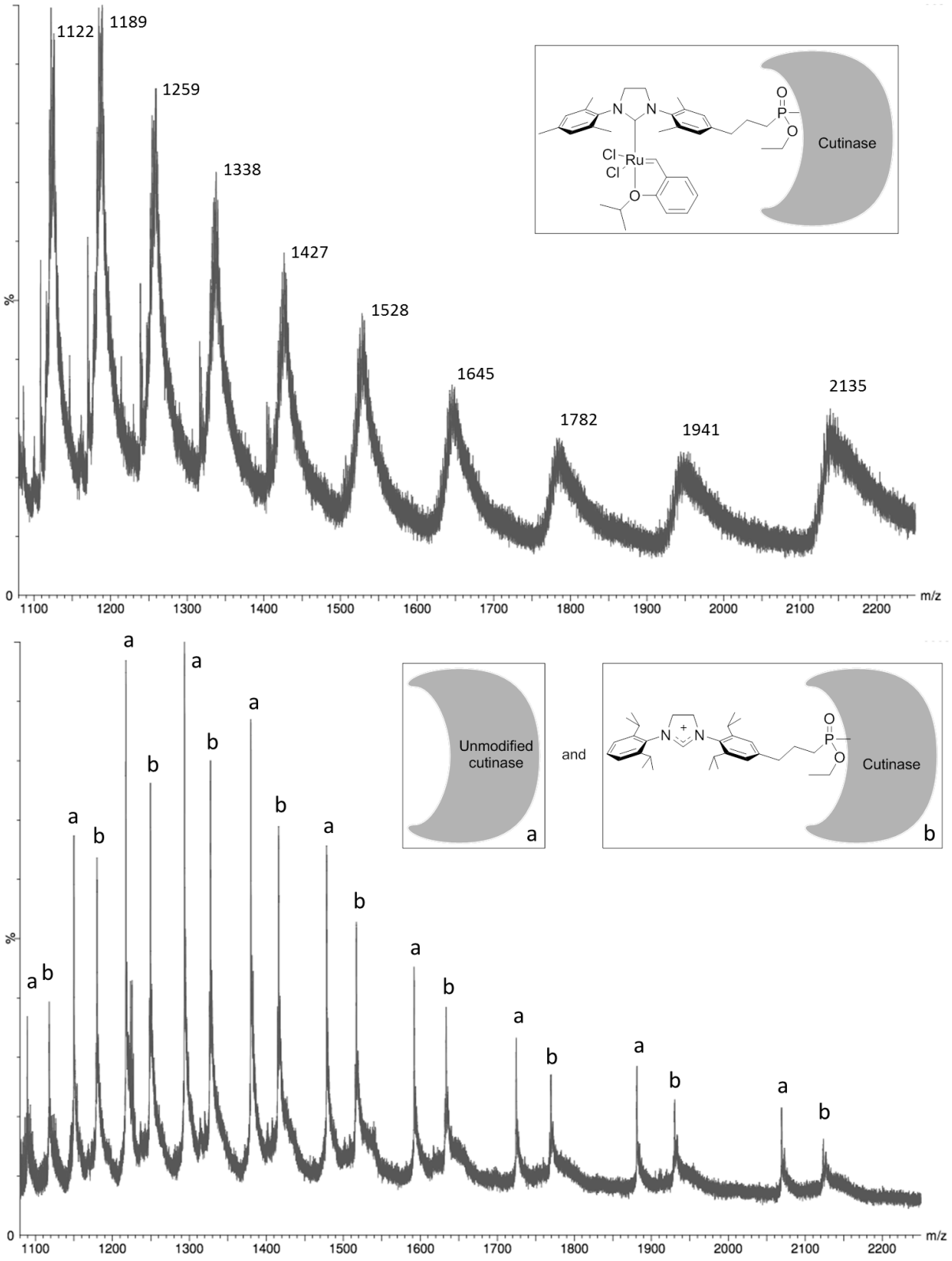


Figure 3. Mass spectra of **Ru1-cut** (*m/z* calc.: 21,348.3, found: 21,352.74 in MeCN/Formic acid) and of **L2-cut** (*m/z* calc.: 21,215.4, found: 21,222.4), top and bottom, respectively.

The observation of the latter ligand-protein hybrid indicates that the unsuccessful formation of the desired **Ru2-cut** conjugate relies on the hindering of the complete inhibitor structure and not only the bis(isopropyl)phenyl groups of the NHC ligand. This insight on the semi-synthetic active site of the hybrids played an important role for the design of a new Grubbs inhibitor, as will be discussed further.

With the new covalent active site-funtionalized hybrid **Ru1-cut**, a series of catalytic tests were conducted (Table 1, vide infra). Since the first reports by Hilvert[7] and Ward,[8] the ring-closing metathesis (RCM) of *N,N*-diallyl-*p*-toluenesulfonamide (DATsA) has become a benchmark for the evaluation of artificial metalloenzymes for olefin metathesis. Our initial experiments for the RCM of DATsA with **Ru1-cut** (5 mol %) in Tris-HCl buffer at pH 8.5 with CH_2Cl_2 (5% *v/v*) at room temperature for up to 40 h, using an excess of $MgCl_2$ as a source of chlorides,[22] resulted in no conversion (Table 1, Entry 1). To avoid interference of the buffer given that previous reports on this reaction with artificial enzymes showed a negative effect of basic pH,[8] the hybrid was dialyzed and tested in aqueous hydrochloric acid at pH 2 but catalysis was still unsuccessful (Table 1, Entry 2). We then suspected a mutual inhibition of the enzyme and the ruthenium compounds since imidazole (from the amino acid histidine) may act as an olefin metathesis inhibitor if coordination with ruthenium occurs.[23] A single histidine residue is present in Cutinase in its catalytic triad Asp_{175}-His_{188}-Ser_{120}. The role of histidine here contributes importantly to the enzymatic activity and retention of the tertiary structure. In the adduct form, histidine is less nucleophillic than in the 'free' form, thus its role and location in cutinase reduces its potential for coordination to the ruthenium center. However, after our experimental results, we chose to exclude the plausible coordinating behavior of histidine by performing the hybridization, i.e. the synthesis of the hybrid, in a medium with lower pH. Importantly, Cutinase has shown to preserve its activity in media of different pH, although with lower catalytic rates.[24] Accordingly, inhibition tests were performed with Cutinase in acetate buffer at pH 5 using inhibitor **1**. The use of longer reaction times of 2 days for the hybridization led again to the successful formation of the hybrid **Ru1-cut**, which was stored at pH 5. In spite of these changes, the hybrid showed a very low yield of 1% towards the desired 1-tosyl-2,5-dihydropyrrole in the tested RCM reaction (Table 1, Entry 3). This experiment was conducted as well using commercial Grubbs-Hoveyda II catalyst in the presence of unmodified Cutinase to determine an intrinsic

incompatibility of this type of catalyst with the enzyme. However, the reaction took place successfully under these conditions (Table 1, Entry 4).

At this point we suspected that the embedment of the catalyst in the pocket of Cutinase would block the metallic center leading to the loss of activity. Noteworthy, Cutinase is a lipase with a relatively open cavity, compared to other known serine hydrolases, and the active serine residue is considered accessible by the solvent.[25] We made use of computational modeling[21] to estimate potential overlapping between the organometallic fragment and the enzyme and thereby to have a better understanding of the sterical demands of the metathesis substrate in this environment. These modeling efforts were performed by the docking of a known Grubbs-Hoveyda II crystal structure, substituted with the phosphorylated propenyl tail, and the known crystal structure of Cutinase. Figure 4 depicts a model configuration of the metalloprotein conjugate, which shows that the Grubbs catalyst is not entirely embedded in the protein but that the ruthenium center is indeed surrounded by amino acid residues known to partially block the entrance of substrates to the cutinase active site (helical flap comprising residues 81-85 and a binding loop involving residues 178-186[26]). Moreover, the alkylidene carbon, the labile site for the substrate, is in close proximity to the protein scaffold surrounding it from opposite directions (5.2 Å to Leu_{81} and 5.4 Å to Leu_{182}). Therefore sterical hindrance brought forward by the protein scaffold is a probable cause of the lack of metathetical reactivity of **Ru1-cut**.

Interestingly, the comparison between the high activity of commercial Grubbs catalyst in presence of unmodified Cutinase against the notably lower activity of the hybrid (Table 1, Entries 3 and 4) points out the little or no risk of leaching of the organometallic fragment from the enzyme, otherwise behaving as a low molecular weight catalyst in presence of protein. Additionally, treatment of the catalysis reaction mixture containing **Ru1-cut** with an excess of organic solvent to extract the product, yet not showing the desired dihydropyrrole product, indicates that the organometallic fragment is also not released after denaturation. These observations stress the importance of prior formation and isolation of the hybrid for the evaluation of its catalytic activity, as we screened here, to avoid interference of unsupported catalytic material in the medium.

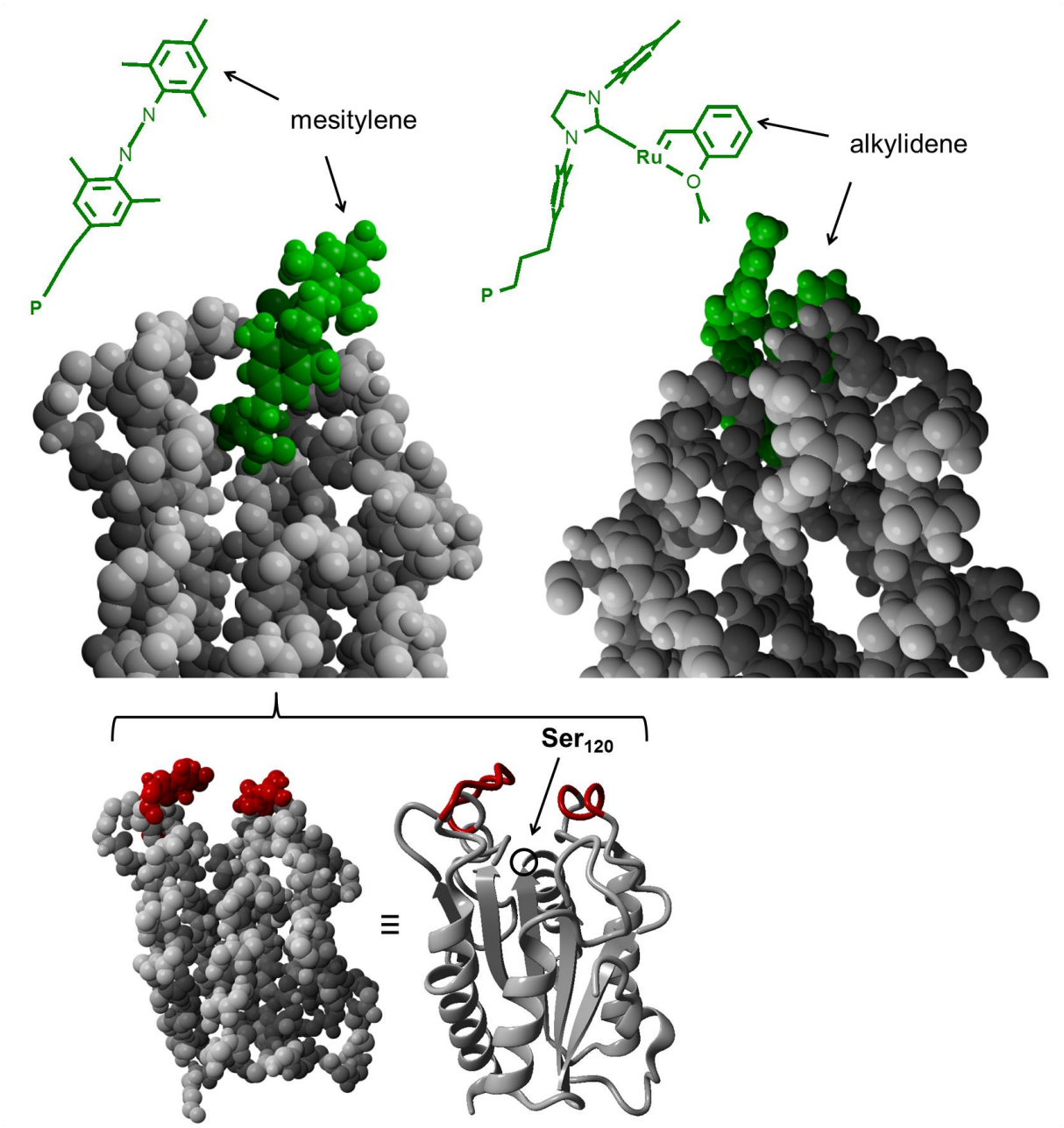
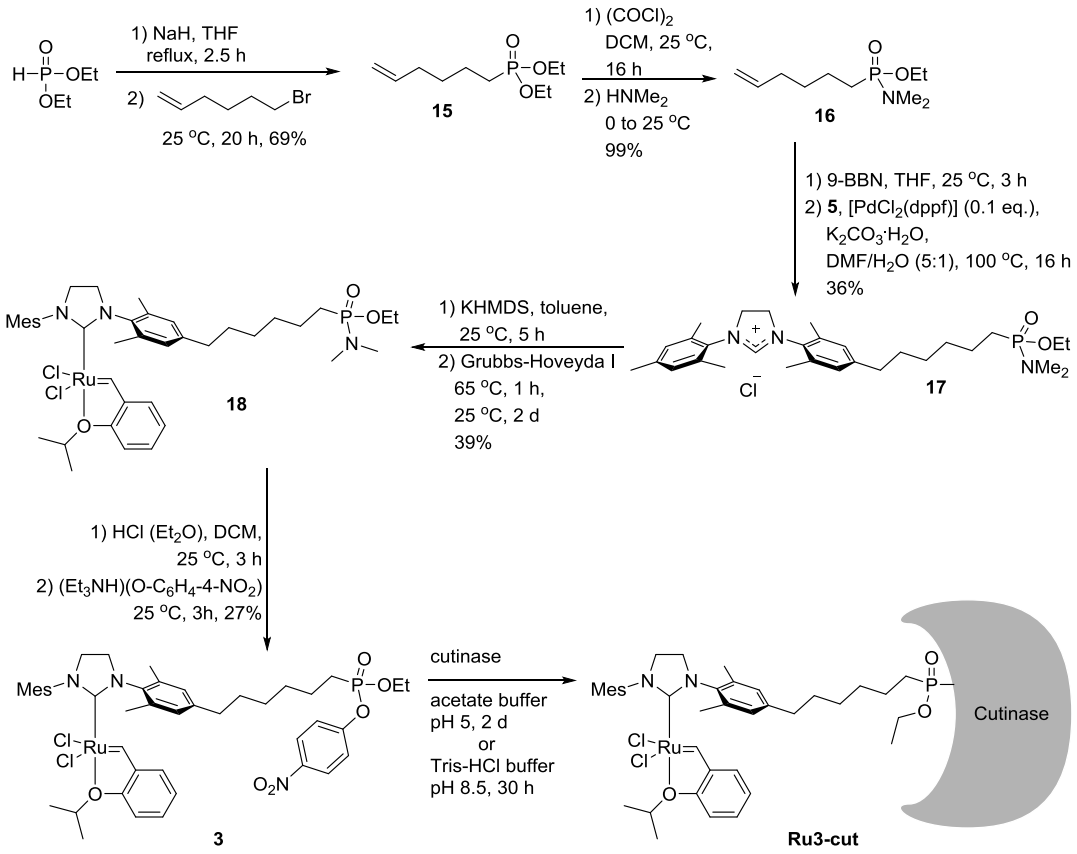


Figure 4. Docking modeling of **Ru1-cut**[21] rear view (left) and side view (right) with the complex in green (top) and cutinase without the complex (bottom) highlighting the blocking residues in red.

In this scenario, and with the aim to reduce the hindrance between the enzyme and the organometallic fragment, instead of the use of a different lipase, we focused on the design of a new Grubbs-containing inhibitor. Due to the unsuccessful hybridization of complex **2**, and assuming excessive sterical hindrance at the metallic center of **Ru1-cut**, a rationalized change in the cofactor was designed so to allow the metallic center for more flexibility. To

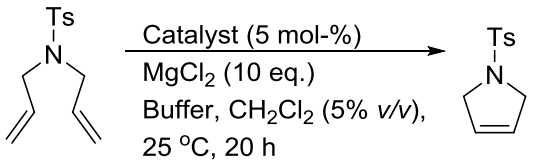
this end, the new inhibitor **3** bearing a larger tether chain in the cofactor, was synthesized. For this, the propylene moiety was replaced by a hexylene moiety, which was incorporated during the synthesis of the phosphonate precursor **15** by alkylation of diethyl phosphite, then transformed into a chlorophosphate and in situ then to the phosphonamidate **16** (Scheme 4). Cross coupling with imidazolinium salt **5** and further metallation steps were equivalent to those of the previous inhibitors. A mixture of isomers was again found after the metallation towards **18** with a ratio of 56:44, suggesting a more homogeneous mixture when compared to the ratio of complexes **9** and **10**. In this case the inhibitor **3** also showed a mixture of isomers with a ratio of 8:2, while for complexes **1** and **2** only one isomer was observed. Inhibition of cutinase was carried out at pH 5 for 30 h using complex **3** in excess. Analysis after dialysis showed the desired hybrid **Ru3-cut** in ESI-MS and no residual hydrolytic activity of cutinase. The complete synthesis of **Ru3-cut** is presented in Scheme 3.



Scheme 4. Synthetic scheme for the synthesis of lipase inhibitor **3** and hybrid **Ru3-cut**.

The catalytic activity of the new hybrid **Ru3-cut** in ring-closing metathesis was screened at pH 5 gratifyingly affording high conversion towards the dihydropyrrole product (Table 1, Entry 5; Figure 5, bottom), whereas at pH 8.5 (or without magnesium chloride) again no conversion was found (Table 1, Entry 6). From these observations it can be deduced that the new ligand along with the acidic conditions and magnesium chloride, are responsible of allowing catalytic performance. Magnesium chloride, as mentioned above, provides a readily source of chlorides, since it has been observed that one chloride ligand bound to the ruthenium center promotes the metathetical reaction[22] (apart from $MgCl_2$ used as well in previous reports of olefin metathesis,[8a] KCl has also been used,[9] both with artificial enzymes).

Table 1. Catalytic evaluation of hybrids **Ru1-cut** and **Ru3-cut** for the ring-closing metathesis of *N,N*-diallyl *p*-toluenesulfonamide.



Entry	**Catalyst**	**Media**[a]	**Yield**
1	**Ru1-cut**	Buffer pH 8.5[b,c]	-
2	**Ru1-cut**	HCl 0.01 M	-
3	**Ru1-cut**	Buffer pH 5	1%
4	Grubbs-Hoveyda II	Buffer pH 5 + cutinase	90%
5	**Ru3-cut**	Buffer pH 5	84%
6	**Ru3-cut**	Buffer pH 8.5[c]	-

[a] Buffer Tris-HCl was used for pH 8.5 and sodium acetate buffer for pH 5. [b] For 20 and 40 h. [c] With and without $MgCl_2$.

Enlargement of the cofactor tether successfully allowed for catalytic activity in the hybrid (Figure 5). There is a longer distance between the metallic center and the protein scaffold in **Ru3-cut** than in **Ru1-cut**, reducing the hindering effect of protein residues to substrate approach. Alternatively, the increment of the aliphatic tether confers flexibility to the inhibitor, possibly producing a different conformation or directionality of the organometallic

fragment in or with respect to the enzyme pocket. The small difference in yield produced by the commercial catalyst and **Ru3-cut** suggests that the active center in the latter is still less accessible than in the unsupported complex (Entries 4 and 5); an interaction of the substrate and the protein scaffold, although reduced, is still possible given that the extention in the catalyst's tether, from **Ru1-cut** to **Ru3-cut** (from a C3 chain to a C6 chain), is smaller in dimension that the length of the substrate.

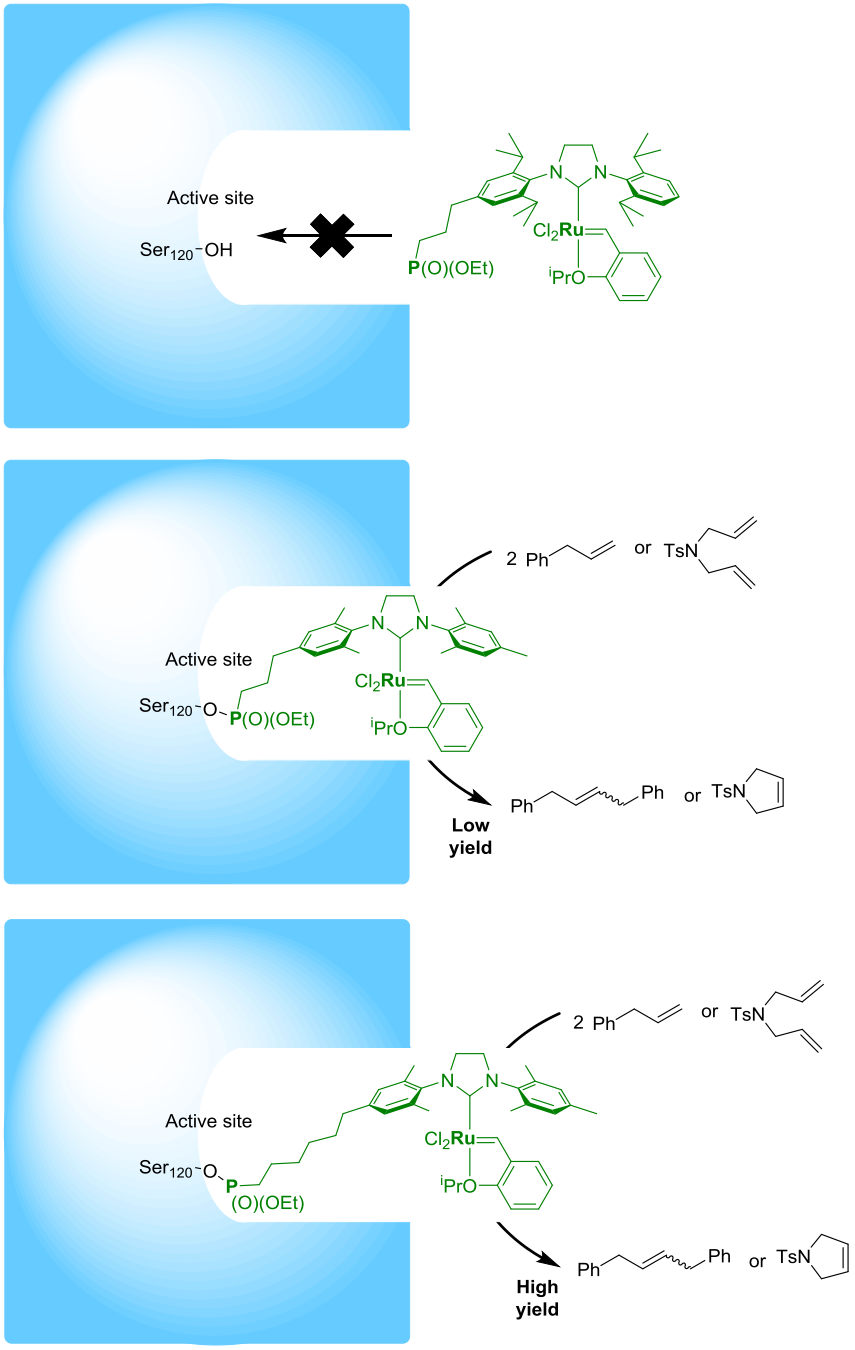
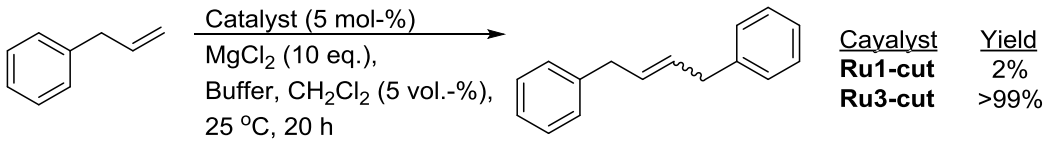


Figure 5. Schematic representation of the targeted Ru-cutinase hybrids showing a trend of steric constraint: **Ru2-cut** (not formed) > **Ru1-cut** (formed but not active) > **Ru3-cut** (formed and active).

The observation that a lower pH buffer facilitates catalytic activity could originate from the lower nucleophilic character that it creates in the amino acid residues, preventing blocking of the ruthenium center. However, it is known that the thermal stability of cutinase is lower at pH 5 than at pH 8.5,[24] denoting a less rigid tertiary structure at these conditions, allowing for structural lability of the protein scaffold to host the catalytic reaction.

Next, we tested the metalloenzymes **Ru1-cut** and **Ru3-cut** as catalysts for a different type of olefin metathesis reaction, i.e. the homocoupling of allylbenzene, a formal cross metathesis reaction (Scheme 5). Under the optimized conditions mentioned above (acetate buffer, pH 5) the desired product 1,4-diphenylbut-2-ene was formed in a very low yield of 2% when **Ru1-cut** was used. On the other hand, catalyst **Ru3-cut** gave quantitative formation of the product within 20 h. Analysis of the reaction outcome showed a product *E/Z* ratio of 5.3 and 4.7 for **Ru1-cut** and **Ru3-cut**, respectively. These are comparable values to the ratio found for the reaction at the same conditions using unsupported Grubbs-Hoveyda II catalyst (*E/Z* = 4.9), indicating that the hybridization of the catalyst did not have an important effect on the selectivity of the catalyst. However, the consistency on the reactivity on metathesis reactions of the two metalloenzymes offers additional proof of the impact of the elongation of the cofactor's tether, i.e. the positioning of the metallic center in the protein. It is known that with unsupported catalysts, the reaction rate of RCM is faster than the CM counterpart, mainly because of the required bimolecular coordination of substrates towards the ruthenium center in the latter reaction.[17] However, our observations point out that, when using **Ru3-cut** as the catalyst at identical reaction times, the RCM reaction is not complete yet, while it is in the case for the CM reaction. This effect could originate from the nature, specifically the structure, of the substrates DATsA vs. allylbenzene and their accessibility to the metallic center, which could not only be hampered due to volume constriction but also due to conformational approach, giving preference to the less bulky allylbenzene to the pocket site. The formation of the desired CM product represents, to the best of our knowledge, the first case of an artificial metalloenzyme used for the cross metathesis of terminal olefins.



Scheme 5. Catalytic evaluation of hybrids **Ru1-cut** and **Ru3-cut** for the crossed metathesis of allylbenzene.

Conclusions

The development of new hybridization techniques represents a fundamental approach to search for selective or versatile semi-synthetic enzymes. The recent reports on the first Grubbs-type protein constructs are an important step in the development of bio-based supported catalysts for olefin metathesis. However, these hybrids do not meet the desirable features of covalent anchoring and an active site-directed hybridization simultaneously; which would allow for robustness, ease of purification and handling of the material with a promising 2nd coordination sphere effect. In the present work the formation of artificial metalloezymes for olefin metathesis meeting these criteria was demonstrated. Following the covalent inhibition of cutinase with Ru(NHC)-phosphonate inhibitors, the formed hybrids allow for purification by dialysis and keep their covalent character even after denaturation, as observed in mass spectrometry. This hybridization technique also produces an important interaction of the pocket around the original active site of the enzyme with the incorporated Grubbs-type catalyst.

The comparison of the successful formation of **Ru1-cut** with the unsuccessful formation of the analog **Ru2-cut**, the latter due to structural changes in the NHC-phosphonate cofactor substituting methyl groups for the moderately bulkier isopropyl groups, proves that the original hybrid design involves high sensitivity of the enzyme at the docking place towards these large complexes. The use of the larger but less rigid inhibitor **3** instead of a bulkier one to overcome the poor catalytic activity of **Ru1-cut** was successful, showing a significant gain of catalytic activity, suggesting not only a sterical demand of the pocket of Cutinase in terms of volume but also in terms of the shape and orientation of the

incorporated organometallic fragment. In the use of Cutinase for the development of artificial metalloenzymes, neither the rejection of a phosphonate inhibitor due to chemical hindrance, nor a facilitation of the hybrid's activity as function of the cofactor bulkiness was earlier observed. This sets a clear example of the need for rationalized cofactor design and states a reference of the sterical limit that Cutinase can accommodate in its pocket. As reported recently for rhodium-protein hybrids,[13] the resulting protein scaffold of Cutinase has promoted catalytic selectivity and this could be enhanced using a different lipase. Nonetheless, changes in activity as a function of the cofactor is a promising tool for the search of new artificial enzymes using Cutinase due to the abundance of this relatively small enzyme.

The difference in reactivity of the hybrids against the unsupported catalyst in presence of the enzyme is a simple proof of the difference of metal-protein interactions inside and outside of a lipase, even with an enzymatic cavity accessible by solvent, as the one of Cutinase. The trend in reactivity between the metallohybrids in RCM is also reflected in CM: in both reactions the short-tethered hybrid **Ru1-cut** is outperformed by the larger **Ru3-cut**, proving a powerful active site-functionalized covalent hybrid for the crossed coupling of allylbenzene and representing the first example of the versatility of artificial metalloenzymes for mono- and bimolecular olefin metahesis.

Experimental Section

Materials and Methods. Chemical precursors were purchased from commercial sources and used without further treatment unless stated otherwise. Cutinase was obtained from Novonordisk®. Diethyl ether, hexane and toluene were dried from a MBraun MB SPS-800 solvent purification system; dichloromethane and tetrahydrofurane (THF) were dried by distillation from $CaCl_2$ and sodium/benzophenone, respectively and stored over 4 Å molecular sieves. Tris(hydroxymethyl)aminomethane buffer (TrisHCl) and sodium acetate buffer were prepared in degassed Milli-Q water and stored in Schlenk flasks. ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded at 298 K using a Varian AS 400 MHz NMR spectrometer at 400,

100 and 81 MHz, respectively. Chemical shifts are reported in ppm and referenced against the residual solvent signal. GC measurements were performed by using a PerkinElmer Autosystem XL gas chromatograph equipped with a PerkinElmer Elite-17 column. GC-MS analyses were performed with a Perkin Elmer gas chromatograph Clarus 680 equipped with a PE Elite 5MS column and coupled to a Perkin Elmer mass spectrometer Clarus SQ 8T with EI ionization. Electrospray Ionization (ESI-TOF) mass spectra of chemical products were recorded with a Waters LCT Premier XE KE317 Micromass Technologies spectrometer; mass analyses of protein and hybrid products are calculated as $[M]^{n+}=(M+n)/n$). Ultrafiltration dialysis of proteomic samples was performed with Vivaspin 6® tubes, 10,000 M.W. C.O. (PEG membrane). All reactions were carried out under standard Schlenk techniques and under inert conditions with N_2.

Synthesis of imidazolinium preligands

Compounds **4, 5** and **7** were synthesized as described before in this thesis.[15]

4-bromo-2,6-diisopropylaniline 11.The synthesis was adapted from a literature procedure by Chow *et al.*[29] To a stirred solution of 2,6-diisopropylaniline (0.74 mL, 3.9 mmol) in 20 mL CH_2Cl_2/MeOH (1:1 v/v), a solution of Br_2 (0.21 mL, 4.1 mmol) in 10 mL CH_2Cl_2/MeOH (1:1 v/v) was added via an addition funnel at room temperature during 1.5 hours. The orange/red solution was stirred for 1 day. Solvents were evaporated and a red solid remained. The solid was recrystallized from hexane and further purified by column chromatography using CH_2Cl_2 as the solvent, resulting in 0.47 g of a light orange/red solid (99% yield). ^{1}H NMR, $CDCl_3$, 400 MHz, δ: 10.09 (bs, NH_2, 2H), 7.35 (s, Ar-H, 2H), 3.69 (m, *J* = 26 Hz, ^{i}Pr-CH, 2H), 1.28 (d, *J* = 6.4 Hz, ^{i}Pr-CH_3, 12H). ^{13}C NMR, $CDCl_3$, 100 MHz, δ: 145.0, 128.0, 123.9, 123.8, 29.0, 24.2. EI-MS $[M]^+$ calc. 255.0623, found 255.2681.

2-((2,6-diisopropylphenyl)amino)-2-oxoacetyl chloride 12. In a 3-neck roundbottom flask under Schlenk conditions, equipped with a condenser and an addition funnel, oxalyl chloride (38.4 mL, 0.44 mol) was placed and cooled to 0 ^{o}C. A solution of 2,6-diisopropylaniline (7.68 mL, 40.7 mmol) in dry CH_2Cl_2 (60 mL) was placed in the addition funnel and added slowly to the oxalyl chloride solution under a gentle stream of nitrogen through the reflux condenser.

Dry CH_2Cl_2 (20 mL) was used to rinse the funnel and the nitrogen flow was stopped. After stirring overnight the solvents were evaporated. Dry Et_2O was added and the solids were filtered off. The solvent was removed under reduced pressure and hexane (60 mL) was added to the residue. The mixture was vigorously stirred before filtration under nitrogen atmosphere and the solids were collected using dry CH_2Cl_2. Evaporation of the remaining solvent resulted in 4.94 g product (46% yield). ^{1}H NMR, $CDCl_3$, 400 MHz, δ: 7.96, (bs, NH, 1H), 7.36 (t, *J* = 7.6 Hz, Ar-H, 1H), 7.22 (d, *J* = 7.6 Hz, Ar-H, 2H), 3.07 (m, *J* = 14.4 Hz), 2.94 (b, ^{i}Pr-CH, 2H), 1.24 (d, J = 7.2 Hz), 1.21 (d, *J* = 6.8 Hz, ^{i}Pr-CH_3, 12H). ^{13}C NMR, $CDCl_3$, 100 Mhz, δ: 168.9, 153.6, 145.7, 129.4, 128.7, 123.9, 29.0, 23.6.

N^1-(4-bromo-2,6-diisopropylphenyl)-N^2-(2,6-diisopropylphenyl)oxalamide 13. A solution of 4-bromo-2,6-dimethylaniline **11**, (5.15 g, 19.3 mmol) and triethylamine (2.67 mL, 19.3 mmol) in CH_2Cl_2 (30 mL) was added drop wise to the solution of **12** (4.9 g, 19.3 mmol) in dry CH_2Cl_2 (100 mL) at 0 °C. At the end of addition, another half equivalent of Et_3N (1.3 mL, 9.7 mmol) was added. The resulting mixture was stirred at 0 ^{o}C for 10 min, then overnight at room temperature. The reaction was filtered and the collected solid was washed with diethyl ether and dried using a rotary evaporator. The remaining solids were dissolved in CH_2Cl_2, washed with water and dried over $MgSO_4$, then concentrated to give a residue, which was precipitated from diethylether. The solids were dried under vacuum to give **13** as a white solid (5.04 g, 53.0% yield). ^{1}H NMR, $CDCl_3$, 400 MHz, δ: 8.8 (bs, 1H, NH), 7.35 (d, *J* = 8.0 Hz, Ar-H, 1H), 7.32 (s, Br-Ar-H, 2H), 7.21 (d, *J* = 7.6 Hz, Ar-H, 2H), 3.01 (m, *J* = 34.4 Hz, ^{i}Pr-H, 4H), 1.21 (d, *J* = 3.2 Hz, ^{i}Pr-CH_3, 12H), 1.19 (d, *J* = 3.2 Hz, ^{i}Pr-CH_3, 12H). ^{13}C NMR, $CDCl_3$, 100 Mhz, δ: 159.7, 159.4, 148.2, 145.7, 129.6, 129.0, 128.9, 127.1, 123.7, 123.1, 29.2, 29.0, 23.5, 23.3. ESI-MS $[M+H^+]^+$ calc. 487.1960, found 489.1948.

N^1-(4-bromo-2,6-diisopropylphenyl)-N^2-(2,6-diisopropylphenyl)ethane-1,2-diaminium 14. $H_3B{\cdot}SMe_2$ complex (4.9 mL, 51.7 mmol) was added slowly to a suspension of the diamide **13** (5.04 g, 10.3 mmol) in toluene (20 mL) at room temperature. The resulting mixture was stirred overnight at 95 ^{o}C under N_2. After cooling to room temperature, $HCl_{(aq)}$ 1M was added to the reaction until the stirred solution tested acidic (pH 3), and the resulting mixture was stirred overnight. The precipitate was collected by filtration, washed with diethylether and dried under vacuum. The solid was suspended in 30 mL of CH_2Cl_2 and the mixture was cooled to 0 ^{o}C. Triethylamine (2.9 mL) was added slowly and the mixture was stirred at room

temperature for 2 h. The solvent was removed under reduced pressure to give a residue, which was purified by silica gel column chromatography using 8:2 hexanes:ethyl acetate as eluent giving 80 mg of **14** (2% yield, because of the low yield, the following product, **6**, is preferably synthesized directly from **13**, see below). ^{1}H NMR, $CDCl_3$, 400 MHz, δ: 7.13 (s, Br-ar-H, 2H), 7.06 (d, *J* = 7.6 Hz, Ar-H, 2H), 6.82 (t, *J* = 15.6 Hz, Ar-H, 1H), 3.73 (bs, NH, 2H), 2.96 (q, *J* = 18.0 Hz, NC*H*$_2$CH$_2$N, 2H), 2.89 (m, *J* = 27.2 Hz, ^{i}Pr-H, 4H), 2.80 (q, *J* = 22 Hz, NCH$_2$C*H*$_2$N, 2H), 1.27 (d, *J* = 6.8 Hz, ^{i}Pr-CH$_3$, 24H). ^{13}C NMR, $CDCl_3$, 100 MHz, δ: 139.3, 134.6, 132.4, 129.0, 128.2, 125.8, 125.3, 122.8, 118.5, 111.1, 52.4, 28.0, 22.2. ESI-MS $[M+H^+]^+$ calc. 459.2375, found 459.2405.

1-(4-bromo-2,6-diisopropylphenyl)-3-(2,6-diisopropylphenyl)-4,5-dihydro-1H-imidazol-3-ium chloride 6 (full synthesis from **13**). To a suspension of **13** (1 g, 2.05 mmol) and $NaBH_4$ (0.47 g, 0.012 mol) in THF (25 mL), $BF_3{\cdot}OEt_2$ (2.62 g, 2.28 mL, 0.0185 mol) was added, and the mixture was heated to reflux for 18 h. After this, the reaction mixture was quenched with MeOH (2 mL) and HCl (1 mL, 10 M), and the solvent was removed under reduced pressure. THF (10 mL) and HCl (1 mL, 4 M) were added and the resulting mixture was stirred overnight, after which the solvent was evaporated leaving an off white solid. Diethyl ether (100 mL) and aqueous NaOH (150 mL, 1 M) were added and the layers were stirred for 10 min to dissolve the solid. The organic layer was separated and the aqueous layer was extracted with diethyl ether (2 x 90 mL). The combined organic layers were dried over $MgSO_4$ and the filtrate was concentrated and dissolved in triethylorthoformate (10 mL). HCl (0.2 mL, 10 M) was added and the mixture was heated to 120 °C for 18 h. The mixture was concentrated, after which diethyl ether (70 mL) was added to collect the precipitation by filtration. The solids were washed using CH_2Cl_2, resulting in 0.82 g (80% yield) of **6**. ^{1}H NMR, $CDCl_3$, 400 MHz, δ: 8.84 (s, NCN, 1H), 7.47 (t, *J* = 15.2 Hz, 1H), 7.38 (s, Br-Ar-H, 2H), 7.27 (d, *J* = 7.6 Hz, Ar-H, 2H), 4.79 (bs, NCH$_2$CH$_2$N, 4H), 2.99 (m, *J* = 32.8 Hz, *i*-Pr, 4H), 1.38 (d, *J* = 6.8 Hz, *i*-Pr-Me, 6H), 1.38 (d, *J* = 6.8 Hz, *i*-Pr-Me, 6H) 1.26 (d, *J* = 6.8 Hz, *i*-Pr-Me, 6H), 1.261 (d, *J* = 6.8 Hz, 6H, *i*-Pr-Me). ^{13}C NMR, $CDCl_3$, 100 MHz, δ: 158.8, 148.4, 146.0, 131.7, 128.5, 125.0, 55.3, 55.1, 53.4, 29.4, 29.3, 25.4, 25.2, 23.7, 23.6. ESI-MS m/z $[M\text{-}Cl^-]^+$ calc. 469.2213, found 469.2233.

1-(2,6-diisopropylphenyl)-3-(4-(3-((dimethylamino)(ethoxy)phosphoryl)propyl)-2,6-diisopropylphenyl) -4,5-dihydro-1H-imidazol-3-ium chloride 8. In a glovebox, **4** (0.24 g, 1.36 mmol) was dissolved in dry degassed THF (10 mL). 9-BBN (0.33 g, 1.36 mmol) was dissolved

in dry degassed THF (15 mL) and slowly added to the solution of **4**. The resulting mixture was stirred for 72 h, after which the solvents were removed in vacuum. The product was directly used for the next reaction.

Compound **6** (0.627 g, 1.24 mmol) was dissolved in degassed DMF (10 mL), [$PdCl_2$(dppf)] catalyst (0.091 g, 0.11 mmol) was dissolved in degassed DMF (20 mL) and $K_3PO_4 \cdot H_2O$ (0.29 g, 1.365 mmol) was dissolved in degassed H_2O (10 mL). The solution with **4** and 9-BBN (in situ, 1.365 mmol) was added to a Schlenk flask and diluted in degassed DMF (20 mL). While stirring, first the solution containing **6** was added, followed by the [$PdCl_2$(dppf)] suspension. Degassed DMF and water were added to obtain a volume of 100 mL of solvent in total with a 9:1 DMF:H_2O ratio before the K_3PO_4 mixture was added. The resulting black mixture was stirred at 100 ^oC for 16 h, and cooled to room temperature. The mixture was filtered over Celite® and washed with DMF (2 x 35 mL). The solvents were removed under high vacuum and the solid was dissolved in CH_2Cl_2 after which the solids were filtered off. Due to the amphiphilic character of the product, this was extracted to an aqueous solution using H_2O (4 x 150 mL). The aqueous layers were dried in vacuum and a dark oil was obtained. This was dissolved in CH_2Cl_2 (15 mL). A spoon of activated carbon (Norit®) was added and the mixture was stirred for 1 h, filtered over Celite®, rinsed with 40 mL CH_2Cl_2 and dried in vacuum. 0.6 g (80% yield) of the product was obtained. ^{1}H NMR, CD_2Cl_2, 400 MHz, δ: 8.17 (bs, 1H, NCHN), 7.46 (t, *J* = 15.6 Hz, 1H), 7.27 (d, *J* = 7.6, Ar-H, 2H), 7.06 (s, Br-Ar-H, 2H), 4.84 (bs, NCH_2CH_2N, 2H), 4.13 (m, $CH_2CH_2CH_2P$, 2H), 3.99 (m, $CH_2CH_2CH_2P$, 2H), 3.84 (m, $CH_2CH_2CH_2P$, 2H), 3.03 (m, *J* = 32.8 Hz, *i*-Pr, 4H), 2.67 (bs, NCH_3CH_3, 3H), 2.64 (bs, NCH_3CH_3, 3H), 1.39 (bs, *J* = 6.8 Hz, *i*-Pr-Me, 6H), 1.28 (d, *J* = 7.2 Hz, OCH_2CH_3, 3H), 1.23 (bs, *J* = 6.8 Hz, *i*-Pr-Me, 6H). ^{13}C NMR, CD_2Cl_2, 100 MHz, δ: 158.1, 146.2, 145.2, 131.6, 129.2, 127.2, 125.0, 125.0, 59.2, 59.2, 55.3, 55.2, 53.4, 36.7, 36.5, 36.1, 36.1, 29.2, 29.1, 25.4, 25.4, 16.3, 16.2. ^{31}P NMR, CD_2Cl_2, 81 MHz, δ: 36.09. ESI-MS m/z $[M\text{-}Cl^-]^+$ calc. 586.4032, found 586.4029.

Diethyl hex-5-en-1-ylphosphonate 15. Sodium hydride (60% disp. in mineral oil, 0.66 g, 15.15 mmol, 1.35 eq.) was suspended in dry THF (6 mL). To this stirred suspension, diethyl phosphite (1.85 mL, 14.37 mmol, 1.28 eq.) was added dropwise via a syringe. After the initial gas evolution had ceased the reaction mixture was stirred for another 30 min at room temperature followed by 2.5 h at reflux. The reaction mixture was cooled to 0 ^oC and 6-bromo-1-hexene (1.5 mL, 11.22 mmol) was added dropwise via a syringe. Upon complete

addition the icebath was removed and the reaction mixture was stirred for 20 h at room temperature. H_2O (50 mL) was added and this mixture was extracted with CH_2Cl_2 (3 x 125 mL). The combined organic extracts were washed once with H_2O and concentrated in vacuo. The residue was purified by column chromatography using Et_2O/CH_3OH 9:1 (*v/v*) as eluent to yield a pale yellow oil (1.71 g, 7.79 mmol, 69% yield). ^{1}H NMR, $CDCl_3$, 400 MHz, δ: 5.70-5.83 (m, 1H), 4.90-5.03 (m, 2H), 3.99-4.15 (m, 4H), 2.01-2.09 (m, 2H), 1.53-1.79 (m, 4H), 1.40-1.51 (m, 2H), 1.30 (t, *J* = 7 Hz, 6H); ^{13}C NMR, $CDCl_3$, 100 MHz, δ: 138.2, 114.8, 61.4, 33.2, 26.2, 24.8, 21.9, 16.4 ppm; ^{31}P NMR, $CDCl_3$, 81 MHz, δ: 32.32.

Ethyl P-(hex-5-en-1-yl)-N,N-dimethylphosphonamidate 16. Oxalyl chloride (1.97 mL, 23.29 mmol, 3 eq.) was added dropwise via a syringe to a stirred solution of **15** (1.71 g, 7.79 mmol) in CH_2Cl_2 (20 mL). The reaction mixture was stirred for 17 h at room temperature and then the volatiles removed under vacuum. This crude chloride was dissolved in CH_2Cl_2 (10 mL) and added dropwise via a syringe to a stirred solution of dimethylamine (33% in absolute ethanol, 27.7 mL, 155.2 mmol, 20 eq) at 0 ^{o}C. Upon complete addition the mixture was stirred for 5 minutes. The ice bath was removed and the reaction mixture was stirred for 20 hours at room temperature. The reaction mixture was concentrated in vacuo and the residue was purified by column chromatography to yield a yellow oil (1.68 g, 7.67 mmol, 99% yield). ^{1}H NMR, $CDCl_3$, 400 MHz, δ: 5.70-5.82 (m, 1H), 4.89-5.01 (m, 2H), 3.95-4.06 (m, 1H), 3.79-3.90 (m, 1H), 2.65 (d, *J* = 9 Hz, 6H), 2.05 (q, *J* = 7 Hz, 2H), 1.38-1.76 (m, 6H), 1.26 (t, *J*=7 Hz, 3H); ^{13}C NMR, $CDCl_3$, 100 MHz, δ: 138.3, 114.7, 59.0, 36.1, 33.2, 29.9, 25.8, 24.5, 21.6, 16.3 ppm; ^{31}P NMR, $CDCl_3$, 81 MHz, δ: 36.90.

3-(4-(6-((dimethylamino)(ethoxy)phosphoryl)hexyl)-2,6-dimethylphenyl)-1-mesityl-4,5-dihydro-1H-imidazol-3-ium chloride 17. 9-BBN dimer (0.46 g, 1.86 mmol, 0.6 eq.) was weighed in the glovebox and added to a stirred solution of ethyl *P*-(hex-5-en-1-yl)-*N,N*-dimethylphosphonamidate (0.68 g, 3.10 mmol, 1.1 eq.) in dry THF (60 mL). The reaction mixture was stirred for 20 h at room temperature and then concentrated under vacuum. To this residue was added a mixture of [$PdCl_2$(dppf)] catalyst (0.16 g, 0.20 mmol, 7 mol%) and compound **5** (1.15 g, 2.82 mmol), followed by addition of degassed DMF (150 mL). After stirring this mixture for 5 min, $K_3PO_4.H_2O$ (0.73 g, 3.44 mmol, 1.11 eq.) was added and stirring was continued for another 10 min. Then degassed H_2O (8 mL) was added and the reaction mixture was stirred for 20 h at 100 °C (conversion followed by TLC with

CH_2Cl_2/CH_3OH 9:1 *v/v*). The reaction mixture was was concentrated under vacuum at 65 °C. The residue was azeotroped once with heptane and purified by column chromatography to yield a yellow oil (0.55 g, 1.0 mmol, 36% yield). ^{1}H NMR, $CDCl_3$, 400 MHz, δ: 8.84 (s, 1H), 6.93 (d, ^3J = 5.5 Hz, 4H), 4.64 (br s, 4H), 3.90-4.02 (m, 1H), 3.75-3.88 (m, 1H), 2.63 (d, ^3J = 9 Hz, 6H), 2.51 (t, ^3J = 7.5 Hz, 2H), 2.39 (d, ^3J = 5 Hz, 12H), 2.27 (s, 3H) 1.41-1.88 (m, 7H), 1.20-1.41 (m, 6H); ^{13}C NMR, $CDCl_3$, 100 MHz, δ: 159.1, 145.4, 140.7, 135.0, 130.1, 129.4, 59.0, 53.4, 52.2, 36.1, 35.4, 30.8, 28.7, 25.9, 24.6, 22.0, 21.1, 18.2, 16.3 ppm; ^{31}P NMR, $CDCl_3$, 162 MHz, δ: 36.94 ppm.

Metallation and synthesis of inhibitors.

Complex 9. As a representative synthesis, in the glovebox, potassium hexamethyldisilazide (KHMDS, 0.5 M solution in toluene, 0.5 mL, 0.238 mmol, 1.1 eq.) was slowly added to a stirred suspension of preligand **7** (110 mg, 0.217 mmol, 1 eq.) in toluene (25 mL) at room temperature and left stirring for 16 h. To the resulting orange solution, Hoveyda-Grubbs I complex (117 mg, 0.195 mmol, 0.9 eq.) in toluene (5 mL) was added. The brown reaction was taken out of the glovebox, stirred and heated to 65 °C for 2 h and then allowed to cool to room temperature and stirred for other 48 h under inert conditions. At this time the solution took a dark green-brown color. The volume was reduced to ca. 10 mL under vacuum and the solution filtered by cannula. All volatiles were evaporated and the product purified as the light green band by column chromatography with degassed acetone as eluent (Rf = 0.7, 58 mg green oil, 74 mmol, 38% yield). ^{1}H NMR, CD_2Cl_2, 400 MHz, δ: 16.51, 16.45, (s, Ru=CH, 1H), 7.54, 7.08, 7.07, 6.97, 6.97, 6.95, 6.93, 6.91, 6.84, 6.82, (ArH, 8H), 4.83 (C*H*$(CH_3)_2$, 1H), 4.52 (C*H*$_2CH_3$, 2H), 4.15 (N$(CH_2)_2$N, 4H), 2.74 (ArCH_2, 2H), 2.69 , 2.67, 2.12 (ArCH_3, 15H), 2.45, 2.43 (N$(CH_3)_2$, 6H), 2.41 (PCH_2, 2H), 1.46, 1.45 (CH(C*H*$_3)_2$, 6H), 1.28 (ArCH_2C*H*$_2$, 2H), 1.22 (CH_2C*H*$_3$); ^{13}C NMR, CD_2Cl_2, δ: 295.2, 210.7, 206.3, 154.8, 151.9, 145.0, 142.6, 138.8, 131.8, 129.5, 129.3, 129.2, 128.7, 122.3, 122.1, 113.0, 112.9, 75.1, 73.6, 70.6, 58.9, 51.4, 36.4, 36.3, 35.9, 30.5, 28.9, 25.6, 24.2, 20.8, 20.7, 19.1, 16.0; ^{31}P NMR, CD_2Cl_2, 162 MHz, δ: 35.83; ESI-MS m/z $[M\text{-}Cl^-]^+$ calc. 754.2478, found: 754.2560.

Inhibitor 1. As a representative synthesis, to a stirred solution of **9** (50 mg, 0.066 mmol) in CH_2Cl_2 (5 mL), hydrogen chloride solution in diethyl ether 1 M (0.65 mL, 0.65 mmol) was

added with syringe via septum and the solution left stirring at room temperature for 3 h. After this time, all the volatiles were removed under vacuum, then CH_2Cl_2 (5 mL) was added and a prepared solution of *p*-nitrophenol (9.2 mg, 0.066 mmol) and triethylamine (0.020 mL, 0.14 mmol) in CH_2Cl_2 (1 mL) was slowly added at room temperature and left stirring for 2 h. After this time, the volatiles were removed under vacuum and the product purified by column chromatography using degassed acetone as eluent collecting the front green band. Evaporation of solvent resulted in a bright green dense oil (70% yield). ^{1}HNMR, CD_2Cl_2, 400 MHz, δ: 16.54 (s, Ru=CH, 1H), 8.10 (d, *J*=8 Hz, O-Ar-NO_2, 2H), 6.94 (d, *J* = 8 Hz, O-Ar-NO_2, 2H), 7.40, 7.38, 7.07, 6.90, 6.87, 6.82, 6.80 (m, Ar-H, 8H), 4.82, 4.81 (C*H*$(CH_3)_2$, 1H), 4.55, 4.51 (m, OC*H*$_2$CH$_3$, 2H), 4.16 (bs, NCH_2CH_2N, 4H), 3.98 (m, $ArCH_2$, 2H), 2.49-2.40 (m, CH_2P, 2H), 2.13, 2.12, 1.47 (s, $ArCH_3$, 12H), 1.45 (d, J = 8 Hz, CH(C*H*$_3$)$_2$, 6H), 1.33, 1.26, 1.23 (m, $ArCH_2CH_2$, 2H). ^{13}C NMR, CD_2Cl_2, 100 MHz, δ: 302.19, 210.57, 206.68, 155.56, 154.93, 151.95, 144.56, 141.65, 138.91, 129.47, 129.26, 128.65, 125.96, 125.59, 122.28, 122.09, 121.04, 121.04, 121.00, 115.56, 112.91, 75.05, 73.62, 70.69, 63.20, 50.39, 30.57, 26.19, 24.79, 20.93, 19.13. 31P NMR, CD_2Cl_2, 162 MHz, δ: 29.90. ESI-MS m/z $[M]^+$ calc. 883.1858, found 883.1739.

Complex 10. A similar procedure as for the synthesis of **9** was followed, here using **8** (78 mg, 0.129 mmol), KHMDS (0.26 mL, 0.5 M solution in toluene), Hoveyda-Grubbs I (59.6 mg, 0.099 mmol), column chromatography using degassed acetone followed by MeOH as the eluent (bright green dense oil, 45 mg, 51% yield). ^{1}H NMR, CD_2Cl_2, 400 MHz, δ: 16.41, 16.36 (Ru=CH), 7.53, 7.52, 7.38, 7.19, 7.04, 6.86 (ArH, 9H), 4.90 (O-C*H*$(CH_3)_2$, 1H), 4.16-3.98 (NCH_2CH_2N), 3.56, 3.44, 3.42, 2.83, 2.81, 2.68 (m, O-CH(C*H*$_3$)$_2$, 6H), 2.12, 1.25 (N(C*H*$_3$)$_2$, 6H), 1.23, 1.22 (ArCHC*H*$_3$, 24H), 1.14 (CH_2C*H*$_3$). ^{13}C NMR, CD_2Cl_2, 100 MHz, δ: 289.30, 213.76, 213.09, 163.45, 152.13, 149.14, 147.67, 147.66, 147.42, 144.03, 142.41, 142.30, 129.13, 124.19, 122.19, 121.95, 112.84, 74.93, 65.593, 58.927, 58.861, 48.892, 36.789, 36.631, 35.923, 35.883, 30.537, 28.664, 26.250, 23.897, 23.046, 21.455, 16.179, 16.114. ^{31}P NMR, CD_2Cl_2, 162 MHz, δ: 37.97. ESI-MS m/z $[M+H^+]^+$ calc. 888.3341, found 888.3325, $[M-Cl^-]^+$ calc. 852.3583, found 852.3556.

Inhibitor 2. A similar procedure as for the synthesis of **1** was followed, here using **10** (15.9 mg, 0.018 mmol), HCl (0.358 mmol, 0.358 mL), *p*-nitrophenol (2.5 mg, 0.018 mmol), triehtylamine (0.18 mmol, 0.025 mL). Column chromatography with degassed acetone (10 mg of a green dense oil, 57% yield). ^{1}H NMR, CD_2Cl_2, 400 MHz, δ: 16.43 (Ru=CH), 8.21 (d, *J* =

8 Hz, O-ArH-NO_2, 2H), 8.12 (d, *J* = 8 Hz, O-ArH-NO_2, 2H), 7.39, 7.37, 7.17, 6.90, 6.85, 6.81 (m, ArH, 9H), 4.87 (m, OC*H*$(CH_3)_2$, 1H), 4.30-4.18 (overlap C*H*$_2$CH$_3$, NCH$_2$CH$_2$N, 6H), 3.56 (m, ArC*H*$(CH_3)_2$, 4H) 3.44, 2.87 (m, ArCH_2, 2H), 2.62, 2.15, 2.13, 1.97 (m, ArCH_2C*H*$_2$, 2H), 1.26 (ArCH(C*H*$_3$)$_2$, 24H) 1.22 (OCH(C*H*$_3$)$_2$, 6H), 1.14 (CH_2C*H*$_3$, 3H). ^{13}C NMR, CD_2Cl_2, 100 MHz, δ: 285.22, 212.97, 162.08, 152.15, 149.40, 148.99, 144.01, 142.00, 129.56, 129.27, 125.96, 125.58, 124.33, 124.11, 122.17, 121.92, 121.05, 121.00, 120.92, 115.52, 112.88, 74.93, 63.24, 52.83, 29.63, 28.96, 28.80, 28.58, 26.51, 26.12, 23.15, 22.91, 21.46, 16.18, 16.12. ^{31}P NMR, CD_2Cl_2, 162 MHz, δ: 30.10. ESI-MS m/z $[M\text{-}Cl^-]^+$ calc. 946.3265, found 946.3100.

Complex 18. A similar procedure as for the synthesis of **9** was followed using **17** (70 mg, 0.127 mmol), KHMDS (0.25 mL, 0.5 M solution in toluene), stirring for 5 h (this first step of the reaction requires less stirring time due to the better solubility of **17** in toluene, in comparisson with **7** and **8**), Hoveyda-Grubbs I (72.0 mg, 0.12 mmol), column chromatography using degassed acetone (bright green oil, 39 mg, 39% yield). ^{1}H NMR, CD_2Cl_2, 400 MHz, δ: 16.52, 16.46 (Ru=CH, 1H), 7.54, 7.07, 6.97, 6.95, 6.90, 6.82 (Ar-H, 8H), 4.88 (m, CH_2CH_3, 2H), 4.15 (s, NCH$_2$CH$_2$N, 4H), 3.95, 3.85 (m, C*H*$(CH_3)_2$, 1H), 2.66, 2.64 (s, ArCH_3, 15H), 2.60 (m, ArCH_2, 2H), 2.44, 2.40 (s, N$(CH_3)_2$, 6H), 1.84-1.64 (tether -CH_2-, 10H) 1.46 (d, J=8 Hz, CH(C*H*$_3$)$_2$, 6H), 1.21 (m, CH_2C*H*$_3$, 3H). ^{13}C NMR, CD_2Cl_2, 100 MHz, δ: 295.15, 211.06, 210.63, 152.02, 151.92, 145.22, 145.06, 143.79, 138.80, 129.52, 129.31, 128.57, 122.46, 122.22, 122.07, 113.00, 112.87, 75.04, 58.76, 52.84, 35.84, 35.53, 30.58, 29.03, 26.27, 25.82, 24.53, 22.17, 20.89, 16.08. ^{31}P NMR, CD_2Cl_2, 162 MHz, δ: 36.37.

Inhibitor 3. A similar procedure for the synthesis of **1** was followed using **18** (11.8 mg, 0.0142 mmol), HCl (0.14 mmol, 0.142 mL), *p*-nitrophenol (2 mg, 0.0142 mmol), triehtylamine (0.18 mmol, 0.025 mL), 97% yield of green oil. ^{1}H NMR (400 MHz, CD_2Cl_2, 400 MHz, δ: 16.55, 16.47 (Ru=CH, 1H), 8.20, 8.09, 7.51, 7.37, 7.06, 6.93, 6.87, 6.82 (Ar-H, 12H), 4.87 (m, C*H*$_2$CH$_3$, 2H), 4.20 (m, C*H*$(CH_3)_2$, 1H), 4.16 s, NCH$_2$CH$_2$, 4H), 2.65 (m, ArCH_2, 2H), 2.44, 2.40 (s, ArCH_3, 15H), 2.01, 1.70, 1.57, 1.49, 1.33 (m, tether -CH_2-, 10H), 1.21 (d, CH_2C*H*$_3$, 3H). ^{13}C NMR, CD_2Cl_2, 100 MHz, δ: 298.14, 210.96, 207.58, 161.64, 152.23, 145.23, 143.46, 141.46, 138.95, 129.66, 129.35, 128.68, 126.14, 125.72, 122.61, 122.20, 121.00, 120.95, 120.40, 115.84, 112.92, 75.00, 63.23, 51.60, 35.50, 30.94, 30.27, 28.86, 26.64, 25.25, 22.19, 21.11, 21.08, 16.34. ^{31}P NMR, CD_2Cl_2, 162 MHz, δ: 30.39. ESI-MS m/z $[M]^+$ calc. 925.2327, found 925.2250.

Hybridization reactions. In a typical procedure, to a stirred solution of Cutinase (50 μM in degassed TrisHCl pH 8.5 or sodium acetate buffer pH 5, 50 mM), inhibitor **1**, **2** or **3** (10 equivalents with respect to cutinase) in degassed CH_2Cl_2 (0.5 mL) was slowly added via septum with a syringe; the volume of CH_2Cl_2 was 1/19 of the volume of buffer so that it accounted for 5% v/v in the mixture. The reaction was stirred vigorously at 25 ^oC for 24 h. Then the content was transferred to a 250 mL round bottom and placed in rotary evaporator with fast spin at 25 ^oC to eliminate the CH_2Cl_2. The solids were filtered off three times and the filtrate was transferred to Vivaspin 6® centrifugation tubes with PEG membrane of 10 kDa M.W. C.O. and centrifuged (3 × 1.5 h at 3500 rpm) for dialysis, restoring the volume each time with Milli-Q water and finally to 9.5 mL with buffer (50 mM). The resulting solutions were stored at – 20 °C in degassed vessels. For ESI-MS analyses, an aliquot of 0.5 mL was further dialysed and treated with formic acid for its denaturation before injection.

Catalysis. To a Schlenk flask charged with a stirring bar and a solution containing N,N-diallyl *p*-toluenesulfonamide –or allylbenzene– and bibenzyl (0.1 mL of 10 mM, 1 μmol), the catalytic hybrid in solution (1 mL of 50 μM, 0.05 μmol) was added. The solution was stirred vigorously for 20 h at room temperature. After this time, the organic fraction was extracted with CH_2Cl_2 (3 x 6 mL), concentrated in rotary evaporator and dissolved in 0.05 mL of CH_2Cl_2 for GC analysis.

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Nature composes some of her loveliest poems
for the microscope and the telescope.
Theodore Roszak

4

Selective C-C coupling with covalent palladium artificial enzymes

Abstract

A series of palladium N-heterocyclic carbene complexes bearing lipase inhibitory tethers have been covalently anchored in the active site of Cutinase as a means to produce a 2nd coordination sphere around the metallic center influencing its selectivity when used as allylic alkylation catalysts. The resulting hybrids did not react with 1,3-diphenylallyl acetate likely due to hampered substrate accessibility, but allowed the reaction of the smaller 1-phenylallyl acetate to take place. The protein scaffold also directed the regioselectivity of the catalyst, from the major linear product when unsupported, to exclusive branched C-C coupling product when hybridized. These catalysts represent the first covalent palladium artificial metalloenzymes and show the suitability of the active site-directed approach to promote regioselectivity in product formation.

Introduction

Homogeneous palladium catalysts have become an essential tool in organic synthesis, particularly for the formation of carbon-carbon bonds.[1] A wide spectrum of C-C reactions are catalyzed by palladium complexes, for example the cross coupling reactions of aryl (or vinyl) halides with organozinc compounds (Negishi), alkenes (Heck), boronic acids (Suzuki), organotin compounds (Stille), organosilicon compounds (Hiyama), alkynes (Sonogashira), amines (Buchwald-Hartwig) and Grignards (Kumada).[2] Widely studied and merited the Nobel Prize, the optimization of catalytic reactivity has not only depended on reation conditions conditions but strongly relied on the coordination sphere of the metallic center, i.e. the ligand environment. It are the ligands that have become the key directing tool for the development of active and selective catalysts, for example, resulting in the development of privileged bulky and chiral ligands.[3]

In spite of the successful advance of palladium catalysis through ligand design, new tools for the promotion of catalyst selectivity and support for their use in alternative reaction media are in demand to facilitate catalyst synthesis and for the use of renewable sources for their building blocks. A relatively new different approach for the creation of selective and water-soluble homogeneous transition metal catalysts is the development of artificial metalloenzymes.[4] These catalysts are semi-synthetic hybrids where an active, yet non-selective, metallic catalyst is supported on a protein scaffold, surrounding the former by an amino acid environment that can generate a so called 2nd coordination sphere and induce catalytic selectivity.[5] Some features of these hybrids are that synthetic chiral components are not needed because of the natural availability of chiral proteins and enzymes that are chosen as hosts. Besides this, the hybridization of the active catalysts grants them new physical properties such as increased size and solubility in aqueous media. As can be anticipated, the conjugation technique used in the construction of such hybrids has a fundamental impact on the catalytic selectivity of the hybrid. Aspects to consider include the nature of the interaction between the organometallic and protein fragments, and the location of modification of the protein.

A number of transition metals has been explored to develop artificial metalloenzymes, including Rh, Ru, Os, Cu, Mn, and Fe.[6] The field represents as well an appealing strategy for the creation of catalytic palladium hybrids. In this respect, our group reported the first examples of structurally defined palladium-based hybrids, produced by the covalent anchoring of ECE-pincer palladium complexes to the active site of a lipase.[7] This active site-directed (ASD) approach made use of the irreversible inhibitory reactivity of phosphonate esters forming a covalent bond with the active site of serine hydrolases and the rationalized functionalization of such phosphonate esters with a pincer ligand coordinated to the palladium center (Figure 1, left). Palladium and platinum hybrids represented the proof of principle of this ASD anchoring strategy, which included the first crystal structure of a Pd-protein species.[8] Other successful covalent approaches by others make use of alkylation of amino acid residues (Cys, Lys), which are sometimes present in more than one specific position in the protein host. On the other hand, other single site-directed methods involve non-covalent inhibitions of enzymes, the use of artificial cofactors (in cofactor reconstitution) and the supramolecular association of (strept)Avidin with functionalized biotin.[5] We believe that the ASD method promises robustness of the constructed hybrid due to the covalent conjugation character facilitating its purification and analysis and, in addition, presents a promising means to confer a 2nd coordination sphere (sterics and chirality) around the metallic center because of its location in a naturally selective pocket, i.e. the active site of the enzyme host.

In spite of the succesful hybridization of the pincer complexes, the activity of the resulting hybrids is reduced in the mild conditions needed to preserve the protein's tertiary structure,[9] since pincer complexes are very stable organometallic species, thus often requiring higher temperatures and activating reagents for their catalytic activity. After these examples, other structurally defined artificial enzymes hosting homogeneous palladium centers have been developed independently by Watanabe[24] and Ward[10] in 2008. The former performed the addition of $[Pd(\eta^3\text{-allyl})(\mu\text{-Cl})]_2$ species with apo-Ferritin leading to the coordination of multiple palladium centers to different His, Glu and Cys residues of the protein host (Figure 1, right). On the other hand, Ward reported artificial enzymes based on the supramolecular biotin-Avidin anchoring strategy (Figure 1, middle), with the biotin moiety bearing phosphine ligands that were also metallated with $[Pd(\eta^3\text{-allyl})Cl]$ species in

situ, achieving the first catalytic hybrids for asymmetric allylic alkylations.[10] Directed evolution of Streptavidin allowed for the promotion of enantioselectivity in this reaction, proving once more the interest in these constructs. Apart from the mentioned reports and to the best of our knowledge, no other studies of active artificial enzymes with homogeneous palladium fragments, in spite of its attractiveness.

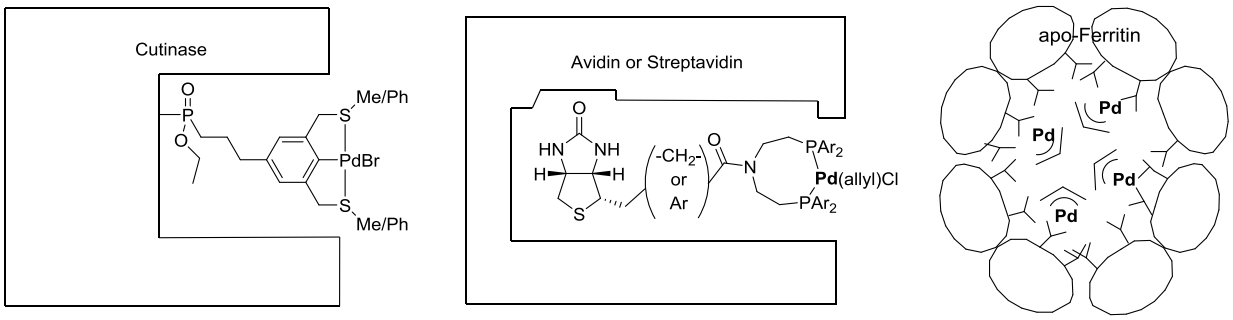


Figure 1. Artificial enzymes with homogeneous palladium centers reported before this work: the covalent ASD protein-SCS metallopincer hybrids (left), supramolecular biotin-Avidin metallophosphine hybrids (center), and apoferritin-Pd hybrids (right).

In the previous chapters, the development of ASD hybrids was continued substituting the pincer ligands with N-heterocyclic carbene (NHC) ligands. The latter motif is well known to form robust bonds with a majority of transition metals using a non-chelating, single σ donating bond towards the metallic center, also resulting in a number of complexes catalytically active in aqueous media.[11] This has led to ASD Rh(NHC)-protein hybrids that showed an increase of the chemoselectivity of the rhodium catalyst compared to unsupported species,[12] and to Ru(NHC)-protein hybrids active in ring-closing and cross metathesis.[13] We believe that the corresponding ASD Pd(NHC)-phosphonate species are interesting target compounds for the construction of active artificial metalloenzymes based on palladium.

The asymmetric allylic alkylation (AAA) is an interesting C-C bond forming reaction that allows for both enantio- and regioselectivity, leading to enantiomeric product excesses and to preferred formation of linear of branched products. Enantiomeric excess comes as a

result of control over the (endo and exo) attack of the nucleophile to a symmetric η^3-allyl fragment at either terminal carbon center (Figure 2, top).

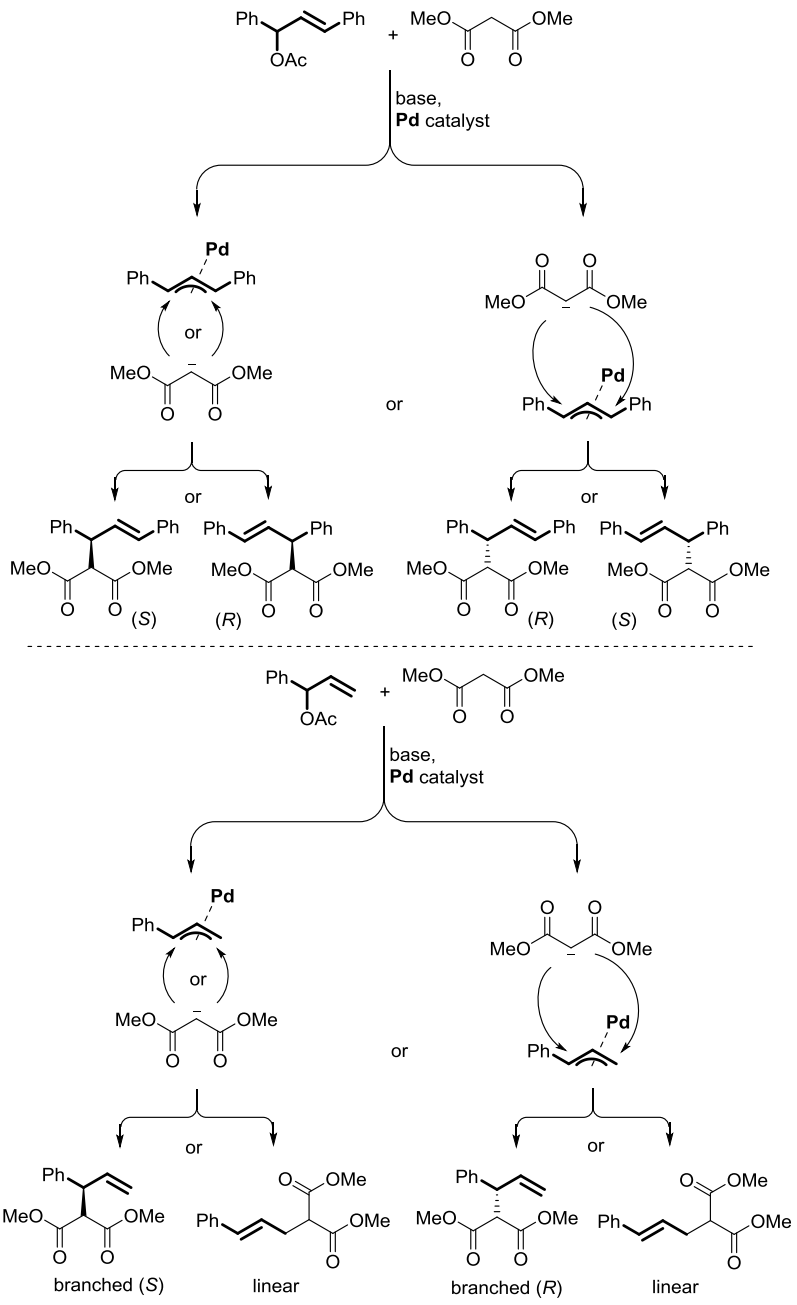


Figure 2. Two mechanistic possibilities (exo, endo) leading to ee in AAA using the substrate 1,3-diphenylallyl acetate (top) and two mechanistic possibilities leading to ee and linear/branched products in AAA using the substrate 1-phenylallyl acetate.

In a similar manner, control over the formation of branched and linear products is achieved in the case of mono-substituted allyl substrates (Figure 2, bottom). To our knowledge, the latter type of product outcome in AAA has not been studied with artificial enzymes nor Pd(NHC) catalysts in general. Nonetheless, in terms of activity and enantioselectivity, this reaction has indeed received attention when conducted with low molecular weight Pd(NHC) complexes, which resulted in excellent catalysts, affording the alkylation of 1,3-diphenylallyl acetate with dimethyl malonate in organic media (CH_2Cl_2), first reported in 2005 by Sato.[14] Under aqueous conditions, the same transformation has been studied with almost quantitative formation of the desired product by Roland and Mangeney.[15] Enantioselective conversions have been achieved with chiral substitutions at the backbone of the carbene ligand by the same authors. Interestingly, Pd(NHC) catalysts also including oxygen, phosphorous or nitrogen donor ligands, additional to the NHC ligand or as part of heterobidentate ligands, show the best activity (Figure 3).

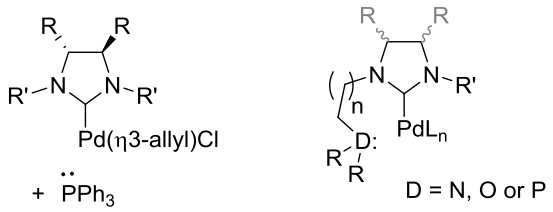


Figure 3. General structure of enantioselective Pd(NHC) catalysts for allylic alkylation.

In the present work Pd(NHC)-phosphonate esters were designed for the formation of artificial Pd-enzymes by covalent inhibition of lipases. Their activity, enantio- and regioselectivity in AAA with 1,3-diphenylallyl acetate and 1-phenylallyl acetate, and the role of a phosphine as aditive was studied (Figure 4). In this study, a limitation in the accommodation of substrates in the active site of the hybrids was discovered, where the former substrate could not be converted while the latter lead to conversion and showed a change in product regioselectivity in comparison with the unsupported catalyst.

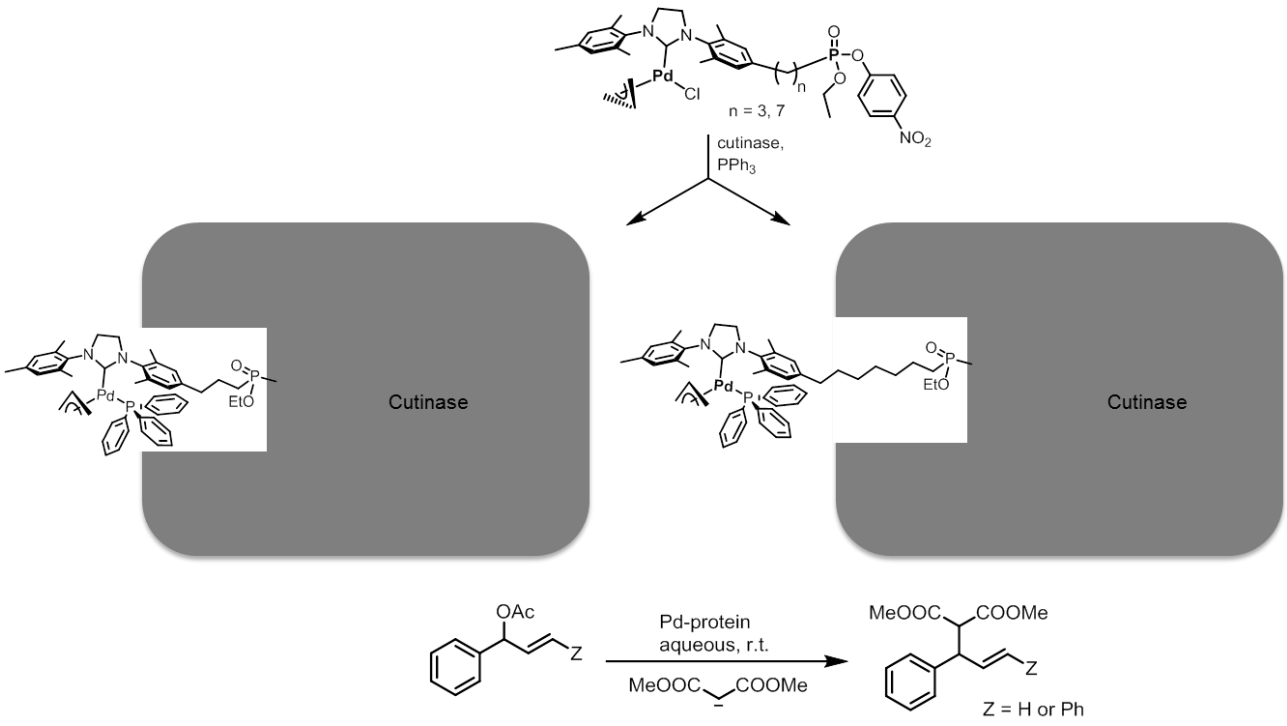
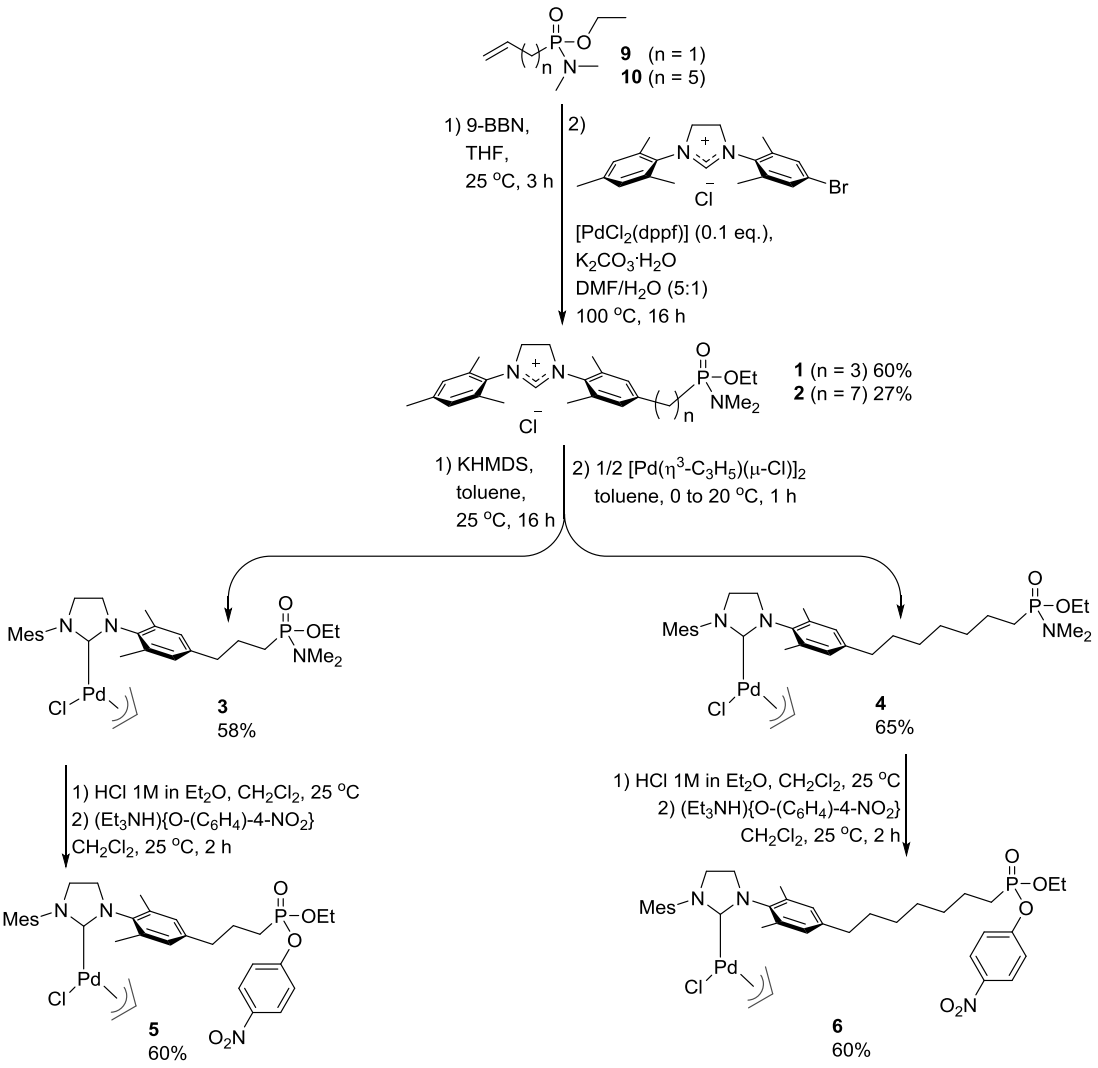


Figure 4. Main study of this work: the allylic alkylation of phenylallyl substrates using artificial Pd-enzymes with the active site in different positions with respect to the protein host.

Results

The design of the hybrids followed the approach that was reported for metal(NHC)-protein hybrids with an orthogonal orientation between the organometallic fragment and the protein backbone.[12,13] For this the NHC-phosphonate moiety plays an important role, thus a series of these cofactors was prepared employing the reported method including the cross-coupling reaction of a halogenated imidazolinium salt (the carbene precursor) and two different olefin-terminated tethered phosphonamidate compounds, producing compounds **1** and **2** (see Scheme 1). Our interest in cofactors of different length comes from recent observations with rhodium and ruthenium derivatives, where in one case a metallic center bound to the protein by a short tether was able to present good catalytic performance (hydrogenation of an olefin),[12] while in another case, a hindering effect of the protein on the short-tethered hybrid was overcome by elongation of the tether allowing for better catalytic performance (olefin metathesis).[13]

The phosphonamidate functionality in **1** and **2** was sufficiently stable in the presence of the organic, non-nucleophillic base KHMDS to allow for selective deprotonation of the imidazolinium moiety to form a carbene-Pd bond upon reaction with $[Pd(C_3H_5)Cl]_2$. The dimeric palladium starting material is split to form two equivalents of $[PdCl(NHC)(\eta 3\text{-}C_3H_5)]$ in each reaction forming products **3** and **4**. The η^3-allyl ligand is common in Pd precatalysts for allylic alkylation studies.[14]



Scheme 1. Synthesis of the Pd-based lipase inhibitors **5** and **6**.

During our metallation studies the production of dimeric Pd species was observed as side products, which was later circumvented by lowering the concentration of reactants in the

reaction medium. Figure 5 exemplifies the dimeric species derived from **3** and **4** observed by ESI-MS. This phenomenon appears more commonly in Pd(allyl)(Cl) species and has also been observed with other Pd(NHC)(allyl) species.[16] A different ratio between the monomeric and dimeric species was noticed in the ESI-MS spectra of **3** and **4**. The lower amount of dimerization of **3** in comparison to **4** may originate from the shorter distance between the Pd center and the phosphonate moiety in **3** hampering its dimerization. The desired monomeric complexes were isolated by column chromatography.

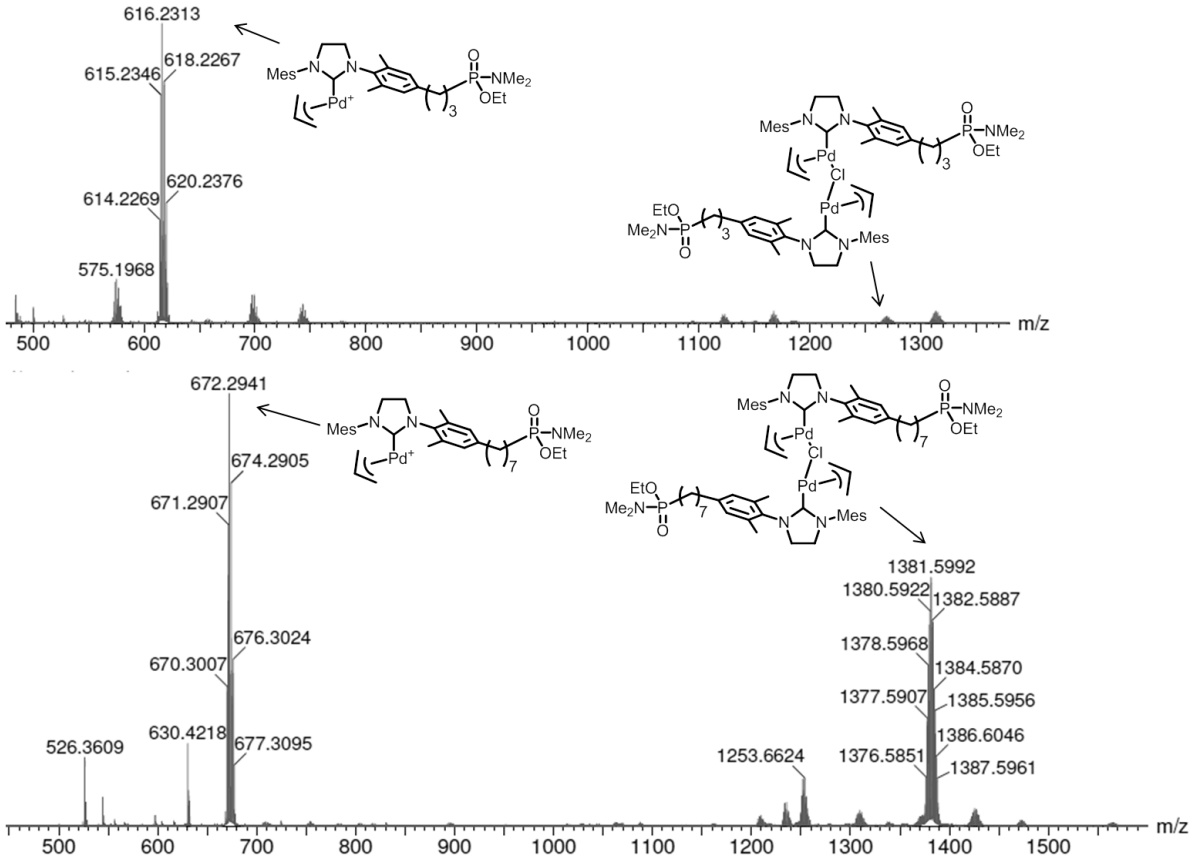
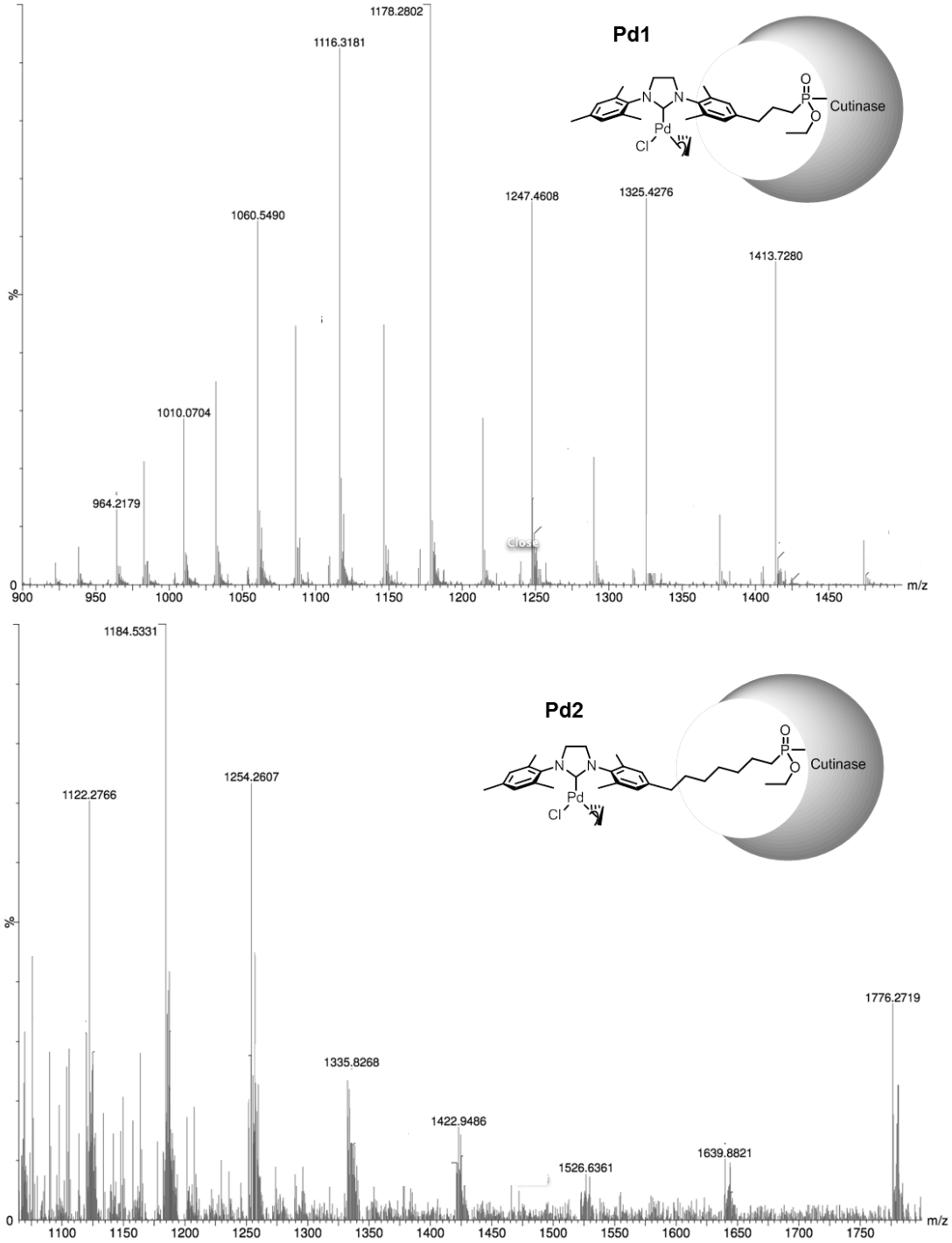


Figure 5. ESI-MS spectra of complexes **3** and **4**.

The final step towards a lipase inhibitor involved conversion of the phosphonamidate tail to a phosphonate tail. For this the dimethylamino group was substituted by a chloride and further substituted again in situ by *p*-nitrophenolate as depicted in Scheme 1. The desired compounds **5** and **6** were characterized by ESI-MS and NMR, although our attempts to

isolate these products by column chromatography were challenging and often resulted in some decomposition according to ^{31}P NMR. Therefore the crude species were used subsequently to modify the enzyme.

In line with the expertise with artificial metalloenzymes derived from Cutinase from *fusarium solani pisi* as the lipase host, this enzyme was again the choice for the formation of palladahybrids. Cutinase features the lack of a lid protecting the active site, which allows easier access of reactants to the active site and yet it shows enantioselectivity when inhibited with racemic phosphonate esters. The modification of Cutinase was performed by its reaction in aqueous TrisHCl buffer at pH 8.5 with an excess of each of the palladium inhibitors **5** or **6** in CH_2Cl_2 (5% *v/v*). This excess was used because of the enantioselective behavior of Cutinase with respect to racemic phosphonate esters, thus stoichiometric use of organometallic inhibitors would result in partial modification only, which is circumvented with a 3-fold excess of the racemic inhibitor. After 24 h at ambient temperature, the organic solvent was eliminated and the solids completely removed from the reaction mixture. The yellow solutions, originating from the formation of *p*-nitrophenolate from the inhibitory reaction, were submitted to repeated centrifugal filtration, restoring the volume to 50 μM with water and then with the desired buffer for catalysis, as will be explained below. This treatment of the hybrids allows the elimination of the excess of metallic inhibitor, as well as *p*-nitrophenolate, buffer and other salts and organic species.



Pd1									
nH^+	23	22	21	20	19	18	17	16	15
Calc.	922.2	964.1	1010.0	1060.5	1116.33	1178.4	1247.7	1325.6	1414.0
Found	922.4	964.2	1010.1	1060.5	1116.25	1178.2	1247.5	1325.4	1413.6
Pd2									
nH^+	19	18	17	16	15	14	13	12	11
Calc.	1124.9	1187.3	1257.1	1335.6	1424.5	1526.2	1643.6	1780.4	1942.2
Found	1122.4	1187.5	1258.6	1335.8	1424.7	1526.6	1643.8	1780.8	1942.3

Figure 6. ESI-MS spectrum of **Pd1** (top), **Pd2** (bottom) and calculated and observed values.

Aliquots in water of the hybrids were denaturated with 10% formic acid and submitted to ESI-MS analyses. The resulting spectra show typical patterns of protonated protein material where each peak belongs to a $[M + nH^+]^{n+}$ species, in each confirming a 1:1 conjugation of the Pd-protein constructs and also proving the covalent stability of the hybrids because of the denaturing conditions for their analysis (Figure 6). This shows the successful modification of Cutinase with the palladium inhibitors **5** and **6** towards the cutinase hybrids **Pd1** and **Pd2**, respectively, and corresponds, next to the protein ECE-metallopincer hybrids,[7] to the only covalent Pd-protein hybrids reported to date.

For the catalytic studies, appropriate reaction conditions were looked for activity, testing the similar but unsupported complex [Pd(SIMes)(C_3H_5)Cl] (**Pd3**), where SIMes is the common NHC ligand 1,3-dimesityl-4,5-dihydroimidazolin-2-ylidene. The above-mentioned allylic alkylation reactions of 1,3-diphenylallyl acetate (**7**) with malonate have been studied in both organic as well as mixed solvent systems. Mori et al. reported that in purely organic solvents like THF, temperatures of 50 ^oC (up to reflux) are needed alongside with deprotonated malonate, e.g. $NaCH(CO_2Me)_2$, as the nucleophile or a base in the reaction system to generate the nucleophile in situ.[14] These are harsh conditions to use in combination with an enzyme. When Cutinase alone was explosed to these conditions a milky colloid was formed, due to denaturation of the enzyme (similar observation when exposing Cutinase to THF). Moreover, these earlier reports speak about the alternative, in situ formation of the Pd(NHC) species from imidazolium salts and a base present in the reaction mixture, which is also not applicable to the current purpose.[14] In aqueous mixtures, malonate and carbonate bases have been combined at ambient temperature, leading to good alkylation yields with phosphine-based complexes or Pd/C with PPh_3 as an additive.[17,18] Good yields with Pd(NHC) systems at ambient conditions in aqueous mixtures have only been met by two strategies: the use of C-N bidentate NHC-amine ligands or by use, again, of a phosphine additive (Figure 3, vide supra).[15] A catalytic experiment with **Pd3** (5% mol) in CH_2Cl_2/H_2O mixtures (1:1 and 1:10, *v/v*) with or without a base (NaOH, 2 eq.) at room temperature for 20 h showed as well no conversion, while the system afforded a high product yield when a 1:1 ratio of **Pd3**/PPh_3 was used (CH_2Cl_2/H_2O 1:10 and NaOH, 2 eq.) and excellent yield when 2 equivalents of PPh_3 were used (Table 1, Entries 1 to 3; see below).

The need of catalytic amounts of PPh_3 could imply a potential mechanistic disadvantage for the eventual promotion of selectivity by the NHC-protein hybrid, namely the substitution of the NHC ligand for PPh_3, resulting in a release of Pd from the hybrid. Alternatively, PPh_3 could substitute the chloride ligand, leading to an activated, [Pd(SIMes)(PPh_3)(C_3H_5)]Cl (**Pd3P**) species. These two pathways are shown in Figure 7.

The reaction of [Pd(NHC)(C_3H_5)Cl] species with PPh_3 was recently studied by Cavell, clearly showing that the formation of [Pd(NHC)(PPh_3)(C_3H_5)]Cl species occurs, according to evidence in solution and solid state (X-ray structures of these structures are available).[19] The reported complexes, however, did not include the SIMes ligand, nor were the isolated species studied for allylic alkylation. We, therefore, conducted a series of experiments to determine the chemical behavior of **Pd3** with triphenylphosphine and determine the activity of the resulting species in the allylic alkylation.

After its reaction of **Pd3** with PPh_3, **Pd3P** was detected by ESI-MS (Figure 7) and NMR in the resulting reaction mixture. The phosphine in this product is labile, as evidenced by the observation of several signals by ^{31}P NMR after a few days. Ion exchange towards [Pd(SIMes)(PPh_3)(C_3H_5)]BF_4 afforded a more robust species in good purity. This complex was characterized by ESI-MS (*m/z* = 715.24), a change in chemical shift for PPh_3 from -5 (free) to 21 ppm in ^{31}P NMR, in accordance to the report by Cavell,[19] and by a non-coordinating tetrafluoroborate anion according to ^{19}F NMR (δ -153.59 ppm). Importantly, no product of Pd-NHC bond cleavage was observed, i.e. no formation of $[Pd(PPh_3)(C_3H_5)]^+$ species was observed by ESI-MS (Figure 7).

We propose that the activation of Pd(NHC) centers for allylic alkylation by coordination with phosphines may originate in the π–accepting character of the phosphine producing a more electrophillic η^3-allyl ligand, which is more prone to nucleophilic attack by forthcoming substrates.

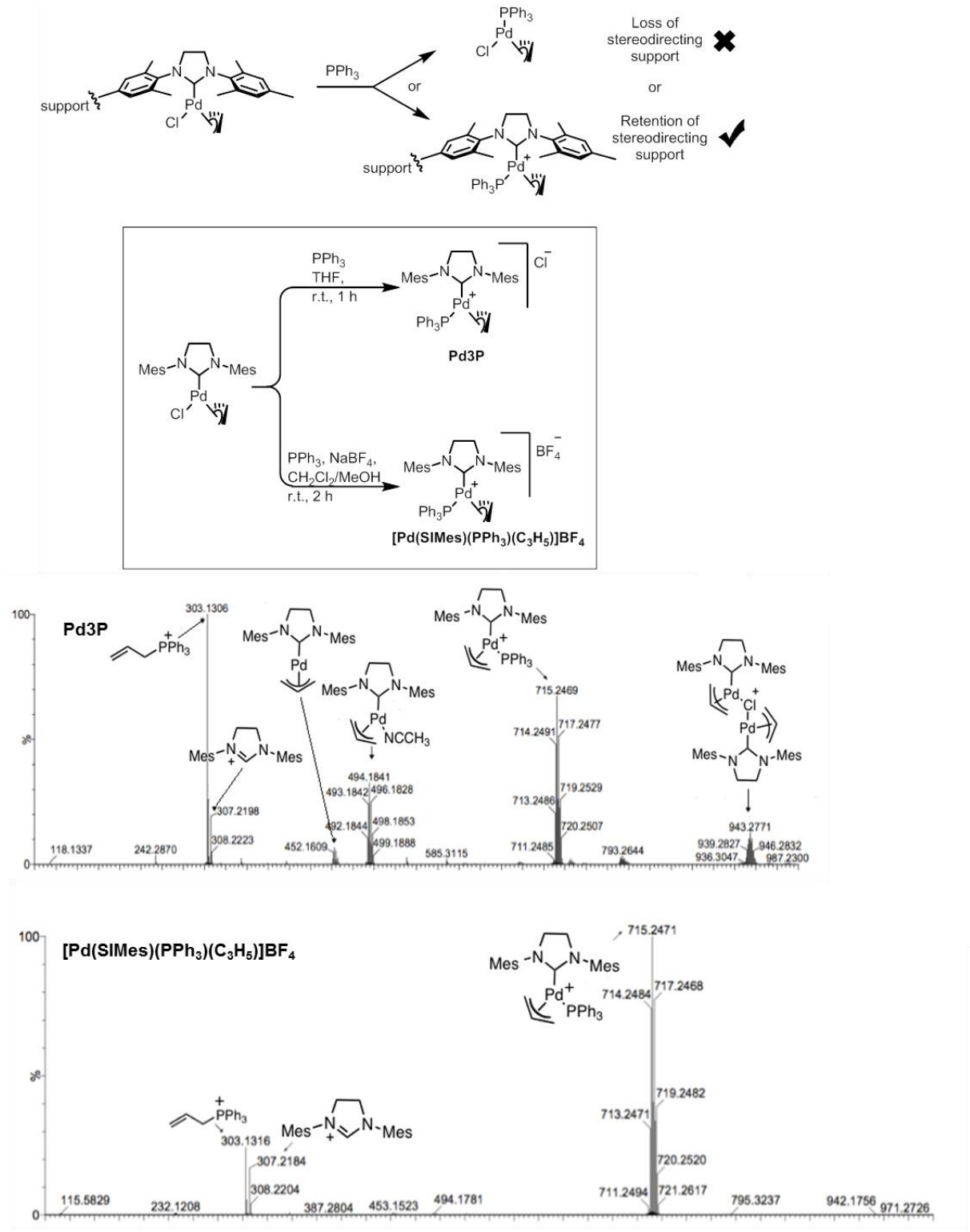


Figure 7. Synthesis and ESI-MS spectra of [Pd(SIMes)(PPh_3)(C_3H_5)]Cl (**Pd3P**) and [Pd(SIMes)(PPh_3)(C_3H_5)]BF_4.

Having established the integrity of the Pd-NHC bond, the catalytic properties of hybrids **Pd1** and **Pd2** were investigated. In these experiments the basicity of the reaction medium was increased with carbonate buffer to pH 10.5 to secure the presence of the malonate anion. A surfactant was also used to promote a proper mixing of the binary solvent system containing a low percentage of CH_2Cl_2 (10%, *v/v*). It has been shown that cutinase still shows activity at this pH, while a higher pH could result in a change of its tertiary structure.[20] Using the new buffer medium, an excellent yield of alkylated diphenylallyl product (99%) was afforded with catalyst **Pd3**. Subsequently, hybrids **Pd1** and **Pd2** were used as catalyst under the same conditions, showing no product formation in either case (Table 1, Entries 4 to 6).

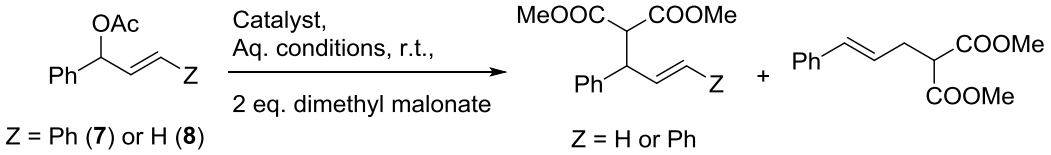
As mentioned before, previous studies with short tethered inhibitors had shown that Ru-protein hybrids had low or no activity due to plausible sterical hindrance at the active site of the protein scaffold.[13] It was also observed that the anchoring of the metallocatalyst in the active site of a lipase with the active pocket in a more constricted invironment (CalB) also resulted in reduced catalytic activity. These considerations could point to the requirement for a lipase with a larger cavity in the active site, but to our knowledge, cutinase presents the most exposed active site among lipases. On the other hand, the solid state structure of metal-Cutinase pincer hybrids[7b] and the catalytic findings with ruthenium-Cutinase hybrids[13] suggest a good accessibility of chemical reactants to the metallic center in these hybrids. Thus, in pursuit of activity with the Pd(NHC)-cutinase hybrids, a different substrate was investigated instead of a different lipase or further elongation of the tether chain.

Accordingly, the allyl substrate with only one phenyl substituent was chosen, i.e. 1-phenylallyl acetate (**8**). This substrate has been widely used as a substrate in allylic alkylation with palladium catalysts, although, to our knowledge, Pd(NHC)-based catalysts have not been tested. Interestingly, iridium and molybdenum complexes have been used as a means to afford the branched catalytic product in reactions using the malonate nucleophile.[21,22]

Pd3 (5 mol%) was tested in the reaction of **8** with malonate in carbonate buffer pH 10.5 with CH_2Cl_2 (10%, *v/v*) and PPh_3 (10 mol%). This led to full conversion (Table 1, Entry 7), showing that this type of palladium system promotes the alkylation of both substrates tested here. A linear/branched product ratio of 18:1 for the latter reaction was found. Under

these conditions, hybrids **Pd1** and **Pd2** also showed activity, albeit that products were only obtained in 8% and 10% yield, respectively (Table 1, Entries 8 and 9). Remarkably, both hybrids selectively catalyze the formation of the branched allylation product only (with only traces of linear product being formed in the case of **Pd2**).

Table 1. Allylic alkylation with dimethyl malonate of 1,3-diphenylallyl acetate (**7**) and 1-phenylallyl acetate (**8**), using Pd catalysts.[a]



Entry	Substrate	Catalyst	PPh_3/Catalyst ratio	Solvent system[b]	Yield (%)[c]	linear:branched ratio
1	**7**	**Pd3**	-	I	-	
2	**7**	**Pd3**	-	II	-	
3	**7**	**Pd3**	1	II	98	
4	**7**	**Pd3**	2	III	>99	
5	**7**	**Pd1**	2	III	-	
6	**7**	**Pd2**	2	III	-	
7	**8**	**Pd3**	2	III	>99	18:1
8	**8**	**Pd1**	2	III	8	only branched
9	**8**	**Pd2**	2	III	10	only branched[d]

[a] All reactions carried for 20 h at room temperature with 2 eq. of dimethyl malonate and 0.05 eq. of catalyst with respect to the allylic substrate.
[b] **I**: CH_2Cl_2:H_2O 1:1, no base; **II**: CH_2Cl_2:H_2O 1:1, 2 eq. NaOH; **III**: CH_2Cl_2:H_2O 1:10, Na_2CO_3/$NaHCO_3$ buffer pH 10.5, with 2 eq. HDTMA as surfactant.
[c] Determined by GC/MS or chiral GC using bibenzyl as internal standard.
[d] Traces of linear observed.

These results represent the second example (after the work of Ward)[10] of (Pd-based) artificial enzymes for allylic alkylations. Notably, the reactivity of **Pd1** and **Pd2** is lower than

that of their unsupported counterpart **Pd3**, while a close to perfect selectivity to branched alkylation product was obtained with the hybrids, i.e. towards the least favored product in the case of **Pd3**. Due to the nature of the two substrates tested, it is believed that the regioselective product formation originates from the easier accommodation of the η^3-allyl-Pd derivative of **8** in the pocket of cutinase because of its smaller size compared to the derivative of **7** bearing one more phenyl ring.

Clearly the volume of the Pd(NHC) inhibitor is larger than the size of either substrate. On accommodating a substrate in the active site, it needs to be considered that the Pd(NHC) inhibitor already takes space in the anchoring site, reducing the available volume for the catalytic reaction. Constriction occurs especially because of the different angle of the Pd(NHC) plane (plane A) and the plane of the substrate (the flat η^3-allyl moiety; plane B); the former carrying the directionality of the tether chain towards the active site. This almost orthogonal angle is found in the crystal structure of [Pd(NHC)(η^3-allyl substrate)(phosphine)]$^+$ species[19] as represented in Figure 8.

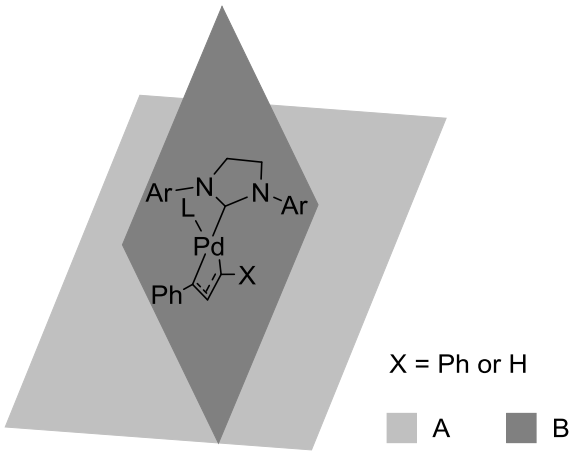


Figure 8. Orthogonality between the NHC–Pd plane (A) and allyl plane (B). Adapted from Cavell[19].

The surrounding of the catalyst by the protein can be conceived to produce a hindrance impact in an area around the metallic center with a plane orthogonal to the direction of the tether chain, i.e. in the allyl plane (plane B). In this scenario, the larger 1,3-diphenylallyl

substrate **7** suffers enhanced blocking by the protein scaffold (see Figure 9). The size of the monophenyl substrate **8** is however smaller than that of **7**, which appears to allow for its coordination to Pd in the hybrids and for its consequent catalyzed transformation. In other words, substrate **8** can coordinate due to the lack of a phenyl substituent at the sterically most demanding position and, accordingly, the remaining phenyl group resides at the least encumbered site, which is also the site where attack of the nucleophile at the η^3-allyl intermediate is most likely to occur. In this manner the sterical environment provided by the lipase host results in the selective formation of the branched product. The lower yields obtained with **Pd1** and **Pd2** can be explained by the overall lower accessibility of nucleophiles to the coordinated allyl moiety, making nucleophillic attack rate-limiting and resulting in lower product formation. The slightly higher product yield and the minor amount of linear product observed for **Pd2** may be due to the longer tether of its artificial cofactor, placing it at a somewhat more remote position with respect to the protein envelope.

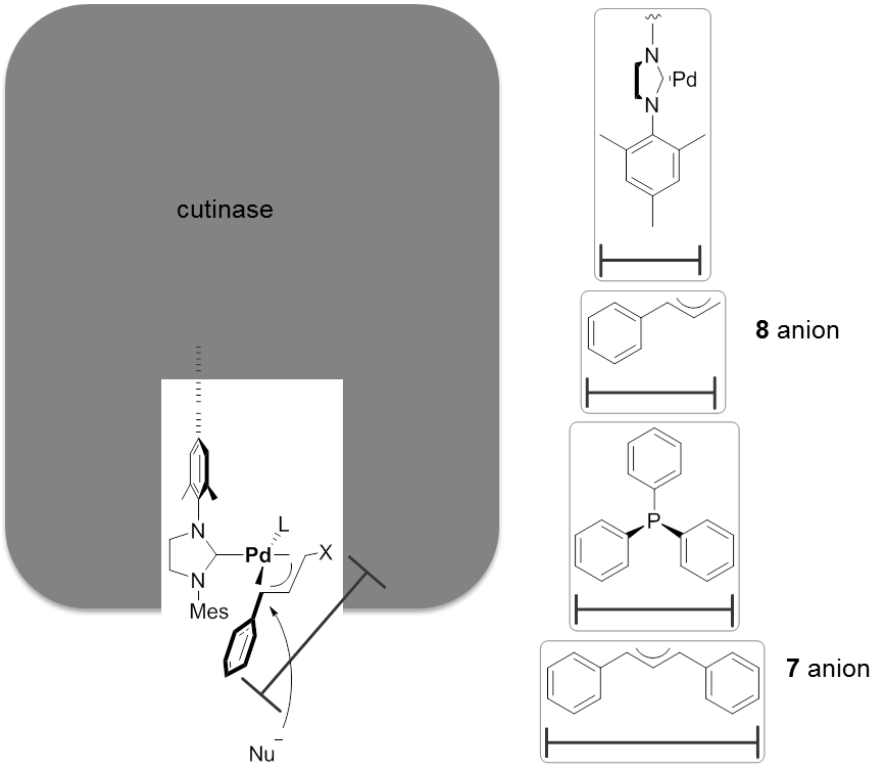


Figure 9. Schematic representation of the orientation of Pd(NHC)(allyl) moieties in the enzyme pocket and relevant coordinating species.

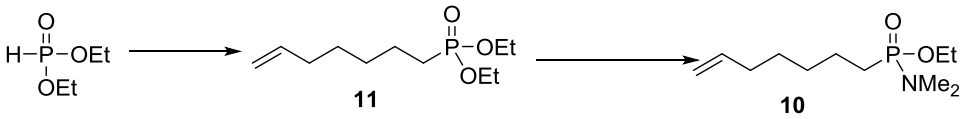
Conclusions

This study shows the synthesis of the first catalytically active palladium artificial enzymes with a covalent constitution. The active site-directed anchoring affords robust hybrids with a bonding tolerant to dialysis, denaturation, and observable by mass spectrometry. This particular conjugation produces a strong sterical effect of the protein scaffold over the organometallic center resulting in clear differences when compared to the activity and selectivity of a parent, unsupported catalyst. In this respect, the hybrids are active in the allylic alkylation with malonate of 1-phenylallyl acetate, but inactive when the larger 1,3-diphenylallyl motif is used due to the increased size of this substrate. Moreover, a change in the regioselectivity is also produced due to this hybridization showing inverse preference of the carbon center attacked by the malonate nucleophile for the hybrid catalysts in comparison with the unsupported catalyst. These observations in catalytic substrate selectivity and product regioselectivity allowed for the monitoring of the importance of the orientation of the catalyst in the enzyme active site. Although no enantiomeric excess was observed in the formation of the branched product, these results represent the first effective artificial enzymes showing inversion of regioselectivity outcome with respect to the unsupported low molecular weight catalyst, expanding the selectivity repertoire of ASD hybrids.

Experimental Section

Materials and methods. Chemical precursors were purchased from commercial sources and used without further treatment unless otherwise stated. Cutinase was obtained from Novonordisk®. Diethyl ether, hexane and toluene were dried from a MBraun MB SPS-800 solvent purification system; dichloromethane and tetrahydrofurane (THF) were dried by distillation from $CaCl_2$ and sodium/benzophenone, respectively and stored over 4 Å molecular sieves. Compund **9** was synthesized as described in reference 12. Tris(hydroxymethyl)aminomethane (TrisHCl), phosphate and sodium acetate and carbonate

buffers were prepared in degassed Milli-Q water and stored in Schlenk flasks. ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded at 298 K using a Varian AS 400 MHz NMR spectrometer at 400, 100 and 81 MHz, respectively. Chemical shifts are reported in ppm and referenced against the residual solvent signal. GC measurements were performed by using a PerkinElmer Autosystem XL gas chromatograph equipped with a PerkinElmer Elite-17 column. Chiral GC analyses were performed with a Perkin Elmer Gas Chromatograph AutoSystem XL equipped with an Agilent Cyclodex B 0.25 micron column and flame ionization detector. GC-MS analyses were performed with a Perkin Elmer gas chromatograph Clarus 680 equipped with a acolumn PE Elite 5MS and coupled to a Perkin Elmer mass spectrometer Clarus SQ 8T with EI ionization. Electrospray Ionization (ESI-TOF) mass spectra of chemical products were recorded with a Waters LCT Premier XE KE317 Micromass Technologies spectrometer; mass analyses of protein and hybrid products are calculated as $[M]^{n+} = (M+n)/n$). Ultrafiltration dialysis of proteomic samples was performed with Vivaspin tubes, 10,000 M.W. C.O. (PEG membrane). All reactions were carried out under standard Schlenk techniques and under inert conditions with N_2.



Diethyl hept-6-en-1-ylphosphonate 11. Sodium hydride (60% disp. in mineral oil, 90 mg, 3.67 mmol, 1.3 eq.) was suspended in THF (15 mL). To this stirred solution was added dropwise diethyl phosphite (0.47 mL, 3.61 mmol, 1.28 eq.) via a syringe. After the initial gas evolution had ceased the reaction mixture was stirred for another 30 minutes at room temperature followed by 1.5 hours at reflux. The reaction mixture was cooled to 0 °C and 7-bromo-1-heptene (0.5 g, 2.82 mmol) was added dropwise via a syringe. Upon complete addition the ice bath was removed and the reaction mixture was stirred for 72 h at room temperature. Analysis by TLC (Et_2O/CH_3OH 9:1) showed a complete conversion. H_2O (25 mL) was added and this mixture was extracted with CH_2Cl_2 (3 x 50 mL. The combined organic extracts were washed once with H_2O (10 mL) and concentrated in vacuo. The residue was purified by column chromatography to yield a pale yellow oil (0.54 g, 2.30 mmol, 82% yield). ^{1}H NMR, $CDCl_3$, 400 MHz, δ: 5.70-5.83 (m, 1H), 4.88-5.00 (m, 2H), 3.97-4.13 (m, 4H), 1.98-2.06 (m, 2H), 1.64-1.75 (m, 2H), 1.51-1.64 (m, 2H), 1.32-1.43 (m, 2H), 1.29 (t, ^{3}J = 7 Hz, 6H);

^{13}C NMR, $CDCl_3$, 100 MHz, δ: 138.7, 114.5, 61.4, 33.4, 28.3, 26.3, 24.9, 22.3, 16.4 ppm; ^{31}P NMR, $CDCl_3$, 162 MHz, δ: 32.49 ppm; ESI-MS $[M+H]^+$ calc. 235.1463, found 235.1439.

Ethyl P-(hept-6-en-1-yl)-N,N-dimethylphosphonamidate 10. Oxalyl chloride (0.57 mL, 6.79 mmol, 3 eq.) was added dropwise via a syringe to a stirred solution of **11** (0.53 g, 2.26 mmol) in CH_2Cl_2 (10 mL). The reaction mixture was stirred for 17 h at room temperature. The reaction mixture was concentrated in vacuo. This crude chloride was dissolved in CH_2Cl_2 (5 mL) and added dropwise via a syringe to a stirred cooled solution (0 °C) of dimethylamine (33% in abs. ethanol, 8.07 mL, 45.2 mmol, 20 eq.). Upon complete addition the mixture was stirred for 5 min. The ice bath was removed and the reaction mixture was stirred for 20 h at room temperature. Analysis by TLC (Et_2O/CH_3OH 9:1) showed a complete conversion. The reaction mixture was concentrated in vacuo and the residue was purified by column chromatography to yield a yellow oil (0.49 g, 2.10 mmol, 93% yield). ^{1}H NMR, $CDCl_3$, 400 MHz, δ: 5.71-5.84 (m, 1H), 4.89-5.01 (m, 2H), 3.95-4.08 (m, 1H), 3.80-3.91 (m, 1H), 2.66 (d, ^3J=8.5 Hz, 6H), 1.97-2.07 (m, 2H), 1.46-1.76 (m, 4H), 1.32-1.43 (m, 4H), 1.27 (t, ^3J = 7 Hz, 3H); ^{13}C NMR, $CDCl_3$, 100 MHz, δ: 138.8, 114.4, 59.1, 36.1, 33.5, 30.2, 28.4, 25.9, 24.6, 22.0, 16.3 ppm; ^{31}P NMR, $CDCl_3$, 162 MHz, δ: 37.09 ppm.

3-(4-(7-((dimethylamino)(ethoxy)phosphoryl)heptyl)-2,6-dimethylphenyl)-1-mesityl-4,5-dihydro-1H-imidazol-3-ium chloride 2. 9-BBN dimer (251 mg, 1.03 mmol, 1 eq.) was weighed in the glovebox and added to a stirred solution of **10** (0.24 g, 1.03 mmol, 2.1 eq.) in THF (30 mL). The reaction mixture was stirred for 20 h at room temperature and then concentrated in vacuo. To this residue was added a mixture of $PdCl_2(dppf).CH_2Cl_2$ (68 mg, 0.92 μmol, 9 mol%) and **11** (0.38 g, 0.94 mmol) followed by addition of degassed N,N-dimethylformamide (75 mL). After stirring this mixture for 5 min an aqueous solution of powdered potassium phosphate tribasic (0.21 g, 1.03 mmol, 1 eq.) in degassed demineralized H_2O (15 mL) was added. The reaction mixture was stirred for 16 h in a pre-heated oil bath at 100 °C. TLC (CH_2Cl_2/CH_3OH 9:1) indicated complete conversion. The reaction mixture was evaporated at room temperature in high vacuo. The residue was diluted with CH_2Cl_2 and the resulting suspension was filtered through a pad of Celite. The filtrate was concentrated to a volume of 20 mL and this was extracted with H_2O (3 x 100 mL). The turbid aqueous extract was concentrated in vacuo to 3 mL and extracted with CH_2Cl_2 (2 x 75 mL). The combined organic extracts were concentrated in vacuo and the

residue was purified by column chromatography to yield a yellow oil (135 mg, 0.26 mmol, 27% yield). ^{1}H NMR, $CDCl_3$, 400 MHz, δ: 9.06 (s, 1H), 6.94 (d, ^{3}J = 4 Hz, 4H), 4.63 (s, 4H), 3.86-3.98 (m, 1H), 3.74-3.86 (m, 1H), 2.62 (d, ^{3}J = 8 Hz, 6H), 2.52 (t, ^{3}J = 8 Hz, 2H), 2.39 (d, ^{3}J=4 Hz, 12H), 2.28 (s, 3H) 1.17-1.75 (m, 15H); ^{13}C NMR, $CDCl_3$, 100 MHz, δ: 159.7, 145.5, 140.7, 135.0, 130.0, 129.4, 59.0, 53.4, 52.1, 36.1, 35.3, 30.8, 30.5, 28.7, 25.9, 24.5, 21.9, 21.0, 18.1, 16.3 ppm; ^{31}P NMR, $CDCl_3$, 162 MHz, δ: 37.16 ppm

[Pd(SIMes)(η^3-C_3H_5)Cl] Pd3. Based on a literature report,[23] 1,3-bis(2,4,6-trimethylphenyl)imidazolinium chloride (SIMesHCl, 0.85 mmol, 290 mg, 1 eq.) was put in a Schlenk flask in the glovebox, toluene (3 mL) was added in the reaction, and potassium bis(trimethylsilyl)amide (KHMDS, 1.7 mL, 0.5 M in the toluene, 1 eq.) was injected dropwise in the Schlenk flask. The solution was stirred for 1 h under room temperature. Allylpalladium chloride dimer (0.39 mmol, 146.8 mg, 0.45 eq.) dissolved in toluene (1 mL) was added slowly to the mixture, which was subsequently stirred for another 80 min at 25 °C. Volatiles were removed under vacuum and the residue was washed with hexane. The product was further purified by column chromatography (CH_2Cl_2:MeOH, 20:1) to give a pale yellow powder (0.2g, 50% yield). ^{1}H NMR, $CDCl_3$, 400 MHz, δ: 6.89 (s, ArH, 4H), 4.75 (m, CH, 1H), 3.95 (s, N-CH_2, 4H), 3.78 (d, CH_2, 1H), 3.24 (d, CH_2, 1H), 2.71 (d, CH_2, 1H), 2.39 (s, $ArCH_3$, 12H), 2.25 (s, $ArCH_3$, 6H), 1.74 (d, CH_2, 1H); ^{13}C NMR, $CDCl_3$, 100 MHz, δ: 211.09 (NCN), 137.97, 136.31, 135.79, 129.29 (Ar), 114.61, 72.93, 51.16 (NCCN), 49.34, 21.02 (Ar-C), 18.38 (Ar-C). ESI-MS $[M\text{-}Cl^-]^+$ calc. 453.1532, found 453.1531.

[Pd(SIMes)(η^3-C_3H_5)(PPh_3)]Cl (Pd3P). Complex **Pd3** (0.02 mmol, 9.8 mg, 1 eq.) and triphenylphosphine (0.02 mmol, 5.2 mg, 1 eq.) were put in a Schlenk flask under nitrogen, and THF (5 ML) was injected into the reaction. The reaction mixture was stirred for 2 h. The organic solvent was removed under vacuum and the residue was washed with diethylether (3 mL). ^{31}P NMR, CD_2Cl_2, 81 MHz, δ: 20.85. ESI-MS $[M\text{-}Cl^-]^+$ calc. 751.2449, found 751.2471.

[Pd(SIMes)(η^3-C_3H_5)(PPh_3]BF_4. Complex **Pd3** (0.08 mmol, 39.2 mg, 1 eq.) and triphenylphosphine (0.02 mmol, 23.1 mg, 1.1 eq.) were put in the Schlenk flask under nitrogen, and CH_2Cl_2 (5 mL) was injected into the reaction. The reaction mixture was stirred for 2 h. Sodium tetrafluoroborate (0.16 mmol, 18mg, 2 eq.) was added into the reaction, and methanol was injected drop by drop until all the solids in the Schlenk flask were dissolved.

After the reaction finished, the mixture was filtered and concentrated. The remained residue was washed with hexane (3 x 3 mL) and purified by column chromatography (CH_2Cl_2:MeOH, 5:1, 48 mg, 75% yield). ^{1}H NMR, $CDCl_3$, 400 MHz, δ: 1.74, 1.98, 2.24, 2.31, 2.33, 2.44 (s, $ArCH_3$, 3H), 2.01 (s, allyl H, 1H), 2.35 (s, allyl H, 1H), 3.00 (d, allyl H, 1H), 4.05-4.23 (m, 4H, NCH_2CH_2N), 4.33 (s, allyl H, 1H), 4.91 (m, allyl H, 1H), 6.49 (s, ArH, 1H), 6.80 (s, ArH, 1H), 6.88-6.96 (m, PPh, 6H), 6.90 (s, ArH, 1H), 7.01 (s, ArH), 7.30-7.45 (m, PPh, 9H); ^{13}C NMR, $CDCl_3$, 100 MHz, δ: 18.31, 18.39, 18.54, 18.88, 20.97, 21.04 ($ArCH_3$), 52.27, 52.61 (NCH_2CH_2N), 69.86 (d, allyl CH_2 *trans* P, J_{CP}=29.5 Hz), 72.49 (allyl CH_2 *trans* SIMes), 119.87 (d, allyl *C*H, J_{CP}=5.3 Hz), 128.83, 128.93 (Ar), 135.07, 135.74, 135.92, 135.95, 136.20 (ArCH3), 138.45, 138.79 (ArN), 209.00 (s, NCN); PPh_3: 129.57 (s, *meta*-Ar), 130.92 (s, *orto*-Ar), 133.26 (d, *para*-Ar, J_{CP}=12.9 Hz); ^{31}P NMR, $CDCl_3$, 81 MHz, δ: 20.86; ^{19}F NMR, $CDCl_3$, 376 MHz, δ: -153.59. ESI-MS $[M\text{-}BF_4^-]^+$ calc. 751.2449, found 751.2471.

Complex 3. As a representative procedure, compound **1** (0.079 mmol, 40 mg, 1 eq) was put in a Schlenk flask in the glovebox, toluene (3 mL) was added and potassium bis(trimethylsilyl)amide (KHMDS, 0.158 mL, 0.5 M in the toluene, 1 eq.) was injected dropwise in the Schlenk flask. The solution was stirred for 16 h. $[Pd(C_3H_5)(\mu\text{-}Cl)]_2$ (0.036 mmol, 13 mg, 0.45 eq.) dissolved in toluene (1 mL) was added slowly to the reaction, and the reaction was stirred for another 80 min. The toluene was removed under vacuum and the residue was washed by hexane. The product was further purified by column chromatography (acetone, yellow band) to give a yellow powder (30 mg, 58% yield). ^{1}H NMR, $CDCl_3$, 400 MHz, δ: 1.27 (dt, CH_2CH_3, *J* = 1.6 and 7.2 Hz, 3H), 1.57-1.89 (m, $ArCH_2CH_2CH_2P$, 6H), 1.79 (d, allyl H, *J* = 12.4 Hz, 1H), 2.25 (s, $ArCH_3$, 3H), 2.40 (s, $ArCH_3$, 3H), 2.41 (s, $ArCH_3$, 6H), 2.42 (s, $ArCH_3$, 3H), 2.59 (d, allyl H, *J* = 7.6 Hz, 1H), 2.64 (d, NCH_3, *J*=2.4 Hz, 3H), 2.66 (d, NCH_3, *J*=1.6 Hz, 3H), 3.42 (d, allyl H, *J* = 6.8 Hz, 1H), 3.76-4.06 (m, CH_2CH_3, 2H), 3.81 (d, allyl H, *J* = 7.2 Hz, 1H), 3.78-4.04 (m, NCH_2CH_4N, 4H), 4.73 (m, allyl H, 1H), 6.89 (s, ArH, 4H). ^{13}C NMR, $CDCl_3$, 100 MHz, δ: 16.26 (OCH_2CH_3), 16.33, 18.66, 21.03 ($ArCH_3$), 23.68 ($ArCH_2CH_2CH_2P$), 23.91 (d, $ArCH_2CH_2CH_2P$, J_{CP} = 53.1 Hz), 25.47 ($ArCH_2CH_2CH_2P$), 36.13, 36.17 (NCH3), 51.14 (OCH_2CH_3), 52.52 (Allyl CH_2), 59.14, 59.21 (NCH_2CH_2N), 72.14 (Allyl CH_2), 114.13 (Allyl *C*H), 128.57, 128.68, 128.71, 128.82, 129.29, 129.37, 135.82, 136.42, 137.99, 141.34 (Ar), 210.96 (s, carbenic carbene), ^{31}P NMR, $CDCl_3$, 81 MHz, δ: 36.65. ESI-MS $[M\text{-}Cl^-]^+$ calc. 616.2292, found 616.2313.

Inhibitor 5. As a representative procedure, complex **3** (0.03 mmol, 19.6 mg 1 eq.) dissolved in CH_2Cl_2 (2 mL) was put in a Schlenk flask (A) under nitrogen, and HCl (0.3 mL, 0.3 mmol, 1M in ether, 10 eq.) was added slowly. The reaction was stirred under room temperature for 1 hr. 4-nitrophenol (0.03 mmol, 4.2 mg, 1 eq.) dissolved in CH_2Cl_2 (2 mL) was put a different Schlenk flask (B) under nitrogen, and triethylamine (0.3 mmol, 85 µL, 10 eq.) was added; the mixture was stirred for 15 min at 25 °C until complete deprotonation towards a yellow solution . The suspension in flask A was filtered and added into flask B. The mixture was stirred for another 2 h at 25 °C. After this time, the product was extracted with CH_2Cl_2 (3 times) from saturated K_2CO_3 aqueous solution and then with ether from water (60% yield). ^{1}H NMR, $CDCl_3$, 400 MHz, selected peaks δ: 3.75-4.05 (m, NCH_2CH_2N, 4H), 4.75 (m, allyl H, 1H), 6.87 (s, ArH, 4H), 6.97, 8.22 (d, POArH, 2H); ^{31}P NMR, $CDCl_3$, 81 MHz, δ: 30.10. ESI-MS $[M\text{-}Cl^-]^+$ calc. 710.1989, found 710.1976.

Complex 4. Following a similar procedure as for the synthesis of complex **3**, and starting from compound **2** (60 mg, 0.107 mmol), product is a yellow oil (48 mg, 65% yield). ^{1}H NMR, $CDCl_3$, 400 MHz, δ: 1.28 (t, CH_2CH_3, *J* = 7.2 Hz, 3H), 1.26-1.71 (m, $ArCH_2(CH_2)_6P$, 12H), 1.73 (d, allyl H, *J* = 12.8 Hz, 1H), 2.27 (s, $ArCH_3$, 3H), 2.41 (s, $ArCH_3$, 9H), 2.43 (s, $ArCH_3$, 3H), 2.51 (t, $ArCH_2(CH_2)_6P$, *J* = 8Hz, 2H) ,2.66 (d, NCH_3, 3H), 2.68 (s, 3H, NCH_3, 3H), 2.98 (d, allyl H, *J* = 12 Hz, 1H), 3.25 (d, allyl H, *J* = 4.8 Hz, 1H), 3.83-4.07 (m, CH_2CH_3, 2H), 3.80 (d, allyl H, *J* = 7.2 Hz, 1H), 3.98 (s, NCH_2CH_2N, 4H), 4.76 (m, allyl H, 1H), 6.89 (s, ArH, 2H), 6.91 (s, ArH, 2H); ^{13}C NMR, $CDCl_3$, 100 MHz, δ: 16.24 (OCH_2CH_3), 16.31, 18.50, 21.01 ($ArCH_3$), 18.38, 22.08, 22.11, 25.90, 29.01, 29.13, 30.61, 30.77, 31.17, 35.44 ($Ar(CH_2)_7P$), 36.13, 36.17 (NCH_3), 51.08 (OCH_2CH_3), 51.20 (Allyl CH_2), 59.01, 59.08 (NCH_2CH_2N), 72.91 (Allyl CH_2), 114.58 (Allyl CH), 128.45, 128.56, 129.71, 135.76, 136.00, 136.23, 136.29, 136.38, 137.97, 142.85 (Ar), 211.14 (NCN); ^{31}P NMR, $CDCl_3$, 81 MHz, δ: 37.07. ESI-MS $[M\text{-}Cl^-]^+$ calc. 672.3010, found 672.2940.

Inhibitor 6. Following a similar procedure as for the synthesis of **5**, starting from complex **4** (40 mg, 0.056 mmol, 1 eq.), product is a yellow oil (60% yield). ^{1}H NMR, $CDCl_3$, 400 MHz, selected peaks: 4.11-4.26 (m, NCH_2CH_2N, 4H), 6.97, 8.21 (d, POArH, 2H); ^{13}C NMR, $CDCl_3$, 100 MHz, δ: 51.09 (OCH_2CH_3), 51.20 (Allyl CH_2), 59.00, 59.07 (NCH_2CH_2N), 72.93 (Allyl CH_2), 114.59 (Allyl CH), 211.14 (NCN); ^{31}P NMR, $CDCl_3$, 81 MHz, δ: 30.47.

Synthesis of hybrid catalysts Pd1 and Pd2. Inhibitor **5** or **6** (7.5 μmol, 30 eq. 30) was dissolved in CH_2Cl_2 (0.5 mL) in a Schlenk flask under inert conditions. Cutinase (5.2 mg, 0.25 μmol, 1 eq.) in buffer solution TrisHCl (5 mL, 50 mM) was added in the reaction. The mixture was stirred under room temperature for 72 h. Volatiles were removed by vacuum, the solids filtered off and the residue submitted to centrifugal filtration using a VIVA Spin® centrifugal concentrator with 10 kDa MWCO until volume was reduced by 90%, then the volume was restored with MiliQ water and the centrifugal filtration repeated enough times until no remaining yellow p-nitrophenolate was observed and the solution tested neutral pH. The final volume was accommodated at the end with the desired buffer (50 mM) for catalysis (see text).

Catalytic experiments. Typical procedure: To a stirred solution of hexadecyltrimethylammonium chloride (6.4 mg, 20 μmol) in Na_2CO_3/$NaHCO_3$ buffer (1 mL, 50 mM, pH 10.5) was added a mixture of substrate, dimethyl malonate and internal standard (0.1 mL stock solution, ratio 100 μmol : 200 μmol : 100 μmol resp. together in 1 mL CH_2Cl_2) followed by a mixture of **Pd3** and triphenylphosphine (50 μL stock solution, 5 mol% Pd catalyst and 10 mol% PPh_3 in 1 mL degassed CH_2Cl_2). The resulting turbid reaction mixture was stirred vigorously at room temperature for 20 h. After this time, a saturated aqueous solution of ammonium chloride (4 mL) was added and this mixture was extracted with CH_2Cl_2 (3 x 4 mL). The combined organic extracts were concentrated under vacuum and the residue was analyzed by GC (see Figure 10).

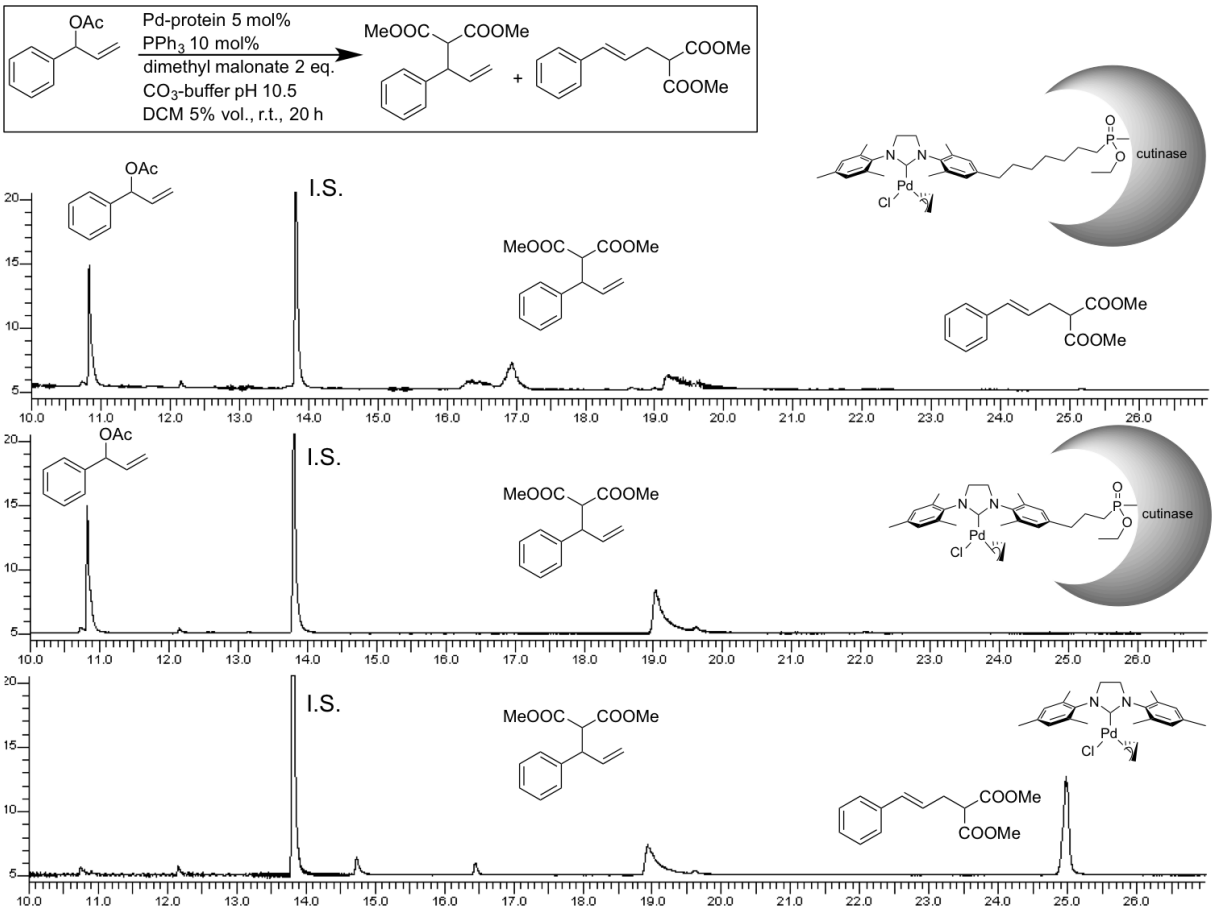


Figure 10. Chromatograms of 1-phenylallyl acetate alkylation.

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Imagination will often carry us to worlds that never were.
But without it we go nowhere.
Carl Sagan

5

An artificial metalloenzyme formed by ditopic hybridization of a Rh(NHC) hydrogenation catalyst

Abstract

An N-heterocyclic carbene (NHC) ligand, functionalized with two phosphonate tails as lipase inhibiting moieties, was covalently anchored to two units of the lipase Cutinase, allowing for the first ditopic protein hybridization of a transition metal catalyst. The new hybrid was studied in the catalytic hydrogenation of acetophenone and methyl 2-acetamidoacrylate.

Introduction

The use of support materials for heterogeneous catalysts is ubiquitous in their preparation processes given that these components provide for a proper bedding of (metallic) catalytic particles. Moreover, the support has significant influence on the activity of the catalyst and this property is taken into account for its selection.[1] On the other hand, the design of homogeneous transition metal catalysts does not involve support materials as a requisite for their preparation, since their stability and solubility in the reaction medium can be granted by their low molecular weight and discrete ligand components. The control of the coordination sphere around the metallic center represents, along with the nature of the metal ion, the conventional way to exert selectivity properties on homogeneous catalysts, for example, by the incorporation of bulky or chiral moieties.[2] Selective catalysts are highly demanded for the production of enantiopure compounds and specific product isomers. Next to these product-selective catalysts, the development of substrate-selective catalysts, e.g. catalysts that can fish the desired substrates in a pool of potential reactive species is of great interest.[3] Besides synthetic ligand design, a number of alternative strategies receive important attention to produce selective homogeneous catalysts.

In more recent endeavors, it has been observed that functionalization of the ligand at greater distances than the coordination sphere can produce a directing effect on its selectivity, e.g. through binding and positioning of the substrate by hydrogen bonding or by blocking of specific transition states and intermediates.[4] The use of supramolecular support materials provides a strategic approach for the generation of a so-called 2nd coordination sphere around the catalyst active site, without fundamental changes in the direct catalyst coordination environment. The encapsulation or caging of active homogeneous catalysts in either homogeneous or heterogeneous 'matrices' represents one of such approaches. Some important examples include the incorporation of molecular catalysts in porous supports like silicates[5] and in supramolecular cages (Figure 1).[6]

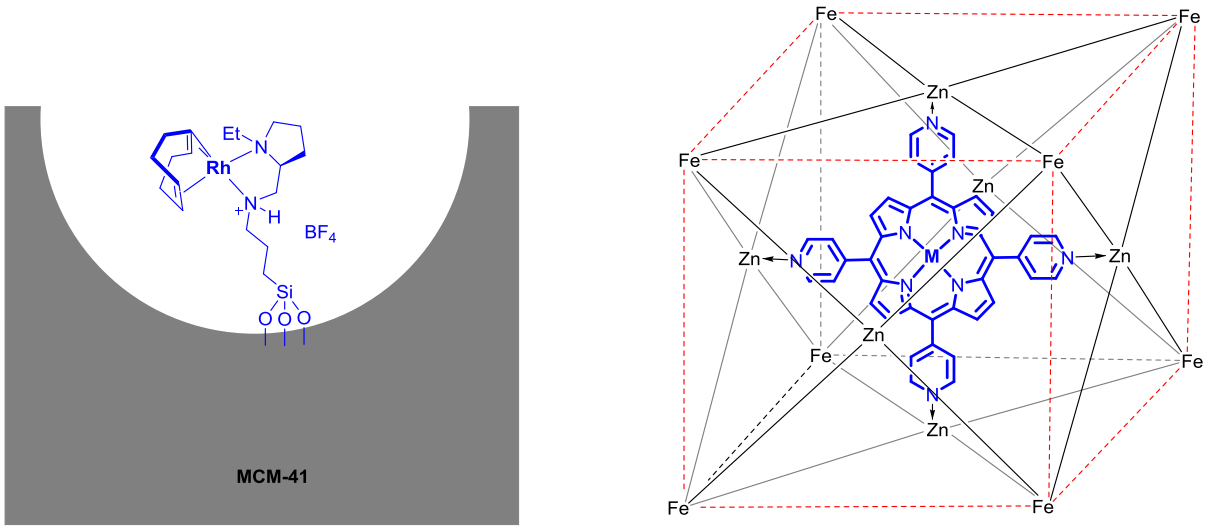


Figure 1. Examples of homogeneous transition metal catalysts encapsulated in mesouporous silica[5b] (left) and supramolecular cages[6e] (right).

Few years ago our group studied the anchoring of transition metal compounds in protein scaffolds via a covalent active site-directed (ASD) hybridization strategy, leading to macromolecular organometallic structures soluble in water[7] and bifunctional hybrids active in, e.g., dynamic kinetic resolution.[8] This hybridization methodology relies on the reaction of serine hydrolases with phosphonate esters bearing the organometallic fragment, covalently binding the phosphorous center of the ester to the serine residue of the active site triad of the lipase, positioning the metallic center at the active pocket of the enzyme, and changing, thereby, the original biological activity of the latter.[9] More recently, as presented in this thesis, the promotion of catalytic selectivity in metal-NHC (NHC = N-heterocyclic carbene) catalysts by their conjugation with protein scaffolds was investigated, following the mentioned hybridization methodology. Therein an effect of the scaffold on the metallic catalyst was observed in terms of enhanced discrimination of the substrate's functional group (chemoselectivity) on catalytic hydrogenation using rhodium(NHC)-protein hybrids,[10] and regioselectivity in catalytic allylic alkylation using palladium(NHC)-protein hybrids.[11] However, no enantioselectivity was observed in these potentially asymmetric reactions (Figure 2).

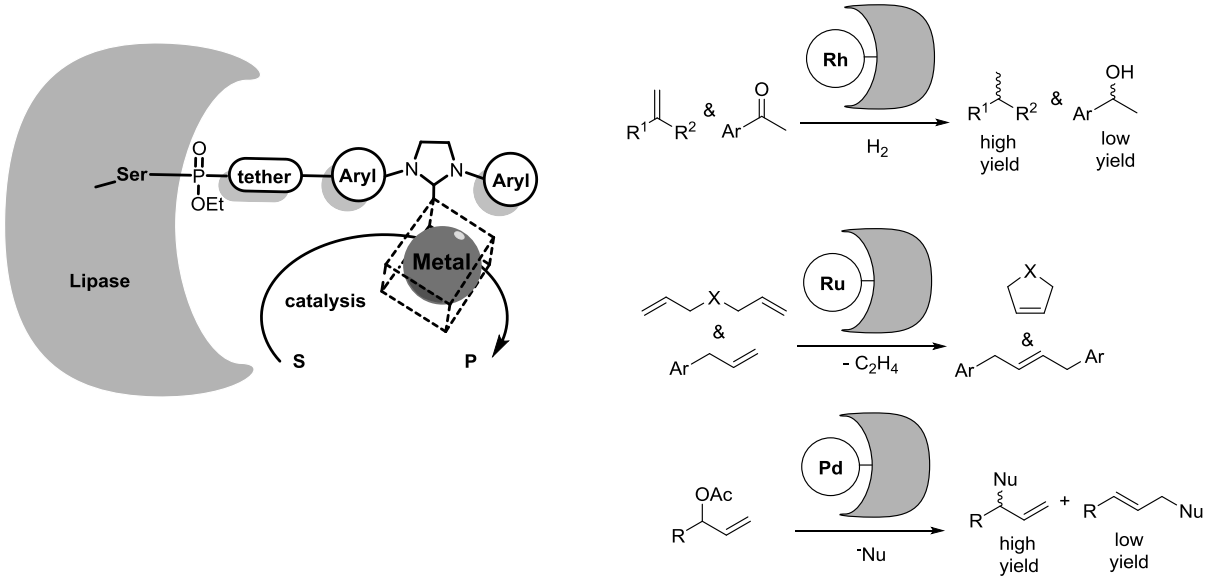


Figure 2. Metallocarbene artificial enzymes based on M(NHC)-lipase hybrids

The organometallic fragment in the pocket of the enzyme host showed a positioning in the outer limits of the protein scaffold. This prompted the possibility of further increasing the sterical hindrance of the 2nd coordination sphere by encapsulation of the metal-NHC catalyst by embedding it in between two protein scaffolds, thereby completely surrounding the organometallic fragment and using the catalyst ligand as a cross-linker between two lipases.

The combination of synthetic transition metal catalysts and protein/enzyme scaffolds to combine the versatile catalytic activity of the former and superb selectivity of the latter is the main objective in the field of artificial metalloenzymes.[12] The encapsulation of transition metal compounds in chosen or designed protein pockets or the surrounding of a synthetic catalyst by protein dimers has also been addressed successfully and increases in the catalytic selectivity due to the spatial restriction of the embedded metal ion and the enhanced rigidity of the protein backbone were observed. This was exemplified by the work of Lu[13] and Roelfes;[14] the former anchored a manganese catalyst by two opposite tethered linkers to cysteine residues in myoglobin and the latter positioned two copper catalysts in the interface of a dimeric protein (Figure 3).

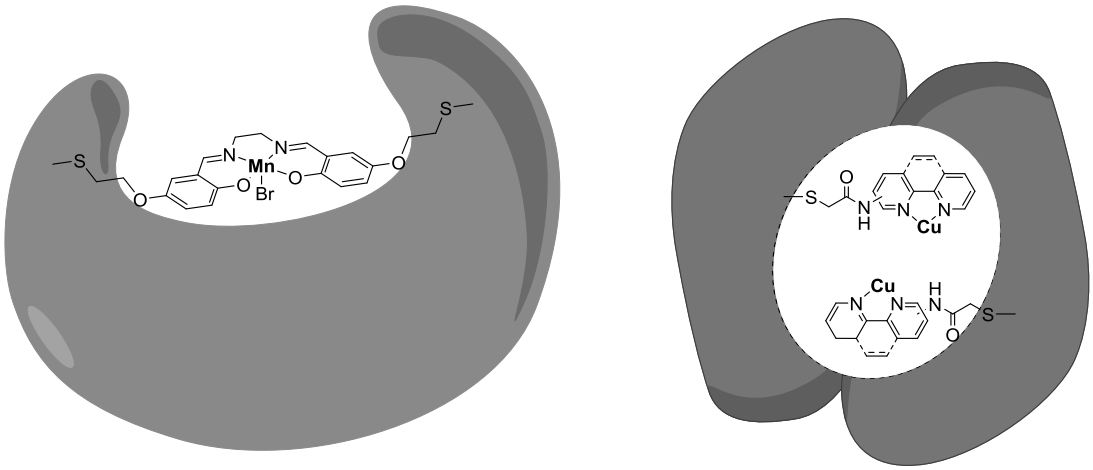


Figure 3. Artificial metalloenzymes with the ditopical anchoring of a catalyst in the protein[13] (left) or its anchoring in dimeric proteins[14] (right).

In the present work a rhodium complex coordinated by a N,N'-disubstituted NHC ligand bearing two phosphonate esters was synthesized to study the possibility of its covalent encapsulation in cutinase units based on a double enzyme scaffold. Differences in the physical properties between single and double protein constructs were found, especially lower solubility of the latter. Moreover, stoichiometrical hybridization was hampered due to the enantioselective inhibition of cutinase by a racemic mixture of phosphonate esters, leading to a mixture of reaction products. Optimization of the pH of the reaction media allowed for the successful formation of the anticipated dimer. A preliminary experiment with the hybrid showed its catalytic activity in the hydrogenation of the olefin methyl 2-acetamidoacrylate and the ketone acetophenone (Figure 4).

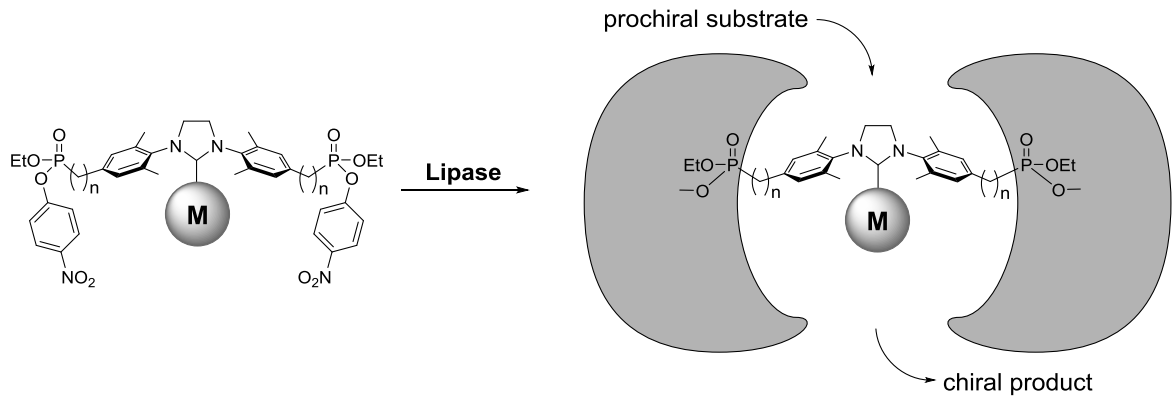


Figure 4. Protein-metal-protein hybrid target in the present study.

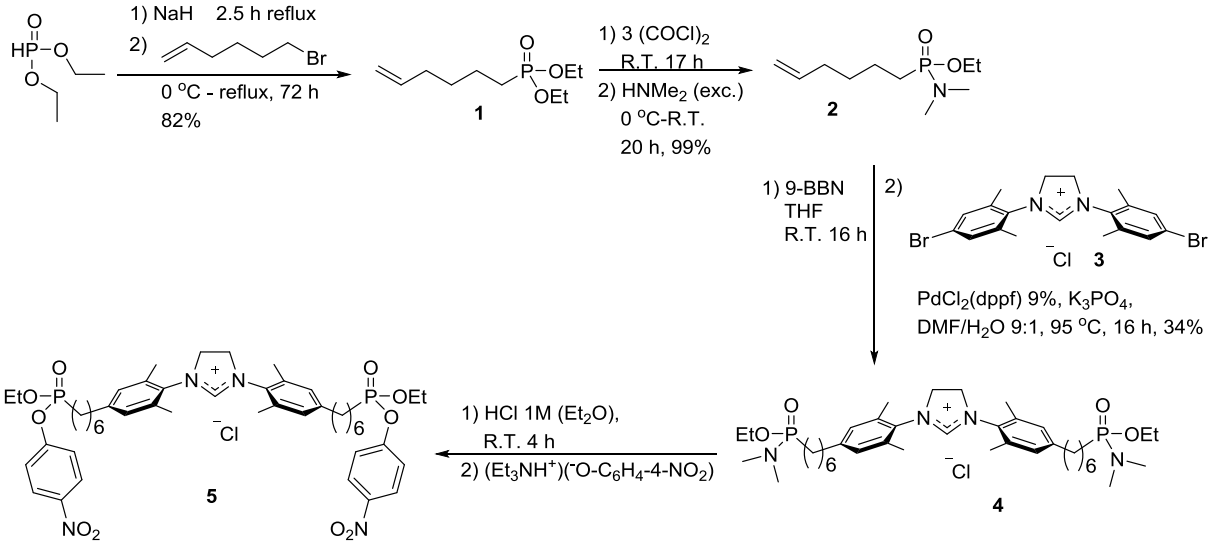
Results

As introduced above, the design of the ligand was based on the experience with metal-NHC inhibitors bearing a single phosphonate tail. The former structure has the potential to allocate the NHC-metal bond in an orthogonal orientation with respect to the protein-NHC anchor, thus promoting a metal-protein 2nd coordination sphere. For this work, two anchoring tails, each with a C_6-aliphatic tether with opposite directionality between each other acting as N,N'-disubstituents of the NHC ligand, were incorporated.

The synthesis involves the formation of the phosphorylated tether arms by the addition of deprotonated diethylphosphite to 6-bromohex-1-ene forming phosphonate **1** bearing a terminal olefin, followed by treatment with oxallyl chloride and dimethylamine to form the desired phosphonamidate **2** (Scheme 1). The latter functionality is resistant to the conditions of palladium-catalyzed cross-coupling reactions, involved in the next step.[7a] Compound **2** was hybroboronated with 9-BBN before it was submitted to Suzuki-type cross-coupling with half an equivalent of bis(bromoaryl)imidazolinium salt **3**[19] towards the dibranched imidazolinium salt **4**. Finally the phosphonamidate functionality was treated with hydrogen chloride followed by *p*-nitrophenolate for its conversion into the corresponding chloro phosphate and to *p*-nitrophenyl phosphonate **5**, respectively (Scheme 1). Note that **5** carries two chiral phosphorous centers and that no attempts were undertaken to obtain **5** in a diastereomericly pure form, i.e. **5** was isolated and used as a mixture of diastereoisomers.

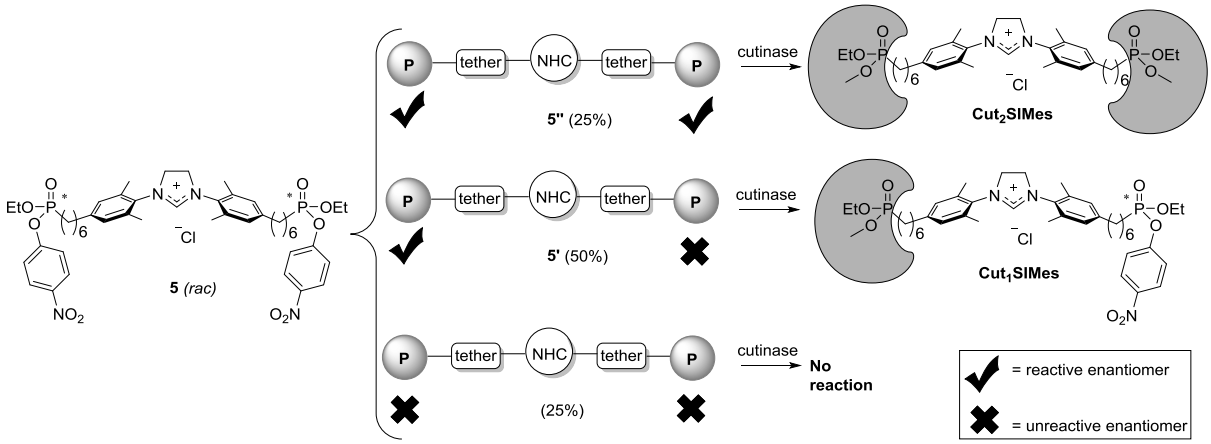
Before the metallation of the bisphosphonate NHC preligand **5**, its inhibitory reactivity towards cutinase was tested. Previous studies showed that Cutinase reacts in an enantioselective manner with phosphonate inhibitors,[15] and that this selectivity is not solely determined by the configuration at the phosphorous center but is also a function of the entire inhibitor structure. For phosphonate inhibitors containing NCN- or SCS-pincer-metal complexes, Cutinase reacts exclusively with the *S*-enantiomer of the NCN-complex and the *R*-enantiomer of the SCS-complex, as observed by X-ray crystallography, even though the different inhibitors had been applied in cutinase inhibition reactions as racemic mixtures. Thus, for full inhibition of the enzyme in these reactions, an excess of the racemic inhibitor is

needed. The latter excess also proved efficient using NHC-phosphonate inhibitors in recent work for the formation of stoichiometric NHC-cutinase hybrids.[10]



Scheme 1. Synthesis of the bis(phosphonate tethered)imidazolinium salt **5**, a NHC precursor with lipase inhibitory activity.

For the present work, two units of cutinase are needed per NHC-inhibitor **5** for the desired **Cut$_2$SIMes** to form. An excess of inhibitor **5** in the reaction mixture would however stir the stoichiometry of the reaction towards a 1:1 product, namely **Cut$_1$SIMes**. To circumvent this, the exact Cutinase/inhibitor ratio required to maximize the yield in **Cut$_2$SIMes** was calculated. The diastereomeric mixture of **5** contains 25% of each of the *S*-*S* and *R*-*R* species, one of these being the active bis-inhibitor **5''**. This will react with two Cutinase units, therefore with 0.5 eq. of Cutinase with respect to the amount of **5**. However, the meso isomer of **5**, **5'**, corresponding to 50% of the diastereomeric mixture of **5**, competes with **5''** in its reaction with Cutinase, forming the monomeric side product **Cut$_1$SIMes** (Scheme 2). This concurrent reaction reduces the availability of Cutinase to react with **5''** if the enzyme is not used in excess. Therefore it was decided to use Cutinase and **5** in a 1:1 ratio, i.e. with a fourfold excess of cutinase with respect to the inhibitor **5''**, in inhibitory reactions aimed at forming **Cut$_2$SIMes**.



Scheme 2. Product formation in the inhibitory reaction of Cutinase with racemic **5**.

Accordingly, the first attempt, Cutinase in TrisHCl buffer at pH 8.5 was slowly added to a concentrated biphasic solution of **5** (1 eq.) and the reaction was allowed to proceed for 24 h. The reaction was then dialyzed to eliminate eventual unreacted **5** and buffer for its analysis by ESI-MS. The analysis showed that the reaction forms both **Cut$_2$SIMes** and **Cut$_1$SIMes**, respectively, as expected (Figure 5). The **Cut$_1$SIMes** mass found by ESI-MS corresponds to the mass of **5** with one arm bound to Cutinase and the other arm hydrolyzed. In other words, after one phosphonate arm has reacted with cutinase, the other phosphonate arm has been converted into the anionic phosphonate derivative either during dialysis or due to competitive hydrolysis of the *p*-nitrophenyl phosphonate group at the inhibition conditions employed. Inspite of this it was clear by ESI-MS that the two units of cutinase could be connected via the NHC linker, prompting the possibility of allocating the organometallic complex between two cutinase units as well.

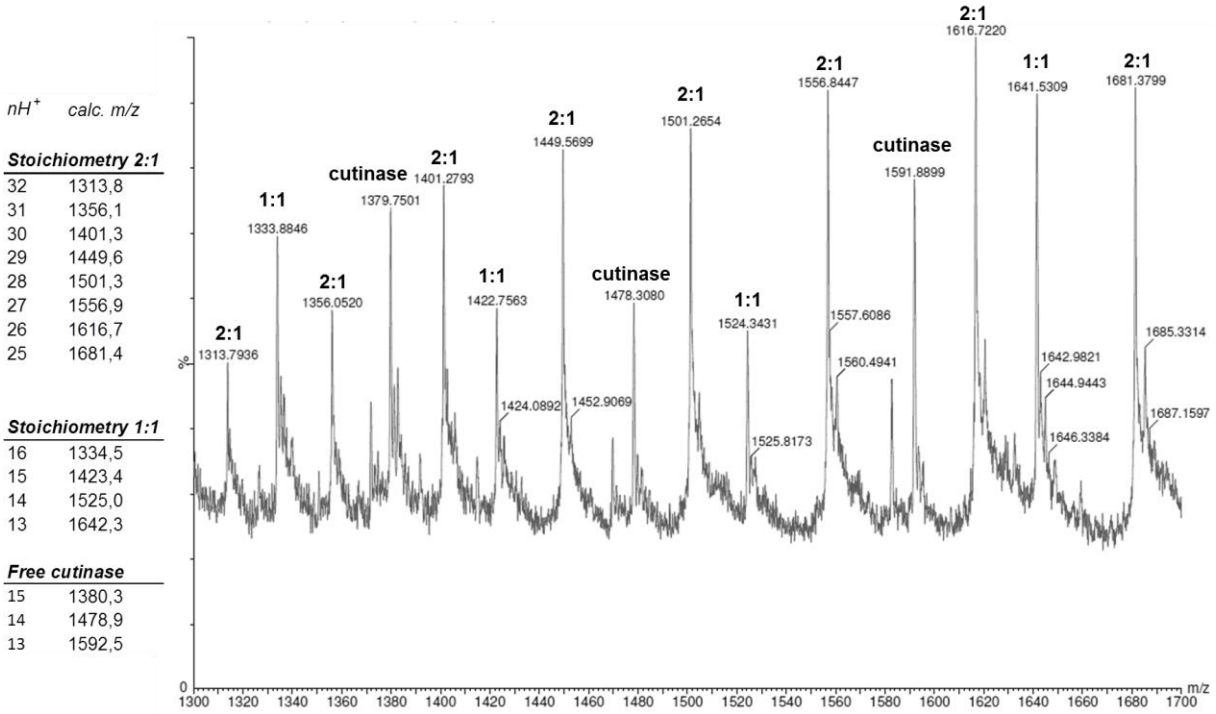
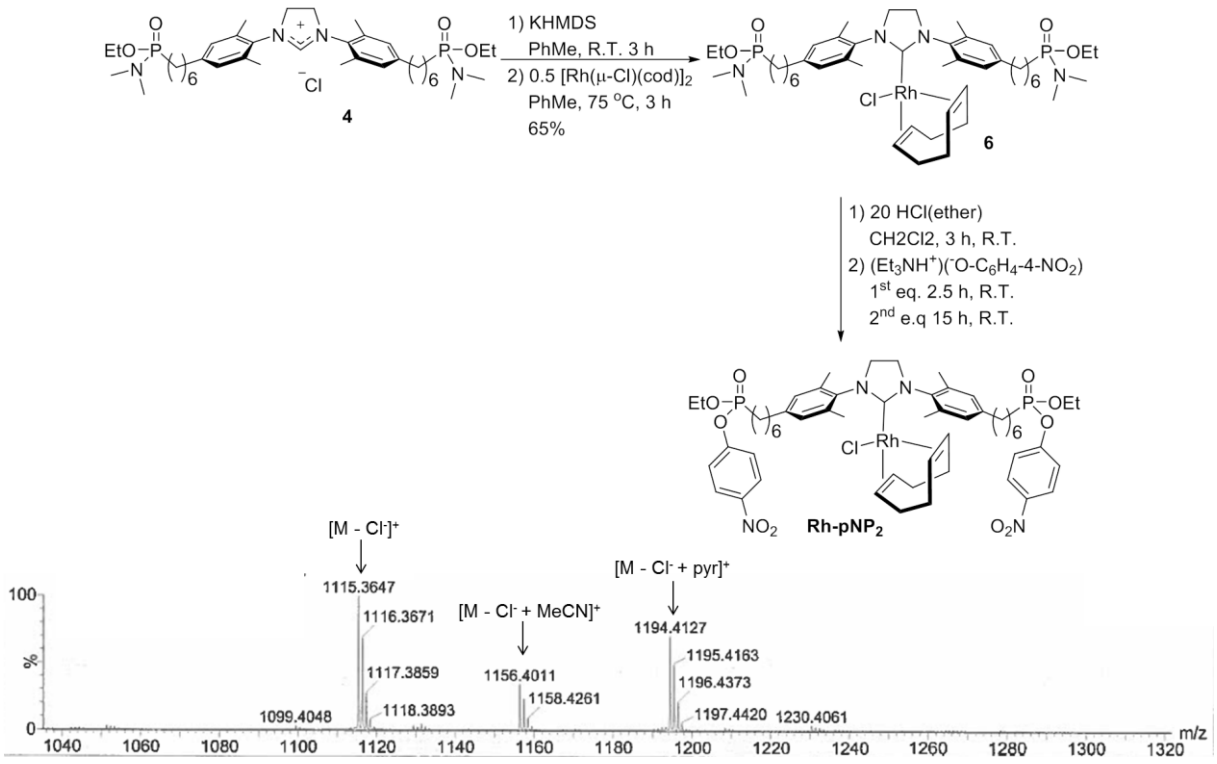


Figure 5. ESI-MS spectrum of the reaction of **5** with cutinase, showing the formation of products with cutinase:ligand stoichiometries of 1:1 (**Cut$_1$SIMes**) and 2:1 (**Cut$_2$SIMes**).

Next, the metallation of the ditopic NHC-ligand was carried out starting from the phosphonamidate precursor **4** (Scheme 3, top). To this end, deprotonation of the imidazolinium moiety with the non-nucleophillic base KHMDS was followed by its reaction with $[Rh(cod)(\mu\text{-}Cl)]_2$ towards Rh(NHC) complex **6**. The conditions were similar to those of the metallation of mono-tethered ligands studied before.[10] Interestingly, analysis by ^{31}C NMR of the latter complex with only one tether showed two signals for the NHC carbene atom because of the presence of isomers of the Rh complex due to positioning of the cyclooctadiene ligand closer or further from the tether chain. However, for the symmetrical NHC ligand with two tether chains, only one ^{31}C NMR signal was observed from the carbene center. For the final synthetic step, a similar procedure as for the synthesis of **5** was followed to convert the phosphonamidate tails into *p*-nitrophenyl phosphonate moieties to arrive at the rhodium lipase inhibitor **Rh-pNP$_2$**. The final inhibitor was characterized by NMR and ESI-MS; Scheme 3 shows the synthetic pathway and ESI-MS characterization of the final inhibitor.

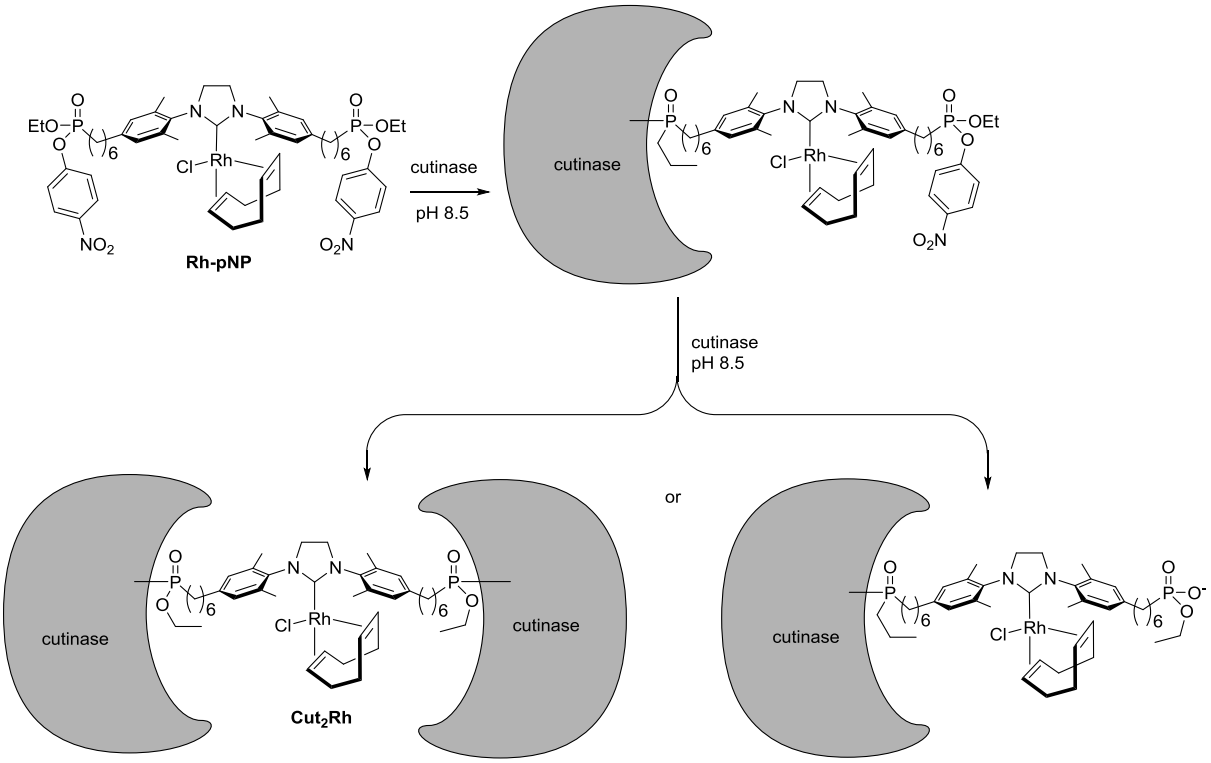


Scheme 3. Synthesis of the Rh(NHC) lipase inhibitor **Rh-pNP$_2$** (top) and ESI-MS from a solution in MeCN and pyridine with formic acid (bottom).

A similar method for the inhibition of Cutinase as mentioned above was followed in TrisHCl buffer at pH 8.5 using compound **Rh-pNP$_2$**. This time, however, after observation of the formation of **Cut$_1$SIMes** with one phosphonate arm hydrolyzed (see above), competition of the second Cutinase inhibition with the hydrolysis of the remaining phosphonate after reaction of the first phosphonate was suspected. For the new reaction, the number of equivalents of Cutinase was therefore doubled (2 eq. with respect to **Rh-pNP**) with the aim to maximize the production of the desired hybrid **Cut$_2$Rh**. This resulted in similar observations as with the organic inhibitor **5**, finding the formation of hybrids with Cutinase/**Rh-pNP$_2$** ratios of 1 and 2, namely **Cut$_1$Rh** and the desired **Cut$_2$Rh**, respectively, again determined by ESI-MS analysis of a dialyzed and denatured sample. This successfully proved that the desired hybrid **Cut$_2$Rh** with encapsulation of the rhodium center by two units of Cutinase can be formed as a stable entity.

Interested in the isolation of **Cut$_2$Rh** for its further study in catalysis, we attempted two purification methods were attempted. First by centrifugal concentration using a polyethersulfone membrane with a molecular weight cut-off (MW CO) of 30 kDa, which would separate **Cut$_1$Rh** (22 kDa) from **Cut$_2$Rh** (43 kDa). This method proved unsuccessful due to blocking of the membrane. Second, size exclusion HPLC for proteins was attempted, however the analyte flow was imperfect and the (UV) absorption of the component around the expected mass was not significant. A closer look at the sample showed a colloidal constitution, which was attributed to the lower solubility of **Cut$_2$Rh** in both buffer solution and MiliQ water, in comparison of the higher solubility of the previously studied Rh-protein hybrids with 1:1 stoichiometry.[10] While the exact concentration of **Cut$_2$Rh** in solution could not be determined, material precipitation was observed soon when this emulsion was concentrated to a theoretical concentration of 50 μM due to the low solubility and likely formation of aggregates of **Cut$_2$Rh**. This behavior could also lead to blocking during size exclusion chromatography (SEC) and the purification attempts were discarded.

The 1:1 hybrids **Cut$_1$SIMes** and **Cut$_1$Rh** formed in the above inhibition experiments contained one hydrolyzed phosphonate arm (ESI-MS analysis). It could not be determined whether the hydrolysis of the non-reacted phosphonate occurs during post-treatment of the resulting material or during the inhibition reaction as a competitive reaction between the hybridization towards a second Cutinase unit and the hydrolysis of the remaining phosphonate arm at the basic reaction conditions. The latter could happen as a result of sterical hindrance over the organometallic moiety from its positioning in the first protein scaffold, hampering the approach of the second Cutinase unit, thus reducing the reaction rate of the second inhibition and resulting in a competing rate of the hydrolysis of this arm (Scheme 4).



Scheme 4. Competition between the desired dimerisation and hydrolysis of half-reacted inhibitor.

As mentioned above, this competitive hydrolysis of the second phosphonate arm would lead to a lower yield of **Cut$_2$Ru**. This possibility was addressed by using non-basic conditions in the inhibition reaction. Although Cutinase shows its higher reaction rate at basic pH values, the enzyme has shown integrity albeit a lower hydrolysis rate at different, even acidic, conditions.[16] Moreover, the use of slightly acidic pH in the inhibition of Cutinase towards artificial metalloenzymes has been recently achieved with Ru(NHC)-phosphonate complexes in an AcONa/AcOH buffer of pH 5.5.[17]

Accordingly, the same protocol for the inhibition of Cutinase (2 eq.) was followed with **Rh-pNP$_2$** but in the mentioned acetate buffer at pH 5.5. Gratifyingly, ESI-MS analysis showed the formation of the expected **Cut$_2$Rh** and, surprisingly, the **Cut$_1$Rh** side product was not observed at all. At the same time, unmodified Cutinase was found as the main component in the resulting mixture (Figure 6). Careful inspection of the sample used for ESI-MS analysis showed this to be an emulsion.

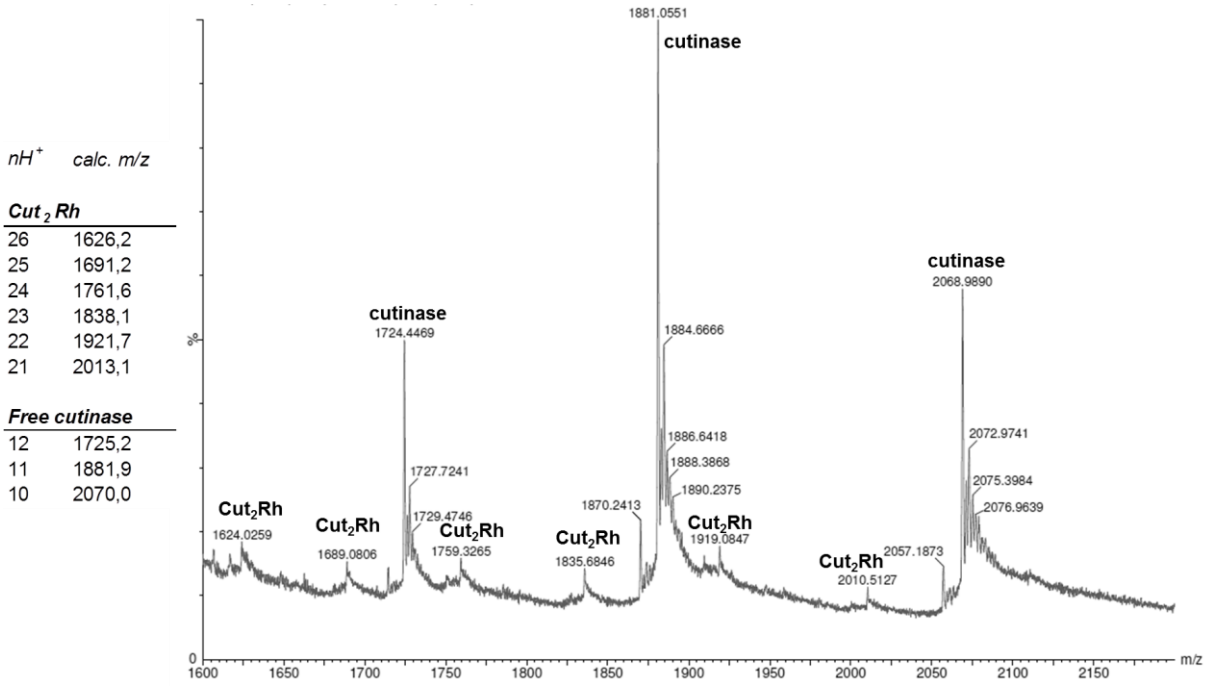


Figure 6. ESI-MS of the reaction towards **Cut$_2$Rh**.

The low solubility of **Cut$_2$Rh** is likely to originate from the enlargement of Cutinase with a second protein unit, resulting in a hybrid that is too hydrophobic for only an aqueous solvent to exert efficient solvation. Cutinase is known to possess some hydrophobic fragments to allow for its natural reactivity towards cutin, a water-insoluble substrate.[18] The latter could also result in samples with lower concentrations of the hybrid, especially for ESI-MS analysis where only water is used as solvent, accentuating the presence of impurities such as unreacted cutinase. Indeed, the intensity of the **Cut$_2$Rh** species was much lower than that of cutinase, suggesting a greater concentration of the latter (strict quantitative comparison of the peak intensity in ESI-MS derived from different species, also with different charges, is not reliable). The fact that the mono-inhibited species **Cut$_1$Rh** was not observed in the analysis of the inhibition reaction carried out at in an acidic medium is unclear at the moment. The observed formation of **Cut$_2$Rh** does imply the intermediate formation of **Cut$_1$Rh**.

A preliminary study of the catalytic activity and selectivity of **Cut$_2$Rh** was conducted using a sample generated under acidic conditions. The hybrid was tested in the competitive hydrogenation of the olefin in methyl 2-acetamido acrylate and the ketone in acetophenone in phosphate buffer at pH 8.5 with 5% *v/v* of CH_2Cl_2, under H_2 pressure (40 bar) at room

temperature for 20 h. These conditions are equal to those successfully explored in previous experiments with a Rh(NHC)-Cutinase hybrid, which showed that the protein scaffold enhanced the chemoselectivity of the rhodium catalyst towards the hydrogenation of the olefin but without enantioselectivity.[10] The experiment was carried assuming a **Cut_2Rh** concentration of 25 μM, which corresponds to an estimated 5% mol loading with respect to each substrate. Under these conditions a poor 2.5-3% yield of the corresponding racemic 1-phenylethanol and methyl acetylalaninate products were obtained. This preliminary result indicates a greatly diminished reactivity of the encapsulated Rh(NHC) moiety in **Cut_2Rh**. It also casts doubts on the presence of (intact) monofunctionalized hybrid **Cut_1Rh** for which a similar catalytic activity is to be expected as for the mono-enzyme hybrids reported in Chapter 2 of this thesis.

In order to investigate the positioning of the rhodium catalyst between the two Cutinase units and estimate a possible selectivity influence by the protein scaffold over the catalytic site, a modeling study of **Cut_2Rh** was conducted. Using the known crystal structures of Cutinase and a Rh(SIMes) motif, and enlarging the latter complex with *P*-terminated hexyl substituents to make a Ser_{120}-O-P-tether bond (1.6 Å), it was found that, independently of a single or double inhibition of Cutinase, a hybridized arm would require an extended conformation of the hexyl chain to avoid overlapping with the amino acid residues around the active site of Cutinase. To simplify the study, a conformation with minimal interaction or overlapping of the protein scaffold units with each other and with the Rh(NHC) moiety was chosen. In this conformation of minimal interactions around the metallic center, the maximal sterical hinderance and restrictions for the rhodium center to interact with substrates could be estimated. The structure resulting from this modeling study is depicted in Figures 7 and 8, in which the cyclooctadiene moiety was not included for clearity.

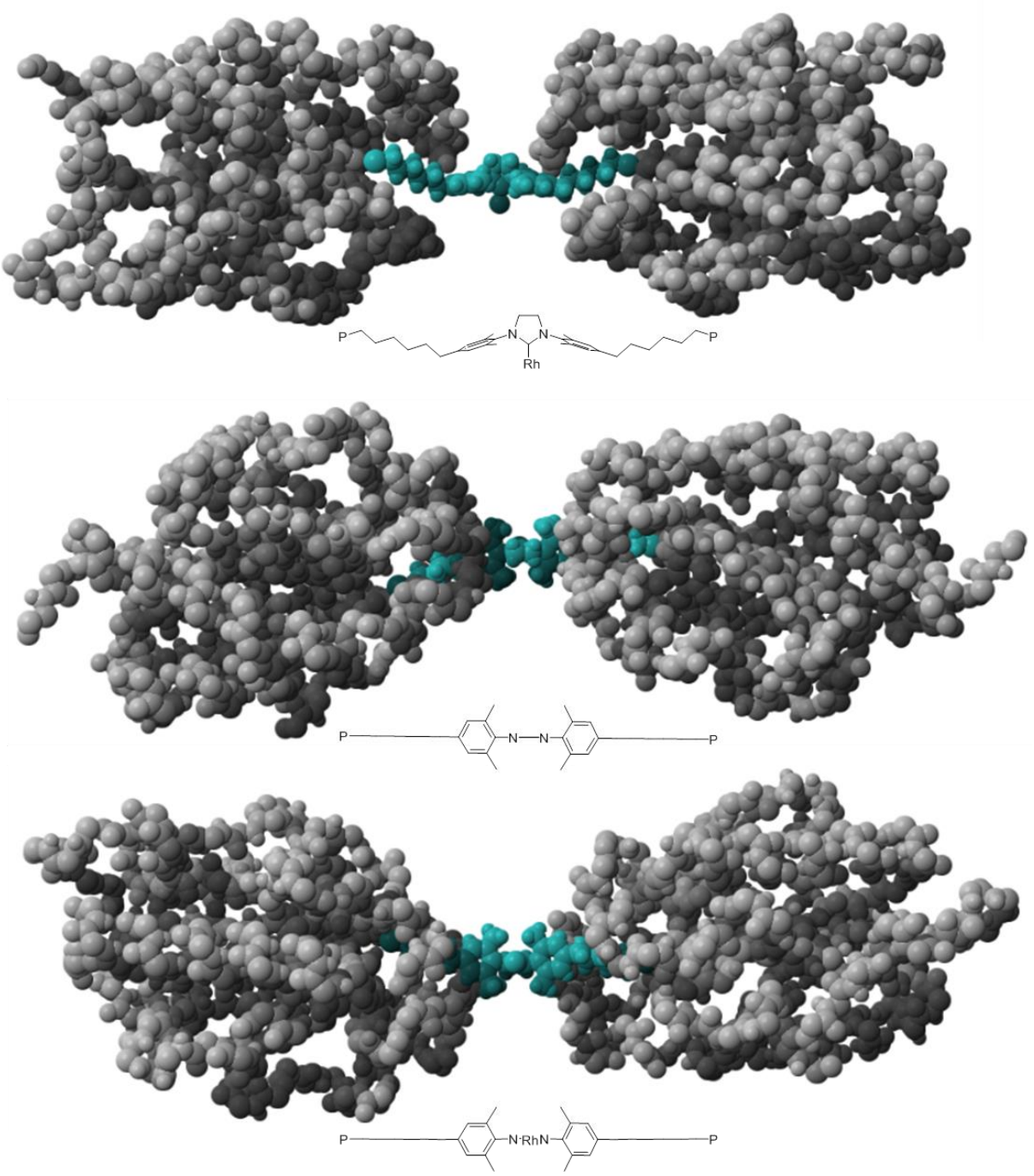


Figure 7. Space-filling representation of the least hindering conformation of **Cut$_2$Rh** through computational modeling.[20] Orthogonal (front) view of the NHC-Rh plane (top), view from the top of the NHC backbone (middle) and from below the Rh-side (bottom). The amino acid side chains have been excluded for ease of representation (see Figure 8 for complementary representation).

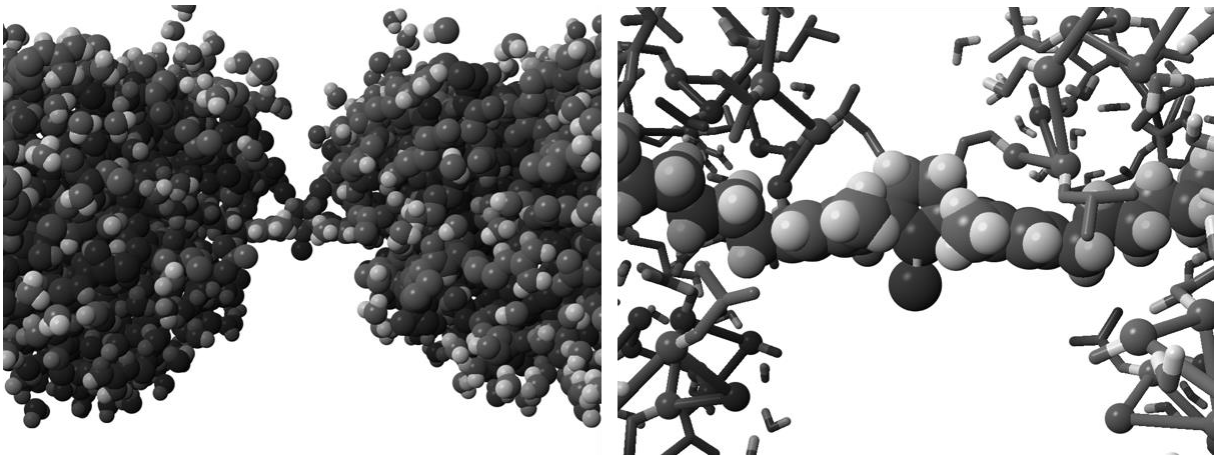


Figure 8. Sterical hindrance around the NHC by the amino acid residues (the Rh center lays in the middle; cyclooctadiene is omitted for ease of representation).

The structure shows that two Cutinase units can indeed bind the inhibitor via each P center and without leading to any sort of overlap between the two protein units. The Ser_{120}-O residue is at a distance of 12.3 Å from the surface of the enzyme in the direction of the rhodium center and the P-P distance in the inhibited Rh(NHC) moiety is 27.2 Å. Each NHC arm fragment is embedded in the helical flap with residues 81-85 and the loop with residues 178-186 Leu_{182} as close as 2 Å to the mesityl moiety of the N-substituents of the ligand, and Ile_{183} at a mere 1.4 Å away of the NHC backbone. The rhodium center is less hindered, being 7.1 Å away from Leu_{81} and 8.4 Å from Ala_{85}; apart from these, most amino acids have a distance of 15 Å between them and rhodium. The two units of Cutinase show their closest interaction between the Ile_{183} residues at 3.6 Å, just above the NHC ligand. As mentioned above, the cyclooctadiene and chloro labile ligands have been removed to observe the room for interaction with substrates. This means that at this open conformation, there is in principle enough room for substrates like acetophenone and methyl 2-acetamide acrylate to interact with the rhodium center (Figure 8). From these calculations it was concluded that the protein scaffold in this hybrid would not exert selectivity over the catalyst and the latter would have a similar catalytic behavior as unsupported species.

Conclusions

The present work shows the synthesis of a ditopic lipase inhibitor bearing two phosphonate tethers for its dual hybridization, covalently bridging two Cutinase enzymes simultaneously through their respective active site. Proof of principle is presented for the formation of an artificial metalloenzyme with the metallic center surrounded by two protein scaffolds. The hybridization of the ditopical inhibitory metallated complex under basic conditions leads to a mixture of hybrids species with either one or both phosphonate arms of the inhibitor bound to Cutinase units. For an inhibition experiment carried out at acidic conditions, only the bis-inhibited hybrid could be identified next to non-reacted Cutinase. Molecular modeling of the anticipated bis-Cutinase hybrid shows a structure in which the NHC ligand connecting the metallic center and the protein scaffolds allows for significant conformational freedom of the surrounding enzymes. This does not seem to result in a considerable 2^{nd} coordination sphere able to induce selectivity over the action of the catalytic metal center. Further studies will have to establish the synthetic procedure towards the anticipated dimeric Cutinase hybrids containing an encapsulated artificial metal(NHC) cofactor. Optimization of the tethering moieties in the ditopic inhibitor used for the preparation of these hybrids is then foreseen to lead to selectivity in catalytic reactions carried out by hybrids of this type.

Experimental Section

Materials and methods. Cutinase was obtained from Novonordisk®. Dry acetonitrile, diethyl ether, hexane and toluene were obtained from a MBraun MB SPS-800 solvent purification system; dichloromethane (DCM) and tetrahydrofurane (THF) were dried by distillation from $CaCl_2$ and sodium/benzophenone, respectively and stored over 4 Å molecular sieves. Tris(hydroxymethyl)aminomethane buffer (Tris-HCl), NaH_2PO_4/Na_2HPO_4 “phosphate” buffer, and AcONa/AcOH “acetate” buffer were prepared in degassed Milli-Q water and stored in Schlenk flasks. Other solvents and regents were purchased from commercial sources and

used without further purification. ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded at 298 K with a Varian AS 400 MHz NMR spectrometer at 400, 100 and 81 MHz, respectively. Chemical shifts are reported in ppm and referenced against the residual solvent signal. Chiral GC analyses were performed with a Perkin Elmer Gas Chromatograph AutoSystem XL equipped with an Agilent Cyclodex B 0.25 micron column and flame ionization detector. Electrospray Ionization (ESI-TOF) mass spectra of chemical products were recorded with a Waters LCT Premier XE KE317 Micromass Technologies spectrometer; mass spectrometry of protein and hybrid products (calculated as $[M]^{n+}$=(M+n)/n) were performed in positive ion mode using the previous apparatus. Ultrafiltration dialysis of proteomic samples was performed with Vivaspin® tubes of 10,000 and 30,000 M.W. C.O. (PEG membrane). All reactions were carried out under standard Schlenk techniques and under inert conditions with N_2.

Diethyl hex-5-en-1-ylphosphonate 1. Sodium hydride (60% disp. in mineral oil, 0.66 g, 15.15 mmol, 1.35 eq.) was suspended in dry THF (6 mL). To this stirred mixture, diethyl phosphite (1.85 mL, 14.37 mmol, 1.28 eq.) was added dropwise via a syringe. After the initial gas evolution had ceased the reaction mixture was stirred for another 30 minutes at room temperature followed by 2.5 h at reflux. The reaction mixture was cooled to 0 °C and 6-bromo-1-hexene (1.5 mL, 11.22 mmol) was added dropwise via a syringe. Upon complete addition the icebath was removed and the reaction mixture was stirred for 20 h at room temperature. H_2O (50 mL) was added and this mixture was extracted with CH_2Cl_2 (3 x 125 mL). The combined organic extracts were washed once with H_2O and concentrated in vacuo. The residue was purified by column chromatography using Et_2O/CH_3OH 9:1 v/v as eluent to yield a pale yellow oil (1.71 g, 7.79 mmol, 69% yield). ^{1}H NMR, $CDCl_3$, 400 MHz, δ: 5.70-5.83 (m, 1H), 4.90-5.03 (m, 2H), 3.99-4.15 (m, 4H), 2.01-2.09 (m, 2H), 1.53-1.79 (m, 4H), 1.40-1.51 (m, 2H), 1.30 (t, *J* = 7 Hz, 6H); ^{13}C NMR, $CDCl_3$, 100 MHz, δ: 138.2, 114.8, 61.4, 33.2, 26.2, 24.8, 21.9, 16.4 ppm; ^{31}P NMR, $CDCl_3$, 81 MHz, δ: 32.32.

Ethyl P-(hex-5-en-1-yl)-N,N-dimethylphosphonamidate 2. Oxalyl chloride (1.97 mL, 23.29 mmol, 3 eq.) was added dropwise via a syringe to a stirred solution of **1** (1.71 g, 7.79 mmol) in CH_2Cl_2 (20 mL). The reaction mixture was stirred for 17 hours at room temperature and then the volatiles removed under vacuum. This crude chloride was dissolved in CH_2Cl_2 (10 mL) and added dropwise via a syringe to a stirred solution of dimethylamine (33% in absolute ethanol, 27.7 mL, 155.2 mmol, 20 eq) at 0 °C. Upon complete addition the mixture

was stirred for 5 minutes. The ice bath was removed and the reaction mixture was stirred for 20 hours at room temperature. The reaction mixture was concentrated in vacuo and the residue was purified by column chromatography to yield a yellow oil (1.68 g, 7.67 mmol, 99% yield). ^{1}H NMR, $CDCl_3$, 400 MHz, δ: 5.70-5.82 (m, 1H), 4.89-5.01 (m, 2H), 3.95-4.06 (m, 1H), 3.79-3.90 (m, 1H), 2.65 (d, *J*=9 Hz, 6H), 2.05 (q, *J* = 7 Hz, 2H), 1.38-1.76 (m, 6H), 1.26 (t, *J* = 7 Hz, 3H); ^{13}C NMR, $CDCl_3$, 100 MHz, δ: 138.3, 114.7, 59.0, 36.1, 33.2, 29.9, 25.8, 24.5, 21.6, 16.3 ppm; ^{31}P NMR, $CDCl_3$, 81 MHz, δ: 36.90.

1,3-bis(4-(6-((dimethylamino)(ethoxy)phosphoryl)hexyl)-2,6-dimethylphenyl)-4,5-dihydroimidazolinium chloride 4. 9-BBN dimer (0.82 g, 3.37 mmol, 2.1 eq.) was weighed in the glovebox and added to a stirred solution of compound **2** (0.74 g, 3.37 mmol, 2.1 eq.) in dry THF (40 mL). The reaction mixture was stirred for 18 h at room temperature and then concentrated in vacuo. To this residue was added a mixture of $PdCl_2$(dppf).CH_2Cl_2 (0.12 g, 0.14 mmol, 9 mol%) imidazolinium salt **3**[19] (0.75 g, 1.60 mmol) followed by addition of degassed DMF (63 mL). After stirring this mixture for 5 min, an aqueous solution of powdered potassium phosphate tribasic (0.72 g, 3.37 mmol, 2.1 eq.) in degassed demi H_2O (7 mL) was added. The reaction mixture was stirred for 15 hours in a pre-heated oil bath at 105 °C. TLC (CH_2Cl_2/CH_3OH 9:1) indicated full conversion. The reaction mixture was evaporated at 70 °C in vacuo and was azeotroped once with heptane. The residue was diluted with CH_2Cl_2 and the resulting suspension was filtered through a pad of Celite. The filtrate was concentrated and the residue was purified by column chromatography to yield a brown oil. This was diluted with CH_2Cl_2 and washed once with brine. The organic layer was concentrated in vacuo to yield a brown oil (406 mg, 0.54 mmol, 34% yield). ^{1}H NMR, $CDCl_3$, 400 MHz, δ: 9.06 (s, 1H), 6.91 (s, 4H), 4.63 (s, 4H), 3.89-4.04 (m, 2H), 3.72-3.89 (m, 2H), 2.63 (d, ^{3}J = 9 Hz, 12H), 2.50 (t, ^{3}J = 8 Hz, 4H), 2.30-2.43 (m, 12H), 1.41-1.74 (m, 12H), 1.12-1.40 (m, 14H); ^{13}C NMR, $CDCl_3$, 100 MHz, δ: 159.6, 145.3, 135.0, 130.3, 129.4, 59.0, 52.2, 36.1, 35.4, 30.8, 28.7, 25.9, 24.6, 22.0, 18.2, 16.3 ppm; ^{31}P NMR, $CDCl_3$, 162 MHz, δ: 36.97 ppm; ESI-MS: $[M+H]^+$ calc.: 717.4637, found: 717.4548.

1,3-bis(4-(6-((R)-ethoxy(4-nitrophenoxy)phosphoryl)hexyl)-2,6-dimethylphenyl)-4,5-dihydro-1H-imidazol-3-ium 5. In a Schlenk flask, compound **4** (100 mg, 0.212 mmol, 1 eq.) was dissolved in CH_2Cl_2 (10 mL), stirred and hydrogen chloride 1 M solution in diethyl ether (4.2 mL, 4.2 mmol, 20 eq.) was added. The reaction was stirred at room temperature for 3 h.

The volatiles were evaporated and the product redissolved in CH_2Cl_2 (10 mL). In a different flask, *p*-nitrophenol (65 mg, 0.47 mmol, 2.2 eq.) was suspended in CH_2Cl_2 (5 mL) and deprotonated with distilled triethylamine (0.15 mL, 1 mmol, 5 eq.) and the yellow solution transferred to the reaction flask and stirred for 20 h. After this time, the volatiles were evaporated under vacuum and the product isolated by wet column chromatography (dense brownish oil, 18% yield). ^{1}H NMR, $CDCl_3$, 400 MHz, δ: 9.21 (s, NCHN, 1H), 8.19 (d, O_2NArH, 4H), 7.37 (d, OArH, 4H), 6.94 (s, ArH, 4H), 4.58 (s, NCH_2CH_2N, 4H), 4.26-4.05 (m, OCH_2, 4H), 2.5 (t, $ArCH_2$, 4H), 2.40 (s, $ArCH_3$, 12H), 2.10-1.76, 1.74-1.49, 1.48-0.96 (m, $(CH_2)_5$), 1.28 (t, CH_2CH_3, 6H); ^{13}C NMR, $CDCl_3$, 100, MHz, δ: 160, 145.2, 135.0, 130.4, 129.4, 125.6, 120.9, 120.9, 77.3, 77.0, 76.7, 62.9, 51.8, 35.3, 30.7, 30.3, 30.1, 29.7, 28.6, 26.7, 25.3, 22.1, 22.0, 18.1, 16.4, 16.3, 14.1; ^{31}P NMR, $CDCl_3$, 162, MHz, δ: 30.30.

Complex 6. In the glovebox, compound **4** (135.8 mg, 0.180 mmol) were dissolved in toluene (20 mL) and to this KHMDS (0.37 mL, 0.185 mmmol) was added under stirring. After 3 h at room temperature, $[Rh(cod)(\mu\text{-}Cl)]_2$ (40 mg, 0.081 mmol) was dissolved in Toluene (10 mL) and added to the reaction mixture. Then the reaction was closed, transferred to the fumehood, stirred and heated at 75 °C for 3 h. The solvent was reduced to 10 mL by evaporation and the reaction mixture filtered via cannula. The solvent was completely removed and the product purified by column chromatography with silica under degassed conditions using acetone as eluent (Rf = 0.25, third yellow component); evaporation of solvent led to a yellow powder (65% yield). ^{1}H NMR, CD_2Cl_2, 400 MHz, δ: 6.99 (d, ArH, 4H), 4.3 (b, cod), 3.93, 3.85 (m, OCH_2, 4H), 3.63 (s, NCH_2CH_2N, 4H), 3.37 (b, cod), 2.64, 2.62 (s, NCH_3, 12H), 2.55, 2.31 ($ArCH_3$, 12H), 2.13 (m, $ArCH_2$, 4H), 1.74-1.40 (m, $(CH_2)_5$, 20H), 1.25 (t, CH_2CH_3, 6H); ^{13}C NMR, CD_2Cl_2, 100 MHz, δ: 212.2 (d, NCN), 142.6, 138.2, 136.6, 135.4, 128.9, 127.8, 96.6, 67.7, 58.8, 51.4, 35.9, 35.4, 32.6, 31.5, 31.2, 30.6, 29.0, 28.8, 28.1, 25.8, 24.5, 22.1, 19.7, 18.2, 16.1; δ:^{31}P NMR, CD_2Cl_2, 162 MHz, δ: 36.42. ESI-MS m/z $[M\text{-}Cl^-]^+$ calc. 927.4553, found 927.4548.

Inhibitor Rh-pNP$_2$. In a Schlenk flask complex **6** (19.5 mg, 0.020 mmol) was dissolved in CH_2Cl_2 (4 mL), stirred and hydrogen chloride 1 M solution in diethyl ether was added (0.4 mL, 0.040 mmol). The reaction was stirred at room temperature for 3 h. The volatiles were evaporated and the product redissolved in CH_2Cl_2 (5 mL). In a different flask, *p*-nitrophenol (2.9 mg, 0.02 mmol) was suspended in CH_2Cl_2 (3 mL) and deprotonated with distilled

triethylamine (0.055 mL, 0.02 mmol) and the yellow solution transferred to the reaction flask and stirred for 2.5 h. Then a second equivalent preparation of the triethylammonium *p*-nitrophenolate was prepared, added to the reaction and stirred overnight at room temperature. The volatiles were evaporated under high vacuum. ^{31}P NMR analysis showed complete conversion towards the desired product. ^{1}H NMR, CD_2Cl_2, 100 MHz, δ: 8.23 (d, OArH, 4H), 7.36 (d, ArH-NO_2, 4H), 6.99 (s, ArH, 4H), 4.18 (m, CH2O, 4H), 4.33-4.13 (m, cod), 3.87 (b, NCH_2CH_2N, 4H), 2.53, 2.33, (s, $ArCH_3$, 12H), 2.00 (m, $ArCH_2$, 4H), 1.73-1.29 (m, $(CH_2)_5$, 20H), 1.23 (m, CH_3CH_2, 6H); ^{31}P NMR, CD_2Cl_2, 162 MHz, δ: 30.62. ESI-MS $[M\text{-}Cl^-]^+$ calc. 1115.3936, found 1115.3933.

Synthesis of hybrids. To a solution of Cutinase (11 mL, 50 μM, 1 eq.) in buffer (TrisHCl pH 8.5 or acetate pH 5.5, 50 mM), inhibitor (compound **5** or complex **Rh-pNP$_2$**, either 0.5 or 1 eq., according to the experiment) in CH_2Cl_2 (0.6 mL, irrespective of the amount of inhibitor) was slowly added under vigorous stirring and the reaction allowed to run at room temperature for 24 h. After this time, CH_2Cl_2 was removed under vaccum and the solids filtered off. The solution was submitted to centrifugal filtration using a membrane of 10 KDa MW CO repetitive times and restoring the volume (11 mL, estimated final concentration 25 μM) with MilliQ water each time, until no residual buffer was resulting in the filtrate by monitoring the pH of this, neither this filtrate was yellow due to *p*-nitrophenolate. An aliquote was taken in water for analysis and formic acid was added (10 % v/v) for denaturation and then submitted to ESI-MS. Then the volume was restored the last time with phosphate buffer pH 8.5 and the hybrid stored in a Schlenk flask under nitrogen at – 20 °C.

Modeling. Computation modeling was performed using Yasara software,[20] positioning the hydrolyzed P center of the inhibitor **Rh-pNP$_2$** at 1.6 A from the O prostetic group of Ser_{120} of the crystal structure of cutinase from *fusarium solani pisi* (pdb 1CUA). Atom distances are from centroids.

Catalytic hydrogenation determination. A pressurization vessel was charged with 0.5 mL stock solution of acetophenone, methyl 2-acetamidoacetate and 1,2-diphenylethane as internal standard (4.3 mM, 4.3 μmol each in CH_2Cl_2). The solvent was evaporated, the vessel refilled with N_2 and CH_2Cl_2 (0.25 mL) added again. Hybrid **Cut$_2$Rh** (4.3 mL, 25 μM, 0.12 μmol, equivalent to 5 mol-%) in buffer (50 mM) was added to the vessel together with a stirring bar

and this was placed in an autoclave chamber. The system was flushed with a gentle stream of H_2 for 1 minute, then closed and pressurized under stirring to 40 bar of H_2 for 20 h. After this time, the chamber was opened. CH_2Cl_2 (3 x 5 mL) was added to extract the organic phase and then concentrated to about 0.05 mL and analyzed by chiral-GC (correlating the retention times with previous analysis of stock material and similar reactions[10]).

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Everything is theoretically impossible until it is done.

Robert A. Heinlein

Summary and Conclusions

Summary

Nowadays, the expected scarcity of fossil fuels has driven the industry to start to incorporate renewable and bio-based components in the materials it produces. A next step in this development may very well be that also the catalysts involved in the manufacturing of these materials are based on abundant or renewable components, resulting in more benign catalytic technologies.

Some important reactions, such as polymerization, cyclizations and isomerizations, to name a few, are efficiently catalyzed by homogeneous transition metal complexes. Selectivity in these types of reactions is of utmost importance, either in terms of substrate selectivity to avoid the need of pre-treatment of reaction feedstock, or in terms of product selectivity to avoid post-treatment of the reaction products to yield pure materials with specific biological or physical properties (enantiomers and isomers in general). The conventional method to promote selectivity in homogeneous transition metal catalysts is significantly dominated by dedicated synthetic ligands. In order to reduce our need for the latter, nature provides superb examples of catalytic selectivity performed by enzymes. Inherently non-toxic, water soluble, abundant and presenting special steric hindrance and chirality around their active site, enzymes have become of interest to function as stereo-directing scaffolds for transition metal catalysts. The study of the combination of active metallic species and protein scaffolds, i.e. artificial metalloenzymes, has flourished alongside other bio-(inspired)-technologies aiming to develop more sustainable chemical synthesis methods. The development of catalyst anchoring methods and the generation of a so-called 2^{nd} coordination sphere by the protein surrounding over the metallic center to confer selectivity to the catalytic guest are the amongst major incentives in this field of research. With the restriction of being water-tolerant (or by using the hybrids in different media), in principle any known homogeneous catalyst can be anchored to a protein scaffold with the aim to generate catalytic selectivity and to develop artificial metalloenzymes with a reaction repertoire extending that of native enzymes.

In the last decade, our group investigated the immobilization and diversification of homogeneous catalysts, and studied, for example, dendritic and compartmentalized catalysts. At the same time, the group became interested in bioinspired technologies for catalyst development. As a means to study artificial enzymes, and thanks to the expertise in ligand design and functionalization, a new hybridization method was developed consisting of the anchoring of organometallic complexes in the active site of enzymes. With the knowledge that serine hydrolases are covalently modified in their active site through the reaction with phosphonate esters acting as suicide inhibitors, organometallic ECE-pincer complexes (Pd and Pt) were furnished with phosphonate tails and irreversibly linked to the serine active residue of the lipase Cutinase (Figure 1). This active site-

directed (ASD) approach produces covalent hybrids that, thanks to their robust conjugation, allow for their analysis by denatured mass spectrometry and for which detailed structural information is available through crystal structures.

The ASD approach also offers a versatile strategy for the generation of a 2nd coordination sphere due to the docking of the organometallic fragment in the naturally selective pocket of an enzyme. The protein-ECE metallopincer hybrids showed a proof of principle of the ASD approach and were, e.g., used for luminescent protein labeling. Their use as catalysts has been hampered due to the generally high stability of pincer complexes at mild conditions such as ones that preserve protein tertiary structures. A more recent report, incorporating a Ru(Cp) racemization catalyst in the immobilized lipase Candida antarctica lipase B (CalB) represents the first example of a catalytically active artificial enzyme created by the ASD approach. In this case however, the protein scaffold did not provide selectivity to the action of the metallic center and the polymer-supported constitution of the enzyme host yielded a heterogeneous catalyst (Figure 1).

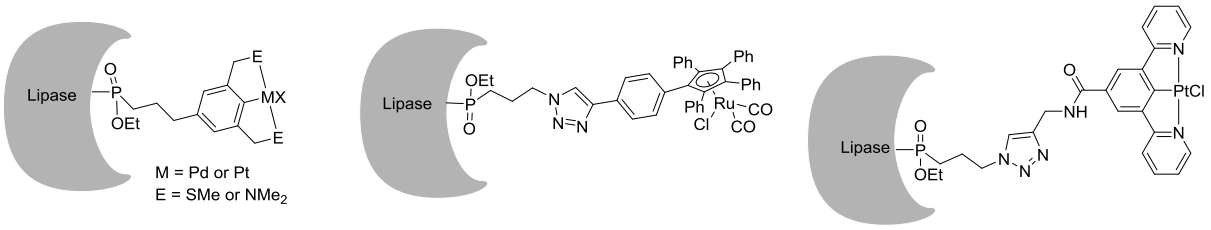


Figure 1. Previous metalloprotein hybrids constructed by the active site-directed approach.

During this time other hybridization strategies in the research community have produced efficient artificial metalloenzymes that are able to catalyze a variety of non-biological reactions, like allylic alkylations, Diels-Alder cyclizations and olefin hydrogenations, and to achieve high (enantio)selectivities in these reactions starting from an inherently achiral transition metal catalyst. In the current thesis, the development of active artificial metalloenzymes using a ASD conjugation strategy was further pursued. This was addressed by choosing a N-heterocyclic carbene (NHC) motif, comprising both a metallic center as well as a phosphonate ester to address the active site of lipases, as the artificial cofactor (Figure 2). The choice for this motif was motivated by the coordination versatility of NHC ligands and the well-known robustness of metal(NHC) complexes, in combination with the numerous catalytical applications of metal(NHC) complexes at mild and aqueous conditions.

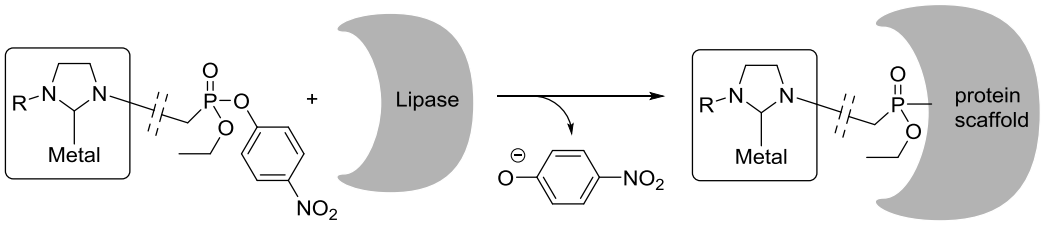


Figure 2. General inhibition protocol pursued in this thesis for the creation of ASD artificial metalloenzymes.

To provide an outlook on the position of the ASD approach in the field of artificial metalloenzymes, the different methodologies for the modification of proteins and enzymes towards catalytic semi-synthetic hybrids are described in **Chapter 1** of this thesis. A number of selected examples of catalytic reactivity and selectivity displayed by artificial metalloenzymes are used to illustrate the extent of their applications in catalysis and the progression in the field. Therein, the anticipated advantages of metal(NHC)-phosphonate inhibitors are also introduced.

Chapter 2 describes the synthesis of a new NHC ligand bearing a propyl phosphonate tail and its anchoring in the lipase Cutinase, a small serine hydrolase with an solvent-accessible active site. This bifunctional ligand was metallated to form a lipase active site-directed rhodium(NHC)-phosphonate inhibitor. ESI-MS studies and residual activity tests showed that this inhibitor can be successfully anchored to Cutinase as well as to CalB, where the latter enzyme is known to bear its active site in a deeper location than Cutinase. This study showed the first covalent active site-directed M(NHC)-protein hybrids and proved for the first time the versatility of the ASD approach by facile screening of irreversible protein scaffolding with a single organometallic inhibitor.

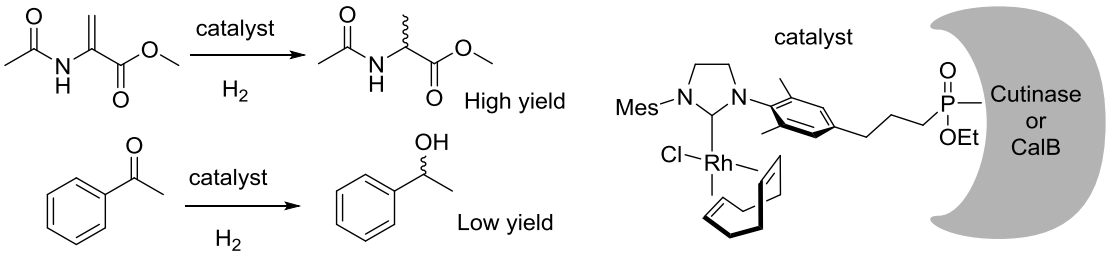


Figure 3. Chemoselective catalytic hydrogenation with Rh(NHC)-protein artificial enzymes.

Moreover, the metalloprotein hybrids were used for the catalytic hydrogenation of the prochiral ketone acetophenone and the prochiral olefin methyl 2-acetamidoacrylate. Catalytic studies with an unsupported parent Rh(NHC) catalyst and the Rh-Cutinase hybrid allowed for an optimization of reaction conditions, resulting in catalytic activity of the hybrid in both reactions and representing the first example of active ASD homogeneous artificial metalloenzymes. While the unsupported catalyst showed a slight preference towards the hydrogenation of the olefin, the hybrids showed an enhanced chemoselectivity, yet no enantioselectivity, towards the conversion of the olefin. Exclusive conversion of the olefin by the Rh-CalB hybrid was observed in competition experiments using both substrates in the same reaction mixture, demonstrating for the first time the generation of a substrate selective 2nd coordination sphere around the metallic center in an artificial metalloenzyme (Figure 3).

Chapter 3 describes the use of the NHC-phosphonate cofactor developed in the previous chapter for the generation of Grubbs-protein metallohybrids for olefin metathesis. For this the NHC moiety was varied to contain differently substituted N-aryl groupings to obtain, after metallation with ruthenium, the corresponding Grubbs-Hoveyda-II type complexes. We observed that Cutinase suffered an impediment to form a Ru-protein hybrid when an inhibitor with bulky substituents on the NHC ligand was used. On the other hand, an inhibitor with sterically less demanding substituents led to the desired hybrid. In the latter case, however, the protein scaffold substantially hindered its activity in ring-closing and cross metathesis (of N,N-dially *p*-toluenesulfonamide and allylbenzene, respectively), again demonstrating an important 2nd coordination sphere achieved by placing the catalyst in the active site of the lipase, in spite of the relatively small size of Cutinase.

In order to achieve catalytic activity, the NHC-phosphonate cofactor was enlarged with a hexyl tether. In the new hybrid derived from this larger inhibitor the steric hindrance of the protein envelop is overcome, resulting in a hybrid active in ring-closing metathesis with good product yields, representing the first covalently attached active site-directed artificial enzyme for olefin metathesis. Gratifyingly, the same hybrid is also active in the cross metathesis of allylbenzene giving the product in excellent yields. To our knowledge, this is the first report of catalytic cross metathesis with an artificial enzyme (Figure 4).

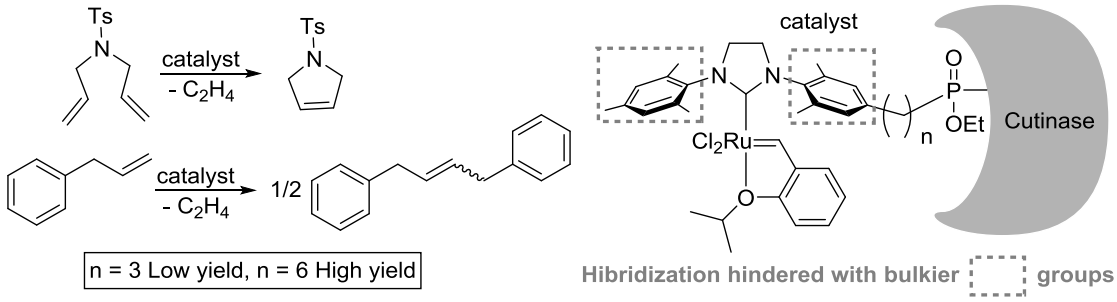


Figure 4. Ring-closing and cross metathesis with covalent active site-directed artificial metalloenzymes

In **Chapter 4**, a series of tethered NHC-phosphonate cofactors were metallated with a Pd(η^3-allyl) motif with the purpose of studying enantioselectivity in asymmetric allylic alkylation reactions catalyzed by the corresponding Pd-hybrids. Preliminar catalytic studies with the unsupported Pd(NHC) complex showed that the presence of PPh_3 was paramount to achieve catalytic activity. A series of experiments were, therefore, conducted to determine whether the Pd-NHC bond is cleaved in the presence of PPh_3, which in the case of the Pd-hybrid would result in release of Pd from the hybrid. The outcome of this study clearly showed the formation of [Pd(NHC)(PPh_3)(η^3-allyl)]X (where X = Cl^- or BF_4^-) species, ruling out Pd-NHC bond cleavage.

Initial attempts to catalyze the allylic alkylation of 1,3-diphenylallyl acetate with dimethyl malonate using the Pd-hybrids showed no product formation by the short-tethered hybrid and a very poor activity for the hybrid derived from a longer (heptyl-)tethered inhibitor. With the smaller substrate 1-phenylallyl acetate, the catalytic activity of both hybrids increased to low-moderate product yields, while product enantioselectivity was not found. This substrate size-selectivity proved the generation of a 2nd coordination sphere, restricting the formation of a catalytic intermediate with larger η^3-coordinated species. Interestingly, a large difference in the linear/branched product ratio in this reaction was found between the Pd-protein the hybrids and the unsupported catalyst, where a shift from mostly linear to almost exclusive branched product occurred by positioning the Pd(NHC) catalyst in the enzyme. This observation represents the first case of altered linear/branch selectivity in Pd-catalyzed allylic alkylations with an artificial enzyme (Figure 5).

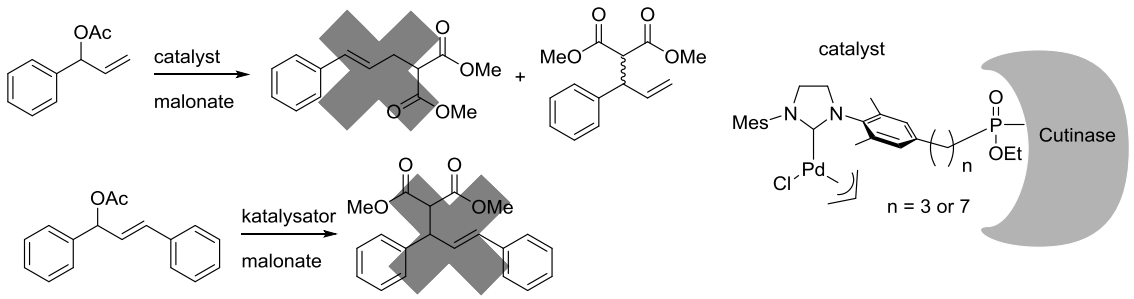


Figure 5. Selective C-C coupling alkylation with Pd(NHC)-Cutinase artificial enzymes.

Chapter 5 describes a different approach for the generation of an artificial metalloenzyme, i.e. by the encapsulation of a Rh(NHC) catalyst via ditopic anchoring in the active site of two Cutinase units. For this study, a bis(phosphonate)-NHC ligand was synthesized and preliminary inhibition experiments using this non-metallated inhibitor showed the formation of a mixture of dimeric and monomeric Cutinase hybrids, even when using an excess of Cutinase. To prevent possible hydrolysis of the second phosphonate tail in the monomeric Cutinase hybrid during the inhibition experiment, a change in pH of the reaction medium to slightly acidic medium allowing the exclusive formation of the desired protein-Rh-protein hybrid (Figure 6). The resulting macromolecular hybrid showed reduced solubility in aqueous media, greatly hampering its purification, analysis and determination of concentration. Preliminary catalytic experiments with this Cutinase-rhodium-Cutinase hybrid showed poor hydrogenation activity in a competition experiment with acetophenone and methyl 2-acetamidoacrylate and no significant difference in the chemoselectivity compared to the unsupported catalyst, nor was any enantioselectivity found. An insight in this behavior was addressed by computational modeling of the dimeric hybrid, where it was observed that the combination of NHC-protein tether chain and the generally globular conformation of Cutinase could not produce enough hindering of the metallic active site in the artificial enzyme (Figure 6).

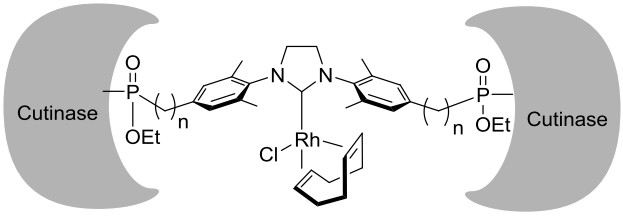


Figure 6. Ditopic hybridization of a Rh(NHC) complex towards hydrogenation artificial metalloenzymes

Conclusions and remarks

The present work represents a study of the suitability and versatility of active site-directed semi-synthetic metalloenzymes for the generation of protein-metal interactions in a second coordination sphere. Not only an extension of the previous advances on hybrids constructed via this hybridization method, the metal(NHC)-lipase hybrids developed in this study provide the first catalytically active soluble ASD-conjugates. The construction of a series of different hybrids also showed the versatility of the phosphonate inhibitor motif to address different lipases and the versatility of the artificial NHC-cofactor to produce different metallic hybrids. Given the nature of the chosen metallic centers, the lightest of the so-called precious metals (Ru, Rh and Pd), the work exemplifies the expansion of enzyme reactivity towards man-made reactions otherwise not performed with purely organic or enzymatic catalysts, i.e. hydrogenations, olefin metathesis, and C-C couplings.

Although originally pursued, enantioselectivity was not found in any of the catalytic reactions of the developed hybrids with prochiral substrates, namely in ketone or olefin hydrogenation and asymmetric allylic alkylation. We can accordingly infer that Cutinase is not a protein scaffold that easily provides an enantioselective 2nd coordination sphere to its corresponding metalloproteins. Further studies along this line using different serine hydrolases could therefore be pursued. Nonetheless, the current study has successfully provided a number of examples of artificial metalloenzymes that are able to display other types of selectivity in catalysis, i.e. competitive substrate selectivity and chemoselectivity and product regioselectivity, showing the potential of artificial enzymes to address a diverse range of selectivity behaviors.

Moreover, besides the observations on the catalytic behavior of the incorporated organometallic fragments in the hybrids, further insight has also been obtained on the inhibition scope of metallated phosphonate esters with Cutinase at different pH-buffered media. Also the more pronounced chemoselectivity found with CalB-based hybrids in comparison with hybrids based on Cutinase is consistent with the deeper location of the active site of the former enzyme. This denotes that rational selection of serine hydrolases can be used, in parallel with computational modeling of structurally known lipases, for further design of covalent active site-directed artificial metalloenzymes.

Samenvatting

De verwachte schaarste van fossiele brandstoffen heeft ertoe geleid dat de chemische industrie begonnen is om duurzame en van biomassa-afgeleidde componenten op te nemen in de materialen die het produceert. Een vervolgstap in deze ontwikkeling zou kunnen inhouden dat ook de katalysatoren die gebruikt worden in de productie van die materialen gebaseerd worden op zulke duurzame componenten.

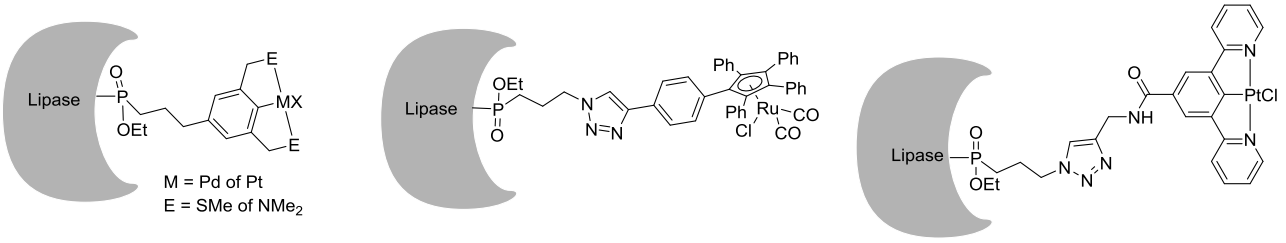
Enkele belangrijke chemische reacties, zoals bijv. polymerisaties, cyclisaties en isomerisaties, worden hedendaags effectief gekatalyseerd met behulp van homogene katalysatoren (oplosbare overgangsmetaalcomplexen). De selectiviteit in dit soort reacties is van uitermate groot belang. Een hoge selectiviteit voor het substraat zelf voorkomt de noodzaak van een voorbehandeling van reactanten, en een hoge selectiviteit naar het eindproduct voorkomt de noodzaak van verdere zuiveringsstappen. Op deze manier kunnen zuivere materialen in hoge opbrengsten worden verkregen, met name producten met specifieke biologische en fysische eigenschappen (zoals zuiver enantiomeren en isomeren in het algemeen hebben). In homogene katalysatoren is het gebruikelijk dat synthetische liganden de selectiviteit van de katalytische reactie sturen. In de zoektocht naar geschikte synthetische liganden stuiten wetenschappers vaak op voorbeelden uit de natuur, aangezien actieve centra in enzymen uitstekende selectiviteit vertonen. Enzymen zijn niet giftig, oplosbaar in water, komen vaak voor in de natuur en hebben een heel specifieke sterische hindering rondom het actieve centrum, wat regelmatig gepaard gaat met chirale inductie. Op die manier kan het actieve centrum in enzymen ook de stereochemie reguleren in asymmetrische reacties.

De combinatie van een actief en synthetisch metaalcomplex dat gebonden is aan een eiwit wordt een kunstmatig metalloenzym genoemd. De studie naar zulke metalloenzymen heeft de afgelopen tijd gefloreert, net als soortgelijke processen om duurzame, bio-geïnspireerde technologieën te ontwikkelen. De literatuur in dit veld heeft zich voornamelijk gebaseerd op het verankeren van een katalytisch metaalcomplexen aan een eiwit of enzym, waardoor een zogenaamde tweede coördinatiesfeer ontstaat en de eiwitomgeving rondom het metaal verantwoordelijk is voor de selectiviteit van het metalloenzym. Bij de constructie van selectieve metalloenzymen kan in principe elke homogene katalysator worden verankerd aan een eiwit en de zo verkregen hybrides kunnen eigenschappen bezitten die niet voorkomen in natuurlijke enzymen.

In de afgelopen tien jaar werd in onze groep onderzoek gedaan naar de immobilisatie en diversificatie van homogene katalysatoren, met dendritische katalysatoren als voorbeeld. Tegelijkertijd groeide ook de interesse in de ontwikkeling van bio-geïnspireerde technologieën. Met als doel om kunstmatige enzymen te bestuderen, werd in dit onderzoek aan de hand van de bestaande expertise in het ontwerp van liganden een nieuwe hybridisatiemethode ontwikkeld. Deze methode is gebaseerd op het

verankeren van organometaalcomplexen in het actieve centrum van een enzym. Met de wetenschap dat serine hydrolases covalent gemodificeerd kunnen worden in hun actieve centrum met behulp van de reactie met fosfonaatesters (deze kunnen als suicideremmers fungeren), werd in dit project een soortgelijke tactiek toegepast. Metaalorganische ECE-pincer complexen (Pd en Pt) werden voorzien van fosfonaatstaarten en irreversibel gebonden aan het serineresidu in het actieve centrum van het lipase Cutinase (Figuur 1). Deze zogenaamde 'active site-directed approach' (ASD) geeft covalente hybrides die dankzij hun robuuste conjugatie ook geanalyseerd kunnen worden met behulp van gedenatureerde massaspectrometrie. Verder is er gedetailleerde structurele informatie over deze hybrides beschikbaar op basis van kristalstructuren.

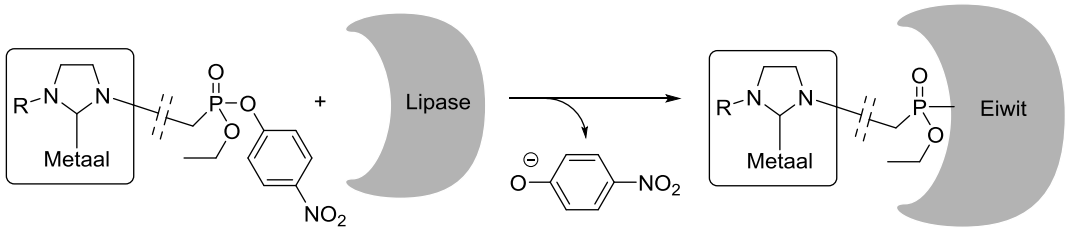
Deze ASD aanpak is ook een veelzijdige strategie om een tweede coördinatiesfeer te creëren, door de bevesting van een organometaalfragment in de natuurlijke, selectieve holte van een enzym. De vorming van eiwit-ECE metallopincerhybrides leverde derhalve het bewijs voor de ASD aanpak. Het gebruik van bovengenoemde metallopincerhybrides als katalysatoren schiet echter nog vaak tekort vanwege de te grote stabiliteit van pincercomplexen onder de milde omstandigheden, welke noodzakelijk zijn om de tertiaire eiwitstructuur te waarborgen. Niettemin werd in een recente studie aangetoond dat het incorporeren van een Ru(Cp) racemisatie katalysator in het geïmmobiliseerde lipase Candida antartica lipase B (CalB) leidt tot een katalytisch actief, kunstmatig enzym, verkregen met behulp van de ASD aanpak. Desondanks droeg in dit geval het eiwitgedeelte niet bij aan de selectiviteit in de katalytische reacties van het metaalcentrum, en heeft de synthese van een polymer-geïmmobiliseerd enzym ertoe geleid dat uiteindelijk een heterogene katalysator verkregen werd (Figuur 1).



Figuur 1. Voorgaande metallo-eiwithybrides verkregen m.b.v. de ASD aanpak.

Ondertussen heeft de ontwikkeling van andere hybridsatiestrategieën in het onderzoeksveld ertoe geleid dat efficiënte kunstmatige metalloenzymen kunnen worden geproduceerd die in staat waren om een variëteit aan niet-biologische reacties te katalyseren, zoals allylische alkyleringen, Diels-Alder cyclisaties en alkeenhydrogeneringen. Hoge (enatio)selectiviteiten kunnen worden bereikt in deze reacties, waarbij wordt uitgegaan van een inherent achirale overgangsmetaalkatalysator. In dit proefschrift is het onderzoek

naar de ontwikkeling van actieve kunstmatige metalloenzymen die gebruik maken van de ASD conjugatiestrategie voort gezet. Daarbij werd gekozen om gebruik te maken van een N-heterocyclisch carbeen (NHC) motief, in combinatie met een metaalcentrum en een fosfonaatester voor de functionalisering van het actieve centrum van lipases (Figuur 2). De keuze voor dit motief kwam voort uit de verscheidenheid in de coordinatiechemie van NHC liganden, de robuustheid van metaal(NHC)-complexen, en de met verschillende katalytische toepassingen van zulke metaal(NHC)-complexen onder milde en waterige omstandigheden.

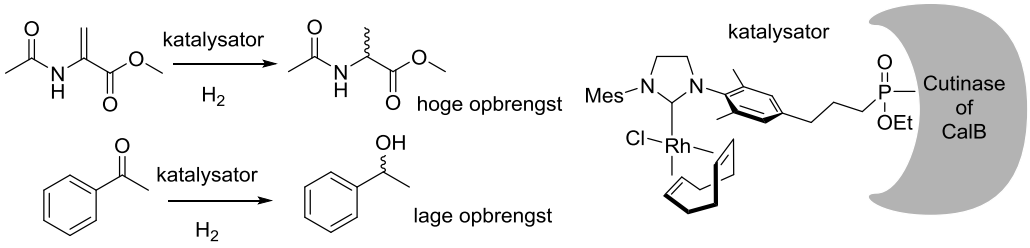


Figuur 2. Het algemene inhibitieprotocol gebruikt voor de vorming van het ASD kunstmatige metalloenzymen in dit proefschrift.

Hoofdstuk 1 van dit proefschrift beschrijft de verschillende methodologieën die ontwikkeld zijn voor de modificatie van eiwitten en enzymen voor de vorming van katalytische semi-synthetische hybrides en geeft zo een overzicht van het onderzoeksveld. Geselecteerde voorbeelden van de katalytische reactiviteit en selectiviteit van dergelijke kunstmatige metalloenzymen zijn daarbij gebruikt om de katalische toepassing van deze hybrides te illustreren, alsmede de vooruitgang in het veld. De geanticipeerde voordelen van metaal(NHC)-fosfonaatremmers worden hierin ook geïntroduceerd.

Hoofdstuk 2 beschrijft de synthese van een nieuw NHC ligand met een fosfonylstaart en het verankeren daarvan aan het lipase Cutinase, een kleine serine hydrolase met een actief centrum dat toegankelijk is voor oplosmiddelen. Dit bifunctionele ligand werd gemetalleerd om een lipase ASD rhodium(NHC)-fosfonaatremmer te vormen. Studies met ESI-MS en testen naar residuële activiteit lieten zien dat deze remmer succesvol aan zowel Cutinase als CalB verankerd kan worden, waarbij laatstgenoemde enzym een actief centrum bevat dat veel dieper in het midden van het enzym gelegen is dan bij Cutinase. Deze studie vertoonde het eerste covalente actief centrum-gestuurde M(NHC)-eiwithybride, en diende als bewijs dat de ASD aanpak veelzijdig van aard is, door middel van eenvoudige ‘screening’ van meerdere eiwit-scaffolds met een enkele organometaalremmer.

De metallo-eiwithybrides zijn bovendien gebruikt voor de katalytische hydrogenering van het prochirale keton acetofenon en het prochirale alkeen methyl 2-acetamidoacrylaat. Katalytische studies werden uitgevoerd met een niet-geïmmobiliseerde Rh(NHC) katalysator en de Rh-cutinasehybride. De hybridekatalysator vertoonde katalytische activiteit in beide hydrogeneringsreacties en is daarmee het eerste voorbeeld van een actieve homogene en kunstmatige ASD metalloenzym. Terwijl de niet-geïmmobiliseerde katalysator een lichte voorkeur voor de hydrogenering van het alkeen boven het keton vertoonde, werd in de hybridereacties een hoge chemoselectiviteit, maar geen enantioselectiviteit, voor de omzetting van het alkeen waargenomen. Met de Rh-CalB-hybride werd uitsluitend de omzetting van het alkeen waar genomen in competitieve experimenten met beide substraten in hetzelfde reactiemengsel. Dit is het eerste voorbeeld van de synthese van een substraatselectieve tweede coördinatiesfeer rondom het metaalcentrum in een kunstmatig metalloenzyme (Figuur 3).

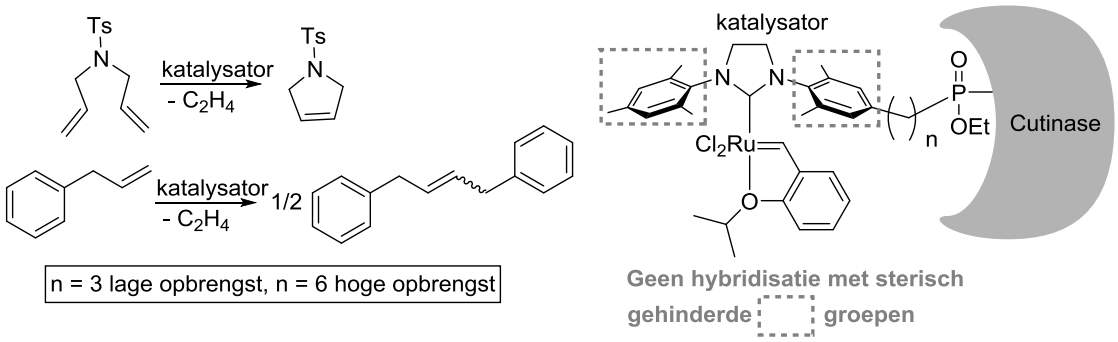


Figuur 3. ASD kunstmatige Rh(NHC)-enzymen voor chemoselectieve hydrogenering.

Hoofdstuk 3 beschrijft het gebruik van de NHC-fosfonaatcofactor gesynthetiseerd in het voorgaande hoofdstuk voor de vorming van Grubbs-eiwitmetallohybrides voor olefine-metathese. Hiervoor werd het NHC-fragment gevarieerd zodat het verschillend gesubstitueerde N-aryl groepen bevat om, na metallering met ruthenium, de overeenkomstige Grubbs-Hoveyda-II-type complexen te verkrijgen. Bij de synthese van Ru-eiwithybrides werd de inhibitie van Cutinase gehinderd bij een remmer met sterisch gehinderde substituenten op het NHC-ligand. Een remmer met minder sterisch gehinderde substituenten leidde wel tot inhibitie, maar het resulterende hydride bezit een verminderde activiteit in ringsluitingsreacties en cross-metatheses. Deze waarnemingen lieten wederom zien dat een belangrijke tweede coördinatiesfeer gecreëerd wordt door de katalysator in het actieve centrum van lipase te plaatsen, ondanks de relatief kleinere grootte van Cutinase.

Om katalytische activiteit te bewerkstelligen werd de NHC-fosfonaatcofactor vervolgens vergroot met een hexyllinker. In de hybride afgeleid van deze grotere remmer, werd de sterische hindering van het eiwit-scaffold overwonnen, hetgeen resulteerde in activiteit in ringsluitingsmetathese met goede

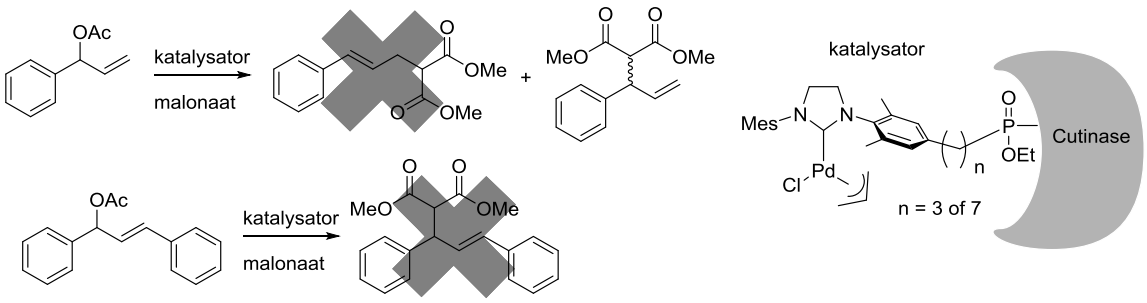
productopbrengsten. Tevens bleek dit hybride actief te zijn in de cross-metathese reactie van allylbenzeen. Dit hoofdstuk beschrijft daarmee de eerste voorbeelden van covalente ASD-hybrides met activiteit in olefine-metathese en het eerste voorbeeld van een cross-metathese reactie gekatalyseerd door een artificieel metallo-enzym (Figuur 4).



Figuur 4. Ringsluitingsmetathese en cross-metathese met een covalent gebonden ASD kunstmatig enzyme

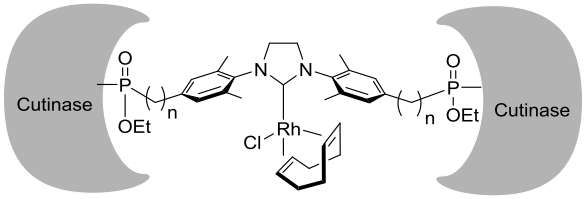
In **hoofdstuk 4** werd een serie van NHC-fosfonaatcofactoren gemetalleerd met een Pd(η^3-allyl) motief. Dit had als doel om de enantioselectiviteit in asymmetrische allylische alkyleringsreacties, gekatalyseerd door de desbetreffende Pd-hybrides, te bestuderen. Initiële katalytische studies met een niet-geïmmobiliseerd Pd(NHC)-complex demonstreerden dat de aanwezigheid van PPh_3 van essentieel belang is voor katalytische activiteit. Derhalve werd een reeks aan experimenten uitgevoerd om te bepalen of de Pd-NHC binding verbroken werd in aanwezigheid van PPh_3, wat in het geval van een Pd-hybride zou leiden tot afsplitsing van Pd van het hybride. De uitkomst van de studie liet echter zien dat de vorming van [Pd(NHC)(PPh_3)(η^3-allyl)]X (waar X = Cl of BF_4^- is) complexen plaatsvond, en sluit daarmee het verbreken van de Pd-NHC binding uit.

Pogingen om de allylische alkyleringsreactie van 1,3-diphenylallylacetaat met dimethylmalonaat te katalyseren met behulp van de Pd-hybrides lieten geen tot matige productvorming zien. Met het kleinere substraat 1-phenylallylacetaat werd echter een hogere katalytische activiteit waargenomen, maar wel met lage tot middelmatige productopbrengsten en zonder product-enantioselectiviteit. Interessant genoeg werd een groot verschil waargenomen in de verhouding van lineair/vertakt product in de reactie met de niet-geïmmobiliseerde katalysator en de Pd-hybrides, waarin een verschuiving van voornamelijk lineair product naar uitsluitend vertakte product plaatsvond. Deze observatie laat het eerste voorbeeld zien van een verschil in de verhouding van lineair/vertakt product in Pd-gekatalyseerde allylische alkyleringsreacties met of zonder een kunstmatig enzym (Figuur 5).



Figuur 5. Selectieve allylische alkyleringsreacties met kunstmatige Pd(NHC)-Cutinase-enzymen.

Hoofdstuk 5 beschrijft een andere aanpak voor het vormen van een kunstmatig metallo-enzym. Hierin wordt een Rh(NHC)-katalysator ingesloten via een ditopische verankering in de actieve centra van twee Cutinase-eenheden. Voor deze studie werd een bisfosfonaat-NHC ligand gesynthetiseerd en initiele inhibitie-experimenten met deze niet-gemetalleerde remmer lieten zien dat de vorming van een mengsel van dimere en monomere Cutinase-hybrides plaatsvindt, ook als een overmaat Cutinase wordt gebruikt. Om een mogelijke hydrolyse van de tweede fosfonaatsgroep in het monomere Cutinase-hybride te voorkomen tijdens de inhibitie, werd een verandering in pH van het reactiemedium naar een licht zuur medium uitgevoerd. Dit had als gevolg dat uitsluitend het gewenste eiwit-Rh-eiwithybride werd verkregen uitgaande van de ditopische Rh-remmer (Figuur 6). Het zo verkregen macromoleculaire hybride vertoonde echter eem matige oplosbaarheid in waterige oplossingen, wat nadelige gevolgen had voor zuivering, analyse, en concentratiebepalingen. Initiële katalytische experimenten met dit cutinase-rhodium-cutinasehybride demonstreerden een slechte hydrogeneringsactiviteit in competitieve experimenten met acetofenon en methyl 2-acetamidoacrylaat. Er werd geen beduidend verschil waargenomen in de chemoselectiviteit in vergelijking met de niet-geïmmobiliseerde katalysator en geen enantioselectiviteit vastgesteld. Bovendien werden computationele modelleringstudies van het dimere hybride uitgevoerd om meer inzicht in dit gedrag te verkrijgen. Daarbij werd het duidelijk dat de combinatie van de bifunctionele NHC-eenheid en de globulaire conformatie van Cutinase weinig afscherming van het metallische centrum in het kunstmatige enzym teweeg brengen (Figuur 6).



Figuur 6. Ditopische hybridisering van een Rh(NHC)-complex voor de constructie van kunstmatige metalloenzymen

Conclusie en opmerkingen

Het onderzoek beschreven in dit proefschrift omhelst een studie naar de geschiktheid en verscheidenheid van ASD semi-synthetische metallo-enzymen in het vormen van eiwit-metaalinteracties, d.w.z. een tweede coördinatiesfeer. De beschreven bevindingen zijn niet alleen een uitbreiding van het aantal beschreven hybrides die gevormd zijn via deze hybridisatiemethode, maar de metaal(NHC)-lipasehybrides die hier ontwikkeld zijn zijn ook de eerste katalytisch actieve, oplosbare ASD-conjugaten. De synthese van een reeks van verschillende hybrides laat de mogelijkheid van het fosfonaatremmersmotief om verschillende lipase te adresseren en de verscheidenheid van de kunstmatige NHC-cofactoren zien. Gezien de aard van de gebruikte metalen, de lichtste van de zogenaamde edelmetalen (Ru, Rh en Pd), laat dit werk ook zien dat de reactiviteit van natuurlijke enzymen kan worden uitgebreid naar reacties, welke normaal gesproken niet met uitsluitend organische of enzymatische katalysatoren kunnen worden uitgevoerd, zoals hydrogeneringsreacties, alkeenmetatheses en C-C-koppelingsreacties.

Hoewel in eerste instantie beoogd, werd in geen van de gevallen enantioselectiviteit in de katalytische reacties van de ontwikkelde hybrides met prochirale substraten waargenomen, zoals in hydrogeneringsreacties of allylische alkyleringen. Er kan derhalve geconcludeerd worden dat Cutinase geen eiwit-scaffold is dat gemakkelijk een enantioselectieve tweede coördinatiesfeer vormt in kunstmatige metalloeiwitten. Niettemin laat de huidige studie een aantal kunstmatige metalloenzymen zien die andersoortige selectiviteit vertonen in katalytische reacties, zoals competitieve substraatselectiviteit, chemoselectiviteit en product regioselectiviteit. Dit laat de potentie zien van dergelijke kunstmatige enzymes om een diversiteit aan selectiviteiten te vertonen.

Tevens werd er, buiten de observaties van katalytische activiteit van de geïncorporeerde organometaalfragmenten in de hybrides, aanvullend inzicht verkregen in het inhibitiegedrag van gemetalleerde fosfonaatesters met Cutinase in verschillende pH-gebufferde media. Bovendien is de verhoogde chemoselectiviteit met CaIB-gebaseerde hybrides in vergelijking met hybrides op basis van Cutinase consistent met het dieper gelegen actieve centrum van het eerstgenoemde enzym. Dit laat zien dat een rationale selectie van serine hydrolases kan worden gebruikt, samen met computationele modelleringsstudies van structureel bekende lipases, voor het verdere ontwerp van kunstmatige ASD metalloenzymen.

When we dream alone it is only a dream, but when many dream together it is the beginning of a new reality.
Friedensreich Hundertwasser

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Manuel

Curriculum vitae

Manuel Basauri Molina was born on January 29th, 1983, in Mexico City. After high school studies in the area of natural sciences, physics and mathematics, he carried bachelor studies in the Faculty of Chemistry of the National Autonomous University of Mexico (UNAM). During these studies he also became a research assistant student at the Institute of Nuclear Sciences, working on kinetic studies of the oxidation of aromatic compounds by iron catalysts and radiation, under the supervision of Prof.dr. M. G. Albarrán Sánchez. In 2007 he completed his bachelor studies with a thesis on supramolecular and catalytic studies of palladium complexes with chelating amines and fluorinated sulfides as ligands, under the supervision of Prof.dr. D. Morales Morales. In the same year he started the master program on chemical sciences at UNAM with a focus on synthesis, analytical and physical chemistry, symmetry, coordination chemistry, bioinorganics and catalysis, and concluded his degree in 2009 with a thesis on the catalytic activity of mercaptane-derived Pd and Ni pincer complexes in carbon, nitrogen and sulfur cross couplings under microwave irradiation; also under supervision of Prof.dr. D. Morales Morales. In 2010 he began his PhD studies at the group of Organic Chemistry and Catalysis, Utrecht University, under the supervision of Prof.dr. R. J. M. Klein Gebbink. The most important results of this multidisciplinary project are presented in this thesis. Several parts of this research have been presented in international conferences, such as the 1st Symposium on Artificial Metalloenzymes, the International Conference on Phosphorous Chemistry, and the Netherlands' Chemistry and Catalysis Conference. Manuel Basauri's passion for science is not limited to his experience in chemistry but, as illustrated in his publications, he enjoys technological innovations, alternative energy-sourced processes and multidisciplinary projects, with a main focus in transition metal chemistry and catalysis.

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