Phosphoramidite ligands and artificial metalloenzymes in enantioselective rhodium-catalysis
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Papain, modified at Cys-25 with a monodentate phosphite ligand and complexed with Rh(COD)$_2$BF$_4$, is an active catalyst in the hydrogenation of methyl 2-acetamidoacrylate.

[...] the pace, at which ‘new’ enzymes for selective biotransformations are appearing, is astonishingly modest. The reasons for this are probably manifold: first, the conservatism of academic and industrial researchers avoiding high-risk projects; second, lack of interdisciplinarity among chemists and biologists; [...]。


5.1 Introduction

There are three main routes for the preparation of enantiomerically pure compounds: (1) separation of racemates; (2) transformation of a precursor provided by natural sources from fermentation or agriculture; (3) asymmetric synthesis from prochiral substrates using both catalytic and stoichiometric methods (Figure 5.1).

![Diagram of Routes to enantiopure compounds](image)

**Figure 5.1** *Routes to enantiopure compounds*

The most convenient and attractive of these methodologies, in terms of atom efficiency and mildness of reaction conditions, are enantioselective chemo-catalysis and biocatalysis.

Enantioselective metal-catalyzed transformations have developed enormously in the last decades, providing fundamental contributions to the establishment of modern organic chemistry. Nevertheless, although homogeneous asymmetric catalysis has reached an advanced stage in the laboratory, the method of choice to obtain enantiopure intermediates, in the production of pharmaceuticals, is still the resolution by crystallization of diastereomeric salts. One of the main reasons for this is the strong time-to-market pressure related to their production. Fortunately, high throughput experimentation can be used increasingly for the rapid identification of a chiral catalyst. The availability of large libraries of chiral catalysts and in particular of the corresponding ligands can be, however, a serious bottleneck. Phosphorus based chiral ligands are arguably the most versatile ligands for asymmetric catalysis. However, the availability of chiral bidentate phosphorus-based ligands is often associated with cumbersome and lengthy synthesis. Recently, there has been a revival of the use of monodentate chiral phosphorus ligands and excellent results have been achieved in different fields of asymmetric catalysis. Moreover, monodentate phosphoramidites, developed in
our laboratories, and monodentate phosphite ligands have the advantage of a simple modular design which allows the versatile synthesis of a large diversity of structures (Scheme 5.1). These ligands can be synthesized in an automated manner, opening the doors to large libraries and truly combinatorial approaches.

Scheme 5.1  Modular structure of phosphoramidites and phosphites

Biocatalysis is also increasingly applied in organic chemistry for a variety of transformations, emerging as a valuable alternative for the preparation of enantiopure compounds. Characteristics such as chemo-, regio- and stereoselectivity manifested by biocatalysts are among the most appealing features connected with their use. Nevertheless, also in this field, despite the continuous efforts, a limited amount of industrial applications has been established. The reasons attributed to the limited implementation of biocatalysis into production seem to be: (1) the commercial availability of the biocatalysts; (2) their limited substrate scope; (3) their operational stability; (4) the reluctance of the chemical community to fully explore their potential.

Inevitably, chemocatalysis and biocatalysis have fundamental differences in their positive and negative features (some of which are listed in Table 5.1) that make them rather complementary.

Table 5.1  Characteristics of homogeneous and enzymatic catalysis

<table>
<thead>
<tr>
<th></th>
<th>homogeneous catalysis</th>
<th>enzymatic catalysis</th>
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<tbody>
<tr>
<td>Enantiomers</td>
<td>both accessible</td>
<td>generally one enantiomer</td>
</tr>
<tr>
<td>Reaction diversity</td>
<td>large</td>
<td>small</td>
</tr>
<tr>
<td>Substrate scope</td>
<td>large</td>
<td>small</td>
</tr>
<tr>
<td>Solvent preference</td>
<td>mostly organic</td>
<td>mostly aqueous</td>
</tr>
<tr>
<td>Optimization</td>
<td>chemical</td>
<td>genetic</td>
</tr>
<tr>
<td>Second coordination sphere</td>
<td>less defined</td>
<td>more defined</td>
</tr>
<tr>
<td>Turnover numbers</td>
<td>smaller</td>
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As summarized in Figure 5.2, although operating in apparently parallel universes, asymmetric homogeneous catalysis and biocatalysis have the preparation of
enantiomerically pure compounds as a common goal. On one side, new routes using metal-catalyzed approaches to target chiral molecules are continuously explored; on the other side, there is a continuous search for enzymes with novel catalytic properties. Similarly, once a hit has been found, improvements of the performance of the catalyst are achieved by its iterative redesign or modification.

Figure 5.2  Chemistry and biochemistry: different approaches, similar goal

Moreover, the idea of cooperation (for example in cascade reactions) between the two fields starts to emerge as an appealing opportunity to expand the toolbox of modern organic chemistry and the implementation of catalysis in the preparation of chiral molecules.12

5.2 Scope of this study

An even more intriguing development, going beyond their simple cooperation, would be the fusion of these two approaches by the insertion of non-chiral metal catalysts into the active site of an enzyme. In this way, the enzyme will provide the chiral environment and the chemo-catalyst will allow the enzyme to perform new types of catalysis, thereby broadening the scope of both fields (Figure 5.3). Once the methodology has been established, this approach would also allow to take advantage of the tremendous advances in molecular biology techniques, such as gene manipulation and functional selection methodologies, for the preparation of large libraries of proteins, expanding even further the combinatorial approach for the identification of suitable catalysts for all kinds of transformations.13,14
Merging Homogeneous Catalysis with Biocatalysis: Papain as Hydrogenation Catalyst

**Figure 5.3** Achiral ligands and proteins scaffolds as source of novel catalysts

Based on the highly successful use of monodentate phosphorus ligands in asymmetric catalysis, this study focused on the challenging goal of building a hybrid enzyme-bound rhodium catalyst with a single phosphorus donor ligand for hydrogenation and hydroformylation.

### 5.3 The pioneering work of Kaiser and Whitesides

The first examples of the preparation of semisynthetic enzymes appeared in 1966 by Bender and Koshland and their coworkers. It consisted in the chemical replacement of the serine residue with a cysteine in the active site of subtilisin. The enzyme lost its ability to hydrolyze amide bonds but maintained the esterase activity.

Chemical modification of residues in proteins was known before these studies and was used to investigate and identify important residues in the proteogenic structures. In this case, the fundamental difference was the intention to actually use the resulting semisynthetic enzymes for biocatalytic transformations. These early attempts not only established the synthetic methodologies, but most importantly demonstrated that, while working with enzymes, researchers could be not only spectators but also designers of enzymatic activities.

Twenty years later, Kaiser and coworkers demonstrated the usefulness of the modified thiol-subtilisin as peptide ligase. The same concept was utilized also by Hilvert and Wu for the preparation of a seleno-subtilisin with peroxidase activity. There have been a number of examples in the literature, in which residues were changed in order to obtain different specificities. During the years, the concept evolved from chemical modifications to even more sophisticated protein engineering such as introduction of non-natural amino acids, peptide ligation and genetic techniques. Nevertheless, these approaches limit the modifications to the
enzyme backbone and to the interconversion of reactivity or specificity among enzymes.

Another fundamental step towards a better understanding of the potential of protein structural manipulation is represented by the milestone work of Kaiser,21 Whitesides22 and their coworkers. They were the first, in the late seventies, to envision the possibility of inducing new catalytic properties by introducing artificial cofactors into protein scaffolds. Nevertheless, although motivated by the same pioneering spirit, they undertook two completely different approaches.

Kaiser and co-workers hypothesized that it was possible to use the cysteine residue in the active site of papain in order to chemically introduce a cofactor able to perform a reaction previously unknown to the enzyme.21 Consequently, the irreversible modification resulted in the complete loss of the original proteolytic activity. Flavins were chosen as cofactors (Scheme 5.2), since they are quite effective oxidation catalysts even in the absence of the protein scaffold.

Therefore, it seemed likely that flavoenzymes could be generated without the involvement of specific α-amino acid functional groups in the protein for the new catalytic reaction to occur. As a result, papain modified with different flavins (5.1a-b) was indeed an active catalyst in the oxidation of NADH analogs 5.2, reaching up to 670-fold rate acceleration compared to the corresponding flavin. Moreover, a preference for the abstraction of the pro R hydride of 5.2 was observed, which would suggest the involvement of the chiral environment of the protein scaffold.23

On the other hand, Whitesides and Wilson described an approach for the construction of an asymmetric hydrogenation catalyst based on the embedding of an achiral catalyst in a protein at a specific site.22 The protein tertiary structure would provide the necessary chiral environment required for the enantioselective hydrogenation to occur. Again as a consequence, the protein would be equipped with new catalytic properties.

Scheme 5.2  Flavopapains 5.1a-b, as first example of covalently attached cofactors

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For this purpose, avidin was chosen because it was a well-characterized globular protein composed of 4 identical subunits. Most important, each subunit was known to bind a molecule of biotin so tightly that the association could be considered effectively irreversible.

\[
\text{NHHN} \quad \text{S} \quad \text{O} \quad \text{HH Ph}_2 \quad \text{P} \quad \text{P} \quad \text{Ph}_2 \quad \text{Rh} \quad + \quad \text{Tf}^+ \quad \text{O} \quad \text{OH} \quad \text{HN} \quad \text{O} \quad \text{OH} \quad \text{HN} \quad \text{O} \quad \text{HN} \quad \text{O}
\]

Scheme 5.3  Biotin-avidin technology used in asymmetric hydrogenation

Therefore, as shown in Scheme 5.3, biotin was modified with an achiral Rh-diphosphine catalyst and the adduct 5.4a was active in the hydrogenation of 2-(acetamido)acrylic acid (5.5). In the presence of avidin, N-acyl alanine (5.6) was obtained with 41% enantioselectivity.

5.4 Artificial metalloproteins: state of the art

The concept of hybrid biocatalysts, as proposed by Kaiser and Whitesides, seemed to have been forgotten for almost twenty years. However, in the last decades, biotechnology, bioengineering and computational techniques evolved tremendously, together with the understanding of why certain protein structures exist and how they operate. Between the different protein families, metalloproteins have been receiving increasing attention as they are among the most efficient and diverse biocatalysts. Consequently, there is a growing interest in bio-inorganic chemistry and the boundary aspects of this kind of research. The modification of the binding properties, metal centers and metal-containing cofactors seem to offer access to different complexes with potentially broad applications. These applications include affinity purification of proteins, improved and metal-mediated protein stability, imaging and therapy, biosensors and possibly new catalysts. Nevertheless, nature offers only a limited amount of ligands, metals and metal-containing prosthetic groups compared with what is provided by metal-coordination chemistry. Therefore, the introduction of non-natural metal containing cofactors seems to be a natural evolution toward novel biocatalysts.

Technical advances and changes in the scientific community’s perspective seem to be at the origin of a renewed interest in the construction of artificial metalloenzymes. The growing efforts towards their creation have been mainly concentrated on either covalent incorporation of cofactors or supramolecular approaches.
5.4.1 The non-covalent approach

Chan and coworkers were the first to reconsider the work of Whitesides. In their extension of this approach, biotin was modified with a chiral bidentate phosphorus ligand 5.4b and used in the hydrogenation of itaconic acid (5.7).30

![Scheme 5.4](image)

Scheme 5.4  Extension of Whitesides approach using a chiral diphosphine based cofactor 5.4b in the hydrogenation of itaconic acid (5.7)

Using the cofactor 5.4b shown in Scheme 5.4 product 5.8 was obtained with 48% ee, whilst using the cofactor containing the ligand with opposite configuration only 16% ee was found. The results, even if not exciting, demonstrated the influence of the tertiary structure within the protein cavity.

More recently, Ward and coworkers31 obtained remarkable results (Scheme 5.5) optimizing the approach of Whitesides by using streptavidin, another biotin-binding protein which displays similar affinity but only 35% sequence homology. The main difference appeared to be the presence in streptavidin of a deeper binding pocket. Furthermore, site specific mutagenesis allowed to obtain N-acetyl alanine (5.6) with an increased 96% ee.

![Scheme 5.5](image)

Scheme 5.5  Optimization of Whitesides approach using a streptavidin adduct of 5.4c and site specific mutagenesis

The authors extended the approach to transfer hydrogenation of methyl aryl ketones (5.12a-c).32 The new cofactor 5.11a was prepared by modification of biotin with an analog of Noyori’s amino sulfonamide scaffold complexed with [η⁶-(p-cymene)RuCl₂]₂ (Scheme 5.6). In the presence of mutants of streptavidin, the hybrid catalyst was able to induce up to 94% ee in the hydrogenation of p-methyl acetophenone 5.12c using formate-boronic acid as reducing agent.
Merging Homogeneous Catalysis with Biocatalysis: Papain as Hydrogenation Catalyst

Scheme 5.6  
**Artificial metallo-enzyme as catalyst in transfer hydrogenation using biotin-streptavidin technology**

Using the same biotin-(strept)avidin technology, Ward and coworkers investigated also the reverse reaction, namely the oxidation of alcohols (Scheme 5.7). In this case, avidin resulted to be the best host for biotin modified with the aminosulfonamide Ru(II) catalyst 5.11b. The hybrid metalloprotein was able to convert phenethyl alcohol (5.13a) into the corresponding acetophenone (5.12a) in the presence of TBHP as oxidizing agent.

Scheme 5.7  
**Artificial metallo-enzyme using biotin-avidin technology as catalyst for alcohols oxidation**

Keinan and Nimri were the first to propose a metalloporphyrin-antibody (SN37.4) assembly 5.14 and to adopt it as model of a heme-dependent enzyme. Using Ru(II) as metal center, 43% ee was obtained in the oxidation of thioanisole (5.18a) using PhIO as oxidizing agent (Scheme 5.8).

More recently, Mahy and coworkers also reported an antibody (3A3) containing a Fe(II)porphyrin complex 5.15. In this case, trying to overcome the intrinsic lower reactivity associated with artificial hemoproteins, they decided to equip the phorphyrin moiety with a heme octapeptide (MP8) containing a histidine able to act as extra ligand for the metal center. The artificial metallo protein was tested in the oxidation of thioanisole (5.18a) and 45% ee was obtained using H2O2 as oxidizing agent. Nevertheless, the activity of this system was still not comparable with the activity of authentic hemoproteins.

In their investigation, Watanabe and coworkers developed mutants of apo-myoglobin (apo-Mb) reconstituted with Cr(III) Schiff base complex 5.16, known to be able to perform oxidation reactions. Apo-myoglobin was chosen as its
reconstitution with heme is well documented. For example, as it is well known that the binding affinity of heme to apo-Mb is caused by hydrophobic interactions, they introduced two t-butyl groups in the 5-, 5'- positions of salophen. The artificial metallo-enzyme obtained was tested in the H$_2$O$_2$-dependent sulfoxidation of thioanisole (5.18a) providing 13% ee.

![Scheme 5.8](image)

**Scheme 5.8**  Use of artificial metallo-enzymes in the sulfoxidation of thioanisole

The most recent artificial hemoprotein was presented by Gross and Mahammed. They adopted Fe(III) and Mn(III) amphiphilic corrole complexes 5.17 conjugated with albumin (HSA). Mn-corroles have been studied in recent years by the authors for their potential in oxidation catalysis; moreover the amphiphilic sulfonated version 5.17, depicted in Scheme 5.8, was shown to form stable conjugates with HSA, which is a cheap and readily accessible protein. Once more, the biomimetic system was tested in the oxidation of a number of aryl methyl sulfides 5.18 reaching up to 74% ee (5.19b) with various degrees of activity.
5.4.2 The covalent approach

In the covalent approach, following the pioneering study of Kaiser, a number of examples of chemical modification of reactive residues in proteins appeared in the literature. In the field of artificial metalloproteins, Meares and Rana attached a Fe-EDTA chelate (Fe-BABE) to Cys-212 of human carbonic anhydrase I (HCAI), the adduct 5.20 was able in the presence of ascorbate and \( \text{H}_2\text{O}_2 \) to cleave selectively HCAI itself after Leu-189 in the peptide chain (Scheme 5.9). The hydrolysis was sensitive to the proximity of the attached reagent more than the presence of specific residues. Moreover, Meares and coworkers applied this approach to \textit{E. coli} RNA polymerase, a DNA-binding protein, which was used in affinity-cleavage experiments in order to identify interaction-sites between nucleic acids and RNA polymerase.

![Scheme 5.9](image)

Scheme 5.9 Artificial iron-dependent protease 5.20

Davies and Distefano introduced a Cu(II) 1,10-phenantroline complex 5.21 in adipocyte lipid binding protein (ALBP); whilst Janda and coworkers proposed an aldolase antibody (38C2) derivatized with a Cu-binding bis-imidazolyl cofactor 5.22; both systems showed hydrolytic activity in the presence of activated esters (A, Scheme 5.10) such as picolinic acid nitrophenyl ester (5.23). Additionally, the system 5.21 adopted by Davies and Distefano showed also to be active in the presence of simple \( \alpha \)-amino acids alkyl esters 5.26 (B) providing enantioselectivities between 31\% and 86\% for the \( \alpha \)-amino acids 5.27 obtained.

![Scheme 5.10](image)

Scheme 5.10 Cu-based artificial metalloproteins 5.21-5.22 with hydrolase activity
Moving away from these interesting but still more traditional examples of hybrid metallo-enzymes, Reetz and coworkers reported the introduction in papain of maleimide based manganese-salen 5.28 and Rh-dipyridin 5.29 complexes as epoxidation and hydrogenation catalysts, exploiting the maleimide unit as Michael acceptor for the reactive Cys-25 (Scheme 5.11). Although no clear experimental documentation was provided, the new biocatalysts are reported to be able to promote hydrogenation and epoxidation reactions, even if the enantioselectivities obtained were less than 10%. They also proposed a diphosphine-based phosphonate inhibitor 5.30 (Scheme 5.11), known to react with the catalytically active serine lipases resulting in covalent inhibition of the residue. Unfortunately, the activity was largely restored within a day, due to the hydrolysis of the phosphonate moiety from the lipase, not surprisingly as such a phenomenon is known and in this case was accentuated by the presence of a labile p-nitrophenol substituent on the phosphonate.

Scheme 5.11 Hybrids biocatalysts 5.28-5.30 envisioned by Reetz and coworkers

Another example of covalent cofactor attachment, in this case of a manganese-salen complex 5.31 into apo sperm whale myoglobin (Apo-Mb), has been recently presented by Lu and coworkers and tested in enantioselective sulfoxidation reactions (Scheme 5.12). The cofactor introduced is similar to the one proposed by Watanabe (5.16, Scheme 5.8) following a supramolecular approach and by Reetz (5.28, Scheme 5.11) using a covalent approach. The special feature of this work consisted in a double mutation that allowed anchoring the cofactor to two cysteine residues, limiting the conformational freedom. The approach resulted to be successful as 51% ee was obtained in the oxidation of thioanisole (5.18a), compared to 12% ee provided by the same cofactor 5.31 but with only one point attachment. Moreover, the double anchoring of the cofactor proved also to be beneficial for the reactivity.
Merging Homogeneous Catalysis with Biocatalysis: Papain as Hydrogenation Catalyst

Recently, van Koten and coworkers\textsuperscript{46} reported the modification of cutinase (a lipase) with "pincer"-type metal complexes 5.32a-c using an approach similar to the one reported by Reetz (5.30, Scheme 5.11).\textsuperscript{43b} The phosphonate adducts described in this work were found to be stable and no dissociation was observed (Scheme 5.13). Nevertheless, these modified proteins have not been reported to possess any kind of catalytic activity.

Scheme 5.13 "Pincer"-type metal complexes 5.32a-c used in the modification of cutinase

5.4.3 Other approaches for the incorporation of metal centers

Beside the two main lines of research presented, there have been reports of different approaches for the incorporation of metal centers in proteins.\textsuperscript{47} For example, using solid phase methodology, Imperiali and Roy prepared a semisynthetic RNase-S protein incorporating an unnatural amino acid with a pyridoxamine moiety 5.33 which displayed transaminase activity (Scheme 5.14).\textsuperscript{48} Suckling and coworkers replaced existing naturally occurring zinc in carboxypeptidase A (CPA) with different metals.\textsuperscript{49} The exchange of Zn with Ni, Co, Rh proved successful, whilst denaturation of the protein occurred with Ru and Pd. The metal-CPA adducts prepared were used in hydrogenation reactions, nevertheless, no activity was observed. Sheldon and coworkers, instead, used different phytases in order to introduce transition-metal oxoanions such as VO\textsubscript{4}\textsuperscript{3-}, taking advantage of their known inhibitory power toward these proteins (Scheme 5.14).\textsuperscript{50} The semi-synthetic vanadium peroxidase 5.34 obtained showed to be...
active, in the presence of H₂O₂, for the oxidation of prochiral sulfides 5.18 reaching up to 66% ee. Marchetti and coworkers used non-specific binding of Rh(acac)(CO)₂ on HSA in order to successfully perform hydroformylation reactions in a two phases system. The use of different proteins such as papain and egg albumin was found to be less efficient.

![Chemical structures](image)

Scheme 5.14  *Alternative approaches for metal-incorporation into proteins*

Finally, theoretical approaches have been developed using automated computer search algorithms, such as Metal-Search and Dezymer, in order to design novel metal-binding sites into proteins.

### 5.5 The importance of the appropriate strategy

At the beginning of this endeavor, the main reference points, among those previously described, were still the studies of Kaiser and Whitesides. The analysis of their approaches helped clarifying the decisions that needed to be made as depicted in Figure 5.4.

![Diagram](image)

*Figure 5.4  The various aspects of the strategy require several choices*
The objective is to equip a protein with an artificial coenzyme that would perform metal-catalyzed transformations unknown to the native protein itself. With this aim in mind, it is necessary to identify suitable proteins with the following properties:

- Good availability and accessible biochemical knowledge.
- Specific (1:1 ratio) and stable adducts between the protein and the cofactor.
- Generality of the approach that could be extended to different proteins as well as different cofactors.
- Minimum destabilization of the protein itself due to the interactions with the cofactor.

### 5.5.1 Protein classes

Proteins can be divided into two main groups: fibrous and globular. Fibrous proteins play a structural function and are characterized by their insolubility which makes them not suitable for this study. Globular proteins are instead globelike proteins more or less soluble in aqueous solution and can be divided according to their functions into messengers, transporters (binding proteins) and enzymes (Figure 5.5). Transport proteins and enzymes are a desirable choice due to their availability and the specificity of either their binding properties or reactivity.

![Protein Classification Diagram](image)

**Figure 5.5 Protein classification**

Among the different transporters of major interest are those proteins with affinity for organic molecules. For example, proteins such as hemoglobin and myoglobin contain a heme unit which is responsible for the actual transport of oxygen. Therefore, the specificity and stability of the binding of the ligand is extremely important and effective. The serum protein albumin is known for being responsible for the transport of a variety among metals and more interestingly lipophilic molecules. Avidin and streptavidin are well known for their high affinity for biotin and are already widely used in immunoassays and affinity chromatography. The replacement or modification of these tightly bound molecules with others with the same binding capabilities, but designed to have interesting catalytic properties, is at the origin of the non-covalent approach of which the work of Whitesides is an excellent example. A desirable feature of the supramolecular approach is the limited amount of protein manipulation required.
On the other hand, enzymes are defined as proteins that catalyze and accelerate a variety of chemical reactions by efficiently bringing substrates together in a so-called enzyme-substrate complex located in a specific region defined as “active site”. The active site of an enzyme also contains the residues (catalytic groups) directly involved in the making or breaking of bonds and possess higher reactivity than any other identical residue present elsewhere in the protein. This specific reactivity in a well-defined position of the enzyme makes these catalytic residues very attractive and practical tools for chemical mutation, which is at the origin of the covalent approach adopted by Kaiser.

The use of enzymes as hosts for artificial cofactors guarantees the availability of an enormous reservoir of potential structures, making them a desirable choice for the establishment of a general method. The success of the introduction of a new cofactor by chemical alteration relies mainly on the reactivity of the residue itself and not on delicate and fundamental non-covalent interactions involved in a strong and selective binding. Moreover, the number of proteins which display this kind of binding for non-proteinogenic molecules is limited. For example, the use of proteins such as avidin or myoglobin would restrict the design to cofactors preserving the natural binding capabilities of biotin and heme respectively.

In consideration of all these factors, it was decided to follow a covalent approach and introduce the artificial coenzyme by selective chemical modification of a specific residue in the active site of a given enzyme.

### 5.5.2 Enzyme families

Enzymes are generally classified according to the kind of reaction performed and the different categories are depicted in Figure 5.6.

![Enzymes classification](image)

Among the different families of enzymes the hydrolases are of particular interest. Their mechanism of action is very similar to a base-catalyzed chemical hydrolysis. Stability, lack of sensitive cofactors and wide substrate tolerance makes them very popular also in organic chemistry applications. Hydrolases are further divided in
Merging Homogeneous Catalysis with Biocatalysis: Papain as Hydrogenation Catalyst

thirteen sub-classes according to their reactivity toward specific bonds and the most common are esterases, glycosylases and peptidases.

Peptidases (or proteases) are the enzymes involved in the hydrolysis of amide (peptide) bonds and they are the most abundant and studied among the hydrolases family as they play a role in a variety of physiological processes. Furthermore, they are commonly used in sequence analysis and domain identification of other proteins. The proteolytic enzymes are further classified, according to the catalytically active residues, mechanism of action and three-dimensional structure. Each family possesses a particular set of residues which arranges similarly to form the active site. The most representative enzymes and their characteristic active site residues are depicted in Figure 5.7. These four families are grouped according to their mechanism of action: serine and cysteine proteases, which form covalent enzyme complexes with the substrate; aspartic and metallo-proteases, which do not, making them less interesting in this context.

Figure 5.7 Proteolytic enzymes divided according to the residues in the active site

Serine and cysteine proteases, which are small monomeric enzymes of $M_r$ between 15,000 and 35,000, are the most well known among the proteolytic enzymes and they share similar mechanism of action and basic characteristics. The involvement of serine proteases in digestion processes made them the object of early extensive studies that provided the basics for most of the current knowledge on protein structure and enzyme function. Investigations on the kinetics, specificity of inhibition, analyses of the $\alpha$-amino acid sequence, X-ray structure and site direct mutation led to the identification of the essential residues and mechanisms of action.

The extensive theoretical and practical knowledge available about these enzymes makes them good candidates for the introduction of an artificial cofactor using a covalent approach. These proteolytic enzymes have strongly nucleophilic serine or cysteine residues in the active site, whose reactivity is enhanced by the presence of a spatially aligned histidine residue (Scheme 5.15).
Modification of the active residues serine and cysteine by acylation or alkylation, taking advantage of their intrinsic reactivity, has been extensively used as a powerful tool to study the mechanism of action, spatial requirement and possible intermediates during catalysis. Therefore, the use of the available knowledge in enzyme inhibition seems to be the most straightforward and reliable way of chemically introducing an organic molecule in a specific position of an enzyme and will be discussed more in detail in a separate section (page 188).

The specificity of the reactivity allows control over the position in which the cofactor is positioned and over the number of cofactors introduced. In this respect cysteine proteases start to emerge as more desirable, due to the limited amount of cysteine residues present in enzymes and proteins, which are generally involved in disulfide bonds of structural importance; so if a single free cysteine is present it becomes a perfect target. With a broader perspective, the higher nucleophilicity of cysteine side chains ($pK_a$ 8.5-9.5) compared to serine ones ($pK_a$ 13), together with their limited presence, allows one to envision their potential artificial introduction in any desired position of generic protein structures maintaining the specificity of the anchoring of the cofactor.
5.6  Cysteine proteases: papain

Cysteine proteases, that are referred to as thiol proteases in the old literature, are enzymes widely distributed in nature and have been found in viruses, bacteria, protozoa, plants, and more recently in fungi. Among the many cysteine proteases isolated from plants the most commonly known are papain (from papaya), ficin (from figs), bromelain (from pineapple), and actinidin (from kiwi fruit) and they are all members of a structurally homologous family.

![Three-dimensional structure of papain with domains and binding cleft](image)

Figure 5.8  Three-dimensional structure of papain with domains and binding cleft

The best understood enzyme in this family is papain (EC 3.4.22.2), which is a small and monomeric protein of 212 residues and molecular weight of 23428 Da. Papain has been extensively studied and it is considered the main representative of its family, also because it was the first cysteine protease structure solved at high resolution (2.8 Å) by Drenth and coworkers and later refined by Kamphuis and coworkers (1.65 Å). The analysis of the x-ray structure shows that the polypeptide is folded into two distinct parts, L and R domains, which are divided by a cleft of about 15 Å with a groove of about 25 Å (Figure 5.8). The groove was found to be able to accommodate peptides constituted of up to seven amino acids residues (P, P') binding to an equal number of subsites (S, S') as depicted in Figure 5.9. The catalytically essential residues His-159 and Cys-25 can also be found in the groove, located on the opposite domains of the cleft. Besides four short α-helical segments and one short segment of β-structure, the conformation of the chain is irregular. An interesting feature of the secondary structure of papain is the presence of 7 cysteine residues, of which 6 are involved in structurally relevant disulfide bonds, making Cys-25 not only the most reactive but also the only available catalytically-active cysteine in the enzyme.
Peptides and esters are hydrolyzed through an acyl-enzyme pathway in the same manner serine proteases are, except that in this case Cys-25 is acylated (Scheme 5.15) and the rate determining step in the hydrolysis of amides and anilides appears to be the general-acid-catalyzed breakdown of this intermediate (Scheme 5.16).

\[
E + S \xrightleftharpoons[k_2]{k_1} ES \xrightarrow{k_2} EA \xrightarrow{k_3} P' \xrightarrow{P_1} E
\]

- E = free enzyme
- S = substrate
- ES = enzyme-substrate complex (or Michaelis complex)
- EA = acyl enzyme
- P' = amine product
- P_1 = acyl product

**Scheme 5.16**  *Intermediates in papain catalysis*

Numerous studies have been conducted on the reaction mechanism of papain and the role of some of the residues. These investigations generated a huge amount of knowledge on this enzyme and other cysteine proteases, although there is still disagreement on many details. Generally speaking, the high nucleophilicity of papain is caused by the thiolate-imidazolium ion pair formed by the interaction of the dissociated Cys-25 (pK_a 8.5) and the protonated His-159 (pK_a 4). This interaction is also suggested by the relative position of these two residues (3.4 Å) in x-ray diffraction studies. The low pK_a of the imidazole group of His-159 is attributed not to the interaction with an aspartic acid as in serine proteases, due to the unfavorable position, but more to the interaction with the side chain of Asn-175 (Figure 5.10). More recently, Ménard and coworkers further confirmed the role of Asn-175 by investigating the reactivity of papain in which the residue was substituted for other residues using site-directed mutagenesis.
Unlike serine proteases, papain has a broad specificity and preference for hydrophobic \( \alpha \)-amino acids. A deep non-polar secondary binding pocket, constituting hydrophobic side chains from Tyr-67, Pro-68 and Trp-69 on one side and those from Phe-207, Ala-160, Val-133 and Val-157 on the other, seems to be responsible for the preferential reactivity toward peptides with hydrophobic \( \alpha \)-amino acids such as phenylalanine, tyrosine and leucine in \( P_2 \) (Figure 5.11).80

The presence of this pocket and steric interference with the bulk of the enzyme are also responsible for stereospecificity observed for L-amino acids. It was experimentally observed that the presence of phenylalanine in the peptide forces

**Figure 5.10** Schematic representation of the active site showing the catalytic triad Cys-His-Asn79

**Figure 5.11** Schematic representation of papain-substrate interactions81
the cleft to open somewhat and increase the strain of the active site. A similar conclusion was reached analyzing the crystal structure of papain inhibited by the chloro-methyl ketone derivative of N-benzyloxycarbonyl-L-phenylalanine-L-alanine (5.37, Figure 5.12) where the estimation of the widening due to the presence of phenylalanine amounted to about 1 Å. In this way, as shown in Figure 5.11, the side chain of phenylalanine would be able to nicely fit in the hydrophobic pocket, which confers extra stabilization to the binding of the substrate and/or to the acyl adduct. Beside this preference for an aromatic residue in P2, papain possesses a rather broad specificity for what the other subsites (S) are concerned.

![Figure 5.12](image-url)  
**Figure 5.12**  
Structure of ZPACK (5.37) used in x-ray diffraction as transition state analogue

### 5.7 Low molecular weight synthetic inhibitors

The use of low molecular weight synthetic inhibitors has had a major impact on the knowledge achieved on the structure, reactivity and specificity of papain and enzymes in general. A valuable example is the just mentioned use of ZPACK (Figure 5.12). Besides being used in exploratory research, inhibitors occupy an important position also in drug discovery as means to understand or interfere with the biological pathway of enzymes. Therefore, there is a huge literature produced on the subject but also controversy on the definitions, which sometimes complicates the assimilation of the information. The following short overview of enzyme inhibitors and their classifications, limiting the examples to cysteine proteases inhibitors, helped identifying the most suitable way of introducing the cofactor into the active site of papain in a convenient, covalent and irreversible manner.

In general, any compound that decreases the measured rate of hydrolysis of a given substrate is, in principle, an enzyme inhibitor which can be chemically reactive, catalytically processed or simply bound to the target enzyme. As shown in Figure 5.13, a first classification divides inhibitors into active site directed inhibitors and allosteric effectors, compounds which interact temporarily with peripheral areas of the enzyme and those are not relevant in this study.
Merging Homogeneous Catalysis with Biocatalysis: Papain as Hydrogenation Catalyst

![Diagram](image)

**Figure 5.13** Classification of inhibitors according to the nature of their interactions

The inhibitors which target the active site can be classified according to their interactions into covalent or non-covalent and reversible or irreversible.\(^{65}\) In the case of reversible inhibition, which usually involves a non-covalent interaction based for example on tight binding, the activity of the enzyme can be regained upon dilution or dialysis. Even if in some cases dissociation is so slow and the binding so strong that it could to be considered ‘pseudo-irreversible’, the non-covalent nature of the interaction makes this class not a desirable choice.

Covalent inhibitors interact with the enzyme via a chemical modification of the active site. These inhibitors are ‘mechanism based’ if they react with the active site following a normal catalytic process.\(^{96}\) Otherwise, they are defined as ‘affinity labels’ if another chemical pathway, which has no connection with the catalytic process, is used (Figure 5.14).

![Diagram](image)

**Figure 5.14** Classification of covalent inhibitors according to the reaction mode

Mechanism-based inhibitors are also defined as ‘suicide substrates’. This means that the reactive functional group is *latent* in the molecule until the enzyme itself upon binding catalyzes its own destruction. As shown in Figure 5.14, these compounds that are converted into inhibitors during catalysis can be classified according to the product obtained after reaction with the enzyme into: transition state analogues when a tetrahedral adduct is formed but does not react further...
(e.g. hemithioacetal with peptidyl aldehydes); dead end inhibitors when a covalent complex which cannot react further is formed (e.g. thioimidate with peptidyl nitriles). Unfortunately, covalent modification of the active site gives no guarantee of irreversibility in all cases. Reversibility can be encountered also when using covalently bound compounds because the bond hydrolyzes over time. Few examples can be found among the mechanism based inhibitors, such as peptidyl aldehydes or nitriles, but also α-keto acids derivatives and semicarbazones (Scheme 5.17). The instability of these adducts, even if covalently bound and frequently used, makes them not suitable.

Scheme 5.17  Covalently bound but reversible inhibitors frequently used

Affinity label inhibitors (Figure 5.14), which in some literature are defined as active site directed inhibitors, are compounds possessing a functionality that reacts with the active site and, upon releasing a leaving group, form adducts which cannot be separated by gel-filtration or dialysis and cannot be hydrolyzed. The reaction pathway is not specific and does not recall the catalytic mechanism. Chemically reactive affinity labels are compounds that would be able to react with other molecules possessing the same reactivity of active site residues (e.g. halo ketones 5.38a); if instead the reagents have no reactivity for non-enzymatic molecules they are called quiescent affinity labels (e.g. acyloxymethyl ketones 5.38c).

Affinity labels 5.38, as many other inhibitors, are characterized by a short peptidyl sequence used for binding recognition but they are divided according to the reactive leaving group present as depicted in Scheme 5.18.
Merging Homogeneous Catalysis with Biocatalysis: Papain as Hydrogenation Catalyst

Scheme 5.18  Generic structure of peptidyl affinity label inhibitors 5.38 and most frequently used reactive leaving groups 5.38a-c

Affinity label inhibitors 5.38 seem to have all the required characteristics. They are reactive compounds and the products of the alkylation reaction are truly irreversible. Halomethyl ketones 5.38a are historically among the first affinity labels developed as a consequence of the high reactivity toward serine and cysteine proteases showed at first by small and simple reagents such as iodo acetic acid and iodo acetamide. Many halomethyl ketones with different peptidyl sequences have been prepared as they played an essential role in the understanding of enzymes characteristics and inhibitors such as TPCK (chymotrypsin) and ZPACK (papain, Figure 5.12). The high reactivity and lower specificity of halomethyl ketones, especially for \textit{in vivo} applications, stimulated the development of the other affinity labels depicted in Scheme 5.18, which were originally intermediates in the synthesis of the halomethyl ketones themselves or are their derivatives (Scheme 5.19).

Scheme 5.19  Chemical connection between the different inhibitors 5.38

Diazoketone-based inhibitors 5.38b are selective for cysteine proteases which makes them useful for \textit{in vivo} inhibition, however, they are more effective at weakly
acidic pH, since protonation is required for the formation of the unstable diazonium ion. Methylsulfonium salts 5.38d, besides having intermediate reactivity between halomethyl ketones 5.38a and diazo-ketone reagents 5.38b, have an intrinsic instability which might result in an internal displacement reaction that regenerates the halomethyl starting material. Acyloxymethyl ketones 5.38c are instead chemically stable compounds, the downside of course is a further reduced reactivity.

Slightly different is the approach of the other two classes of important affinity labels comprising epoxysuccinyl and α,β-unsaturated derivatives (Scheme 5.20). After E-64 (5.41) was isolated as a naturally occurring inhibitor of papain in 1978, many analogues have been prepared to study the function of the different groups present. It became evident that the configuration of the epoxide is essential and changes resulted in a consistent decrease in the inhibitory power. Inspired by E-64 it was also envisioned that unsaturated substrate analogues such as 5.43 would have had similar alkylating power acting as nucleophile trapping Michael acceptors. In general these reagents exhibit very specific but somewhat lower inhibitory power due to the slow alkylation reaction, even if showing high affinities. Modification of thiols employing N-alkyl maleimides 5.45 is often used in protein sequencing and analysis.

Epoxysuccinyl derivatives:

Michael acceptors:

Scheme 5.20  Epoxysuccinyl and Michael acceptor based inhibitors

A good part of the inhibitory power of most of the compounds presented is due to the binding affinity of the peptidyl portion of their structure to the active site. It represents the driving force for the formation of the non-covalent enzyme-inhibitor complex as a recognition factor. However, during the alkylation step this part of the inhibitor is already irrelevant and the rate determining step of the inhibition is the
alkylation reaction itself. Therefore, the electrophilicity and leaving ability of the reactive group becomes an important factor. The use of any of these affinity labels would guarantee the specificity and irreversibility required for the introduction of an artificial cofactor. Maleimide and halomethyl ketone groups seem to be the most reliable alternatives. Eventually, a halomethyl ketone moiety was considered to be the best choice. Its use would allow maintaining generality in the approach, as both serine and cysteine proteases can be alkylated, and probably also other cysteine residues eventually engineered in a given protein. This choice seemed to be a good compromise of reactivity, stability and simplicity of synthesis among the different options. The intrinsic affinity of thiols for these electrophilic reagents would also hopefully allow the design of a cofactor lacking a peptidyl portion.

5.8 Cofactor design

So far we have identified a suitable enzyme whose unique reactive cysteine can be used as chemical recognition site for the irreversible and specific introduction of the artificial cofactor using a halomethyl ketone moiety as linker.

As the most reactive residue of the enzyme is used to covalently immobilize the cofactor, the expectation is that the active site of the enzyme will not directly participate in the new catalysis that will be performed. It will provide water solubility and chiral environment, becoming the scaffold for the transition-metal catalysis to take place. Therefore, the cofactor should have the potential to act as a catalyst without any requirement for the enzyme to participate to the catalytic act in a specific or predetermined way.

The cofactor, which does not resemble a natural substrate or inhibitor of the enzyme, should possess sufficient water solubility to allow a homogeneous reaction to take place and to overcome the lack of specific binding provided by a peptidyl moiety. The introduction of the cofactor should not alter the structural stability and spatial requirements of the enzyme which could be the cause of possible denaturation or impossibility for the substrate to effectively coordinate to the newly introduced artificial catalytic site.

The design of the cofactor should also provide effective coordination with a metal, small interference of the chemistry involved in its preparation with the reactivity of the labeling moiety and stability of the adduct obtained after enzyme modification. Last but not least, the unfavorable molecular weight ratio between the enzyme and the transition metal requires a highly active transition metal catalyst.

The introduction of an artificial cofactor is meant to broaden the range of catalytic reactions naturally available to enzymes with reactions more characteristic of transition-metal catalysis. Therefore, the core of the cofactor should be constituted by the structure of ligands which, upon coordination with a suitable metal center,
would allow performing reactions like hydrogenation of alkenes,\textsuperscript{99} hydroformylation, allylic substitution, hydrosilylation and so on. Phosphorus based ligands are popular for this kind of reactions in combination with Pd, Rh, Ru as metal centers.

In order to meet as many of these requirements as possible, a bulky achiral monodentate phosphite ligand \textbf{5.47} was chosen as core of the cofactor (Figure 5.15). The \textit{t}-butyl substitution and the use of a bisphenol skeleton were chosen for reactivity and stability reasons.

![Figure 5.15](image)

\textbf{Figure 5.15} General structure of the core of the cofactor \textbf{5.47}  
Phosphite ligands have been extensively studied due to their potential in rhodium-catalyzed hydroformylation reactions (Scheme 5.21).\textsuperscript{100} The use of monodentate phosphite ligands such as \textbf{5.50a}, as effective alternative to phosphines, was first reported in the late sixties by Pruett and Smith in connection with studies conducted by the Union Carbide Corporation (UCC).\textsuperscript{101} Further investigations reported by van Leeuwen and Roobeek showed that the increasing steric hindrance of the ligands \textbf{5.50b} resulted in higher reaction rates and reactivity toward substrates considered unreactive.\textsuperscript{102} It appeared that bulky ligands possess a large cone angle\textsuperscript{103} which prevents the coordination of a second ligand. In such a complex the metal center is electron poor which implies a faster dissociation of CO and alkenes coordination and therefore faster catalysis.\textsuperscript{104} The same phenomenon has been reported also for Pd-catalyzed aromatic substitution.\textsuperscript{105}
Merging Homogeneous Catalysis with Biocatalysis: Papain as Hydrogenation Catalyst

Scheme 5.21  Hydroformylation and early monodentate phosphite ligands

More recently, monodentate phosphite ligands 5.53 have also been introduced in asymmetric Rh-catalyzed hydrogenation reactions by Reetz as well as monodentate phosphoramidite ligands 5.54 by Feringa-Minnaard-de Vries and their coworkers (Scheme 5.22). In this field a less systematic study has been conducted on the possibility and effect of metal unsaturation by coordination of a single monodentate ligand.

Scheme 5.22  Hydrogenation using monodentate phosphite and phosphoramidite ligands
Both groups performed hydrogenation reactions in the presence of a 1:1 ratio between metal center and ligand, as in the preliminary investigation of the new systems.\textsuperscript{6b,106} The reactions proceeded equally well and in the case of MonoPhos\textsuperscript{TM} (5.54 where \( R \) is a methyl group) a slight increase in reactivity was also observed. Whether the active catalysts contained one or two ligands was, nevertheless, not clear. Rhodium is also known to be able to coordinate up to four equivalents of MonoPhos\textsuperscript{TM} and the resulting catalyst is not active, which means that this ligand is definitely not bulky enough. A study on non-linear effects using MonoPhos\textsuperscript{TM} confirmed that at least under those conditions two ligands were present on the metal center. Recently both groups also showed that it is possible to perform highly enantioselective hydrogenation reactions using a combination of two different ligands.\textsuperscript{107} The influence of the additional ligand arises clearly from the results, confirming that the metal center coordinates two ligands if given the possibility. It seems that so far no ligand reported was bulky enough to ensure a 1:1 ratio with the metal center in the complex. Recently, van Maarseveen and coworkers prepared a dendritic version of MonoPhos\textsuperscript{TM} 5.55 (Scheme 5.22). They reported an active catalyst with up to 4 equivalents of ligand and \(^{31}\text{P}-\text{NMR}\) evidence of the partial formation of a 1:1 complex which means that the ligand was bulky enough to induce metal unsaturation.\textsuperscript{108} Unfortunately, no hydrogenation reaction was performed to investigate this possibility. The introduction of the phosphite cofactor 5.47 in the active site of papain is an excellent opportunity to use a ligand that will provide a 1:1 catalytic species with the metal center, as the rhodium is expected to be able to coordinate only one enzyme-cofactor adduct due to steric hindrance. Moreover, the catalyst should be active enough to overcome the unfavorable molecular weight difference between the enzyme and the catalytically active center.\textsuperscript{109}

Although less prone to oxidation than phosphines, phosphites might undergo few side reactions which cause their degradation and the most frequently occurring ones are depicted in Scheme 5.23.\textsuperscript{110}

\[ \text{Scheme 5.23} \quad \text{Possible phosphite decomposition pathways} \]
Arbusov rearrangements are usually metal-catalyzed and typical for aliphatic phosphites. A systematic study conducted by Billig and coworkers (UCC) to understand the causes of ligand decomposition showed that bulky phosphite ligands such as 5.50c, besides providing very reactive catalysts, are also less prone to hydrolysis. The stability seems to further increase when using bisphenols like 5.56a-b (Figure 5.16).

Figure 5.16 Bulky phosphites are more resistant to hydrolysis

5.9 Cofactor synthesis

The size of the phosphite ligand 5.47 was reduced as much as possible to minimize water solubility problems. The first two cofactors 5.47a-b synthesized are depicted in Figure 5.17.

Figure 5.17 Phosphite based cofactors 5.47a and 5.47b prepared

A bromobenzyl phenol was at first considered as labeling moiety in order to keep the linker as short as possible. The alkylation was expected to be the result of the nucleophilic attack of Cys-25 to the bromide being the leaving group. Alkylation of papain using a benzylic bromide was also reported by Kaiser and Levine (Scheme 5.2). Among the different halides, bromide was chosen because of its intermediate reactivity (>Br>Cl) but also better stability toward degradation than iodide. In order to have a possible variation both 3-bromobenzyl (5.58a) and 4-bromobenzyl (5.58b) phenols were prepared following a literature procedure starting from the corresponding hydroxy-benzyl alcohols 5.57a-b as shown in Scheme 5.24.
A solution of hydroxyl-phenol 5.57a-b in dry THF was added dropwise at -5 °C to another THF solution containing PBr₃ and pyridine. The reaction mixture was left at room temperature overnight and then filtered over celite. After removal of the solvent, toluene was added; the solution was maintained at -20 °C for a couple of hours and filtered again over celite. In the literature procedure, products 5.58a and 5.58b are reacted without further purification as a toluene solution that can be stored in the fridge. However, both solutions started to darken and purification was attempted. Column chromatography allowed the isolation of 5.58b as an oil solidifying on standing. The solution of 5.58a could not be purified because of the presence of considerable amount of dark and insoluble material. The decomposition of 5.58a seems to be caused by easy HBr elimination and formation of p-quinone methide (5.59). More recently p-quinone methide (5.59) has been reported to be a short-lived reactive species, which might explain the formation of insoluble degradation products. Due to the clear instability, the synthesis of 5.58a was abandoned and a slightly different literature procedure was used to obtain 5.58b (Scheme 5.25).

The bisphenols 5.62 were prepared according to literature procedures based on the oxidative dimerization of the corresponding phenols 5.60 (Scheme 5.26). The tendency of phenols to oxidation has been extensively studied as they are considered important antioxidants. There is quite an amount of early literature...
dealing with the response of differently substituted phenols to various oxidizing agents.\textsuperscript{115}

\begin{align*}
\text{ortho, para substituents:} \\
\begin{array}{c}
\begin{array}{c}
\text{5.60} \\
\text{R} \\
\text{OH} \\
\end{array} \\
\begin{array}{c}
\begin{array}{c}
\text{R} \\
\text{OH} \\
\end{array} \\
\text{R} \\
\end{array} \\
\begin{array}{c}
\begin{array}{c}
\text{R} \\
\text{OH} \\
\end{array} \\
\text{R} \\
\end{array} \\
\begin{array}{c}
\begin{array}{c}
\text{R} \\
\text{OH} \\
\end{array} \\
\text{R} \\
\end{array} \\
\end{array}
\end{align*}

\begin{align*}
\text{no substituents:} \\
\begin{array}{c}
\begin{array}{c}
\text{O} \\
\text{O} \\
\text{O} \\
\end{array} \\
\begin{array}{c}
\begin{array}{c}
\text{O} \\
\text{O} \\
\end{array} \\
\end{array} \\
\begin{array}{c}
\begin{array}{c}
\text{O} \\
\end{array} \\
\end{array} \\
\end{array}
\end{align*}

Scheme 5.26 \textit{The presence of substituents limits the oxidation products}

In all the procedures adopted, the first step seems to be the abstraction of the phenolic hydrogen of \textit{5.60} and the oxidation is a one electron process. The radical \textit{5.61} formed is stabilized by resonance in \textit{ortho} and \textit{para} positions and the reactivity of these three positions is responsible for the potentially wide number of products. Out of all the oxidation products it is possible to mainly obtain symmetric bisphenols \textit{5.62} depending on the substitution. Bulky groups in \textit{ortho} and \textit{para} positions limit the number of oxidation products and polymer formation.\textsuperscript{116}

The first procedure applied involved the use of CuCl\textsubscript{2} (0.5\%) in the presence of TMEDA (1:2) under an atmosphere of oxygen (Scheme 5.27).\textsuperscript{117} The reaction was rather slow and was eventually stopped after 5 days providing \textit{5.62a} from \textit{5.60a} in a disappointing 20\% yield after column chromatography.

\begin{align*}
\text{Scheme 5.27 \textit{Synthesis of bisphenol 5.62a using Cu(II)-diamine complex}}
\end{align*}

Analysis of the \textsuperscript{1}H-NMR crude of the reaction mixture showed mainly the presence of product \textit{5.62a} and starting material \textit{5.60a}. Two other oxidation side compounds were clearly visible on TLC but present in small amount. The use of copper-amine complexes is reported to be important not only in the initiation of the reaction but
also for the coupling pattern and the reaction was proposed to proceed by coordination of the reagent to the complex (5.63 and 5.65), as shown in Scheme 5.28.118,119

![Scheme 5.28 Some intermediates using Cu(II)-diamine complex](image)

In retrospect, a faster reaction following this procedure might have been obtained by increasing the amount of catalyst, as elsewhere it was reported that using similar Cu-diamine complexes the reaction rate had a linear dependence with the concentration of the catalyst.118 Bubbling of molecular oxygen directly in the reaction solution might have also increased the reactivity, improving its solubility in the reaction media. A higher yield (69%) of the desired bisphenol 5.62a was obtained by using FeCl₃ (25%) in the presence of t-BuOOH under a nitrogen atmosphere, as described in a literature procedure.120
In the third procedure used for the preparation of 5.62b, alkaline ferricyanide was employed as oxidizing agent and the product was isolated in 60% yield after recrystallization (Scheme 5.30). In this case, an equimolar amount of Fe(III) is necessary as the obtained Fe(II) cannot be oxidized again to Fe(III) in situ.\textsuperscript{121}

Following a modified literature procedure reported by van Leeuwen and coworkers,\textsuperscript{122} bisphenols 5.62a-b were used to prepare the corresponding phosphorus chlorides 5.66a-b by reacting them with PCl\textsubscript{3} in the presence of base using toluene as solvent. The reaction of 5.66a-b with benzyl bromide 5.58b delivered the desired phosphites 5.47a-b (Scheme 5.31).
Phosphorus trichloride was added dropwise to a solution of NEt₃ in dry toluene at 0 °C. A toluene solution of bisphenols 5.62a or 5.62b was also added dropwise and the mixture was stirred at room temperature. The progress of the reaction was monitored by ³¹P-NMR following the disappearance of the starting material signal (202 ppm) and the appearance of the phosphorus chloride 5.66a-b signals (~172 ppm). During the preparation of 5.47a, when PCl₃ had disappeared, another 2 equivalents of NEt₃ were added to the reaction mixture at 0 °C, followed by a toluene solution of benzyl phenol 5.58b. The reaction was again checked by ³¹P-NMR following the appearance of product 5.47a (~138 ppm). Work up consisted of the filtration of the triethylamine salts after the addition of Et₂O and removal of the solvent. Phosphite 5.47a was obtained in 50% yield after purification by column chromatography in the presence of 0.5% of NEt₃ and under nitrogen atmosphere using distilled solvents. Column chromatography performed in dry and slightly basic conditions have been reported to minimize the degradation of phosphites during the purification step.¹¹⁰ A slightly different procedure was used for the preparation of phosphite 5.47b. The triethylamine salts formed during the first step were removed by quick filtration under nitrogen atmosphere and volatiles removed before adding benzyl phenol 5.58b. The resulting reaction mixture was stirred overnight and the product 5.47b was isolated in 41% yield. It was not clear if the lower yield should be attributed to the intermediate salts filtration or to the longer reaction time which could have compromised the stability of the phosphite or of the benzyl bromide. However, when the second step of the reaction was stopped after 3 h, 5.47b was isolated in 50% yield after column chromatography using again

**Scheme 5.31 Synthesis of phosphites 5.47a and 5.47b**

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202
distilled solvents but without the presence of NEt₃. Both compounds were stored at -12 °C.

5.10 Cofactor solubility tests

Although keeping the size of the cofactor as small as possible, phosphites 5.47a-b were assumed to be fairly hydrophobic molecules and solubility in water was already considered to be an issue. It was desirable to be able to dissolve enough cofactor in aqueous solution to obtain a good inhibition profile of papain. A few organic solvents miscible with water were screened in order to find the best compromise between the use of a minimum amount of organic solvent, which would avoid possible destabilization of papain, and best solubility of the cofactor. The tests were first performed on 5.47a.

This test was based on visual observation of turbidity of the solution containing the cofactor in different mixtures of organic solvents in water. At first a 3.7 mM stock solution of 5.47a in CH₃CN was prepared and 50 µL of this solution were added to the water / organic solvent solutions obtaining a final concentration of ligand of 180 µM. The results of the test are shown in Table 5.2.

Table 5.2  Solubility of 5.47a in water / organic solvents mixtures

<table>
<thead>
<tr>
<th>solvent</th>
<th>volume (%) in H₂O</th>
<th>solution appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₃CN</td>
<td>10</td>
<td>quite turbid</td>
</tr>
<tr>
<td>DMSO</td>
<td>10</td>
<td>turbid / precipitate</td>
</tr>
<tr>
<td>DMF</td>
<td>10</td>
<td>turbid / precipitate</td>
</tr>
<tr>
<td>DMF</td>
<td>20</td>
<td>turbid / precipitate</td>
</tr>
<tr>
<td>iPrOH</td>
<td>10</td>
<td>turbid</td>
</tr>
<tr>
<td>iPrOH</td>
<td>20</td>
<td>almost clear</td>
</tr>
<tr>
<td>acetone</td>
<td>10</td>
<td>quite clear</td>
</tr>
<tr>
<td>1,4-dioxane</td>
<td>10</td>
<td>clear</td>
</tr>
<tr>
<td>MeOH</td>
<td>50</td>
<td>quite clear</td>
</tr>
</tbody>
</table>

After this first qualitative examination, 1,4-dioxane appeared to be the best solvent. Therefore, it was decided to test different concentrations of 5.47a in different percentages of solvent adding it to a 100 mM phosphate buffer solution at pH 7 that will be used in all papain manipulations. Unfortunately, it became immediately clear that, using buffered aqueous solutions, the cofactor 5.47a was not soluble enough anymore and started precipitating. Therefore, the test was performed starting from a more diluted 1 mM stock solution of 5.47a and changing the final percentage of 1,4-dioxane present in the buffered solution from 5% up to 50%.
Moreover, analysis of the UV-Vis spectra of \textit{5.47a} in the different solutions was also diagnostic as turbidity resulted in disturbed baselines. Final phosphite concentrations of 50 µM, 25 µM and 12.5 µM, respectively, were considered and the resulting UV profiles obtained for the highest concentration used are shown in Figure 5.18.

![UV spectra of phosphite 5.47a (50 µM) in 100 mM phosphate buffer with different amount of 1,4-dioxane](image)

Figure 5.18 \textit{Uv spectra of phosphite 5.47a (50 µM) in 100 mM phosphate buffer with different amount of 1,4-dioxane}

At a final cofactor concentration of 50 µM, a clear solution was obtained using 50% 1,4-dioxane in buffered solution. The UV-Vis spectra showed that with 45% organic solvent the solution started to be slightly turbid but it could be considered still acceptable, whereas a lower amount of 1,4-dioxane (40%) was clearly too little. Similarly, it was found that clear solutions could be obtained using 40% of 1,4-dioxane if the final concentration of \textit{5.47a} was 25 µM and 30% for 12.5 µM.

A similar test was performed with phosphite \textit{5.47b}, which was prepared in the hope that it would have better solubility in water; however, it behaved exactly in the same way, so \textit{5.47b} was no longer considered in this investigation.

5.11 Papain pretreatment and activity test

Papain is commercially available as a buffered aqueous suspension in 0.05 M sodium acetate at pH 4.5 and containing 0.01% of thymol.\textsuperscript{123} Papain needs to be activated using reducing agents such as cysteine, dmercapto propanol or DTT (dithiothreitol). The mechanism and the reason for this required procedure are not well understood.\textsuperscript{124} The use of thiol-based reducing agents is a standard protocol when using papain and the source of inactivation seems to be directly connected with the way in which the enzyme is produced.\textsuperscript{125} More insight into this subject can be found later in this chapter (page 215).
Activated papain solutions usually contain stabilizing agents such as EDTA and the previously mentioned reducing agents, used to avoid inactivation of the enzyme by heavy metal complexation or oxidation, respectively. For the same reasons, the buffered solution should be prepared using bidistilled water and all manipulations should be performed using carefully degassed solutions. Adapting a protocol reported by Albeck and Kliper, papain was activated by incubation at room temperature in 100 mM potassium phosphate buffer at pH 7.0, in the presence of DTT and EDTA, for 20 minutes.\textsuperscript{126}

Another advantage in using an enzyme as protein scaffold for this study is the possibility of monitoring the progress of the cofactor introduction using standard activity tests which measure the hydrolytic activity of the enzyme (Scheme 5.32).

![Scheme 5.32 Papain activity test using 5.67 as standard substrate](image-url)

The introduction of the cofactor 5.47a by covalent modification of Cys-25 results in the inhibition of papain that will no longer be able to hydrolyze ester or amide bonds. Conveniently, ester 5.67 (used as test substrate) upon hydrolysis releases a p-nitrophenolate (5.68) that can be easily monitored by UV-Vis absorption spectroscopy.\textsuperscript{126,127} This test was used to assess papain activity after the activation and in different moments during the introduction of 5.47a to check the progress of the inactivation. Upon incubation with 5.47a, the inability of papain to hydrolyze the standard substrate 5.67 would provide the first evidence that the alkylation proceeded successfully (Scheme 5.33).

![Scheme 5.33 Assessment of papain activity as the first proof of successful alkylation](image-url)
There were some concerns that the addition of a too high percentage of organic solvent might compromise the stability or reactivity of papain. Therefore, the hydrolysis of Z-Gly-p-NPE (5.67) was used to determine the influence of 1,4-dioxane on the enzyme catalysis. To avoid the inactivation of papain due to oxidation of the reactive Cys-25 also 1,4-dioxane was carefully degassed and a protocol compatible with the UV-Vis analysis was prepared.

**Table 5.3 Composition of solution A used for papain activation**

<table>
<thead>
<tr>
<th>-component</th>
<th>volume (μL)</th>
<th>final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-buffer (100 mM)</td>
<td>976</td>
<td>-</td>
</tr>
<tr>
<td>DTT (0.1 M)</td>
<td>10</td>
<td>1 mM</td>
</tr>
<tr>
<td>EDTA (0.5 M)</td>
<td>4</td>
<td>2 mM</td>
</tr>
<tr>
<td>papain (1.1 mM)</td>
<td>10</td>
<td>11 μM</td>
</tr>
</tbody>
</table>

An aliquot (50 μL) of the solution used for the activation (solution A, Table 5.3) was diluted to 1 mL with phosphate buffer and the required amount of 1,4-dioxane (solution B). A sample (90 μL) of this dilution solution was added to a quartz cuvette containing phosphate buffer (890 μL). The substrate 5.67 (20 μL, acetone) was added as last to this activity test solution C and its hydrolysis was followed by UV. The results shown in Figure 5.19 were quite surprising.

![Graph](image.png)

**Figure 5.19 Effect of organic solvents on papain activity: normalized p-nitrophenolate (5.68) absorbance (λ= 404 nm) after 10 min. reaction vs. 1,4-dioxane (%) present in solution.**

The addition of 5% or 10% of 1,4-dioxane resulted in a slightly higher value for the absorbance of the p-nitrophenolate (5.68) released during the hydrolysis, which was considered an artifact due to interactions with the organic solvent. However, increasing the amount of 1,4-dioxane considerably decreased the hydrolytic
activity of papain. In another set of experiments papain, after the addition of 5% or 20% of 1,4-dioxane, was either immediately used or first incubated for half an hour in the presence of the solvent. As a result, the incubated papain was respectively 30% and 43% less active than when directly used. The increasing presence of 1,4-dioxane could have destabilized and therefore inactivated the enzyme on a structural level. However, when a decrease in activity was noticed also using 5% organic solvent followed by an incubation period, the possibility that 1,4-dioxane was contaminated with potential inactivating agents started to arise. It was found that commercial 1,4-dioxane might contain acetaldehyde, ethylene acetal and peroxides. All these compounds might indeed form adducts or oxidize the Cys-25 thiol and consequently deactivate the enzyme. Purification of 1,4-dioxane by percolation through a column of activated alumina (80 g per 100-200 mL) efficiently solved the problem. This was proven by following the hydrolysis of 5.67 with and without 50% of purified 1,4-dioxane and the release of p-nitrophenolate (5.68) showed a very similar profile in both cases (Figure 5.20).

![Figure 5.20](image)

Figure 5.20 Hydrolysis of 5.67 catalyzed by papain followed by monitoring the release of p-nitrophenolate (5.68) by UV-Vis absorption spectroscopy (λ = 404 nm) with (▲) or without (■) the presence of 50 vol% purified 1,4-dioxane.

Alternatively, 1,4-dioxane was also distilled from sodium under N2 atmosphere. In both cases, the purified solvent was directly used or stored at 4 °C. This allowed the use of a phosphate buffer containing 50 vol% 1,4-dioxane and therefore a higher concentration of cofactor 5.47a during the alkylation procedure.

### 5.12 Alkylation of papain using linker 5.58b and cofactor 5.47a

The linker part of cofactor 5.58b was initially used as model for the preparation of the inhibition protocol and to test the inhibitory power of the labeling compound itself. In the protocol used to test the influence of 1,4-dioxane on papain the dilution
step was transformed into an incubation step as shown in Table 5.4. As before, a sample (90 µL) of this incubation solution B was used in the activity test for the hydrolysis of Z-Gly-p-NPE (5.67).

**Table 5.4 Composition of incubation solution B**

<table>
<thead>
<tr>
<th>volume</th>
<th>final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-buffer (100 mM)</td>
<td>450 µL -</td>
</tr>
<tr>
<td>papain (solution A)</td>
<td>50 µL 0.55 µM</td>
</tr>
<tr>
<td>5.58b (dioxane)</td>
<td>500 µL 0.5, 2.3, 4.6 mM</td>
</tr>
</tbody>
</table>

At first, the incubation of the activated papain (0.55 µM) with the linker 5.58b (0.5 mM) was performed at room temperature. The extent of the inhibition was checked by comparing the ability of papain in catalyzing the hydrolysis of 5.67 before and during the incubation with 5.58b (Figure 5.21).

![Figure 5.21](image)

**Figure 5.21** Hydrolysis of 5.67 catalyzed by papain followed in time by monitoring the release of p-nitrophenolate (5.68) by UV-Vis absorption spectroscopy (λ= 404 nm): before (●) and after incubation of papain at rt with 5.58b (0.5 mM) after 0:30 h (♦), 1:20 h (■), 2:15 h (▲), overnight (x).

The alkylation seemed to proceed very slowly during the first couple of hours and it was decided to continue the incubation overnight. However, as shown in Figure 5.21, even after one night in the presence of 5.58b, papain appeared to be still quite active in catalyzing the hydrolysis of 5.67. Therefore, in another set of experiments, maintaining 50% of 1,4-dioxane as cosolvent, papain was incubated at room temperature for 3 h in the presence of an increased amount of labeling compound 5.58b (2.3 and 4.6 mM), anticipating that faster inhibition might occur. Papain was also incubated for one hour in the presence of the same concentrations of 5.58b but at higher temperature (37 °C). A control was also prepared in which papain was left at 37 °C in the absence of the labeling
compound 5.58b, in order to check if an increased temperature was compromising the catalytic activity of the enzyme. The influence of the different conditions was once more indirectly monitored following the catalyzed hydrolysis of 5.67. The residual catalytic activity of papain after incubation under the different conditions is shown in Figure 5.22 in relation with the untreated and fully active enzyme.

![Graph showing the relative hydrolytic activity of papain under different conditions](image)

**Figure 5.22** Maximum release of p-nitrophenolate (5.68) measured by UV-Vis absorption spectroscopy (λ = 404 nm) due to the hydrolysis of 5.67 catalyzed by papain: **A)** without any treatment used as reference; **B)** after 1 h at 37 °C; **C)** after 3 h incubation with 2.3 mM 5.58b at rt; **D)** after 1 h incubation with 2.3 mM 5.58b at 37 °C; **E)** after 3 h incubation with 4.6 mM 5.58b at rt; **F)** after 1 h incubation with 4.6 mM 5.58b at 37 °C.

The incubation of papain in the presence of a higher amount of labeling reagent 5.58b (4.6 mM) at 37 °C induced a reduction of the release of p-nitrophenolate (5.68) of around 70% (Figure 5.22, F). However, under the same conditions even without 5.58b a considerable decrease of activity was also observed (B). Alkylation of the enzyme at room temperature seemed to be a more reliable procedure, as it would avoid deactivation not related to the introduction of the cofactor.

At this point the complete phosphite cofactor 5.47a was also tested in the alkylation of papain. Due to the lower solubility of 5.47a compared to the labeling reagent 5.58b, a lower concentration of 0.5 mM in 50% 1,4-dioxane was used. The solution was incubated for two nights and checked for activity after each night. In one experiment untreated papain was also left at room temperature for two nights. The solutions were tested for residual enzymatic activity as previously described and the resulting profiles of the release of p-nitrophenolate (5.68) are depicted in Figure 5.23.
After one night incubation with \(5.47a\), the test showed a residual activity comparable to the one observed for the linker \(5.58b\) under the same conditions (Figure 5.21). This gave the impression that at least the complete cofactor was accepted as much as the smaller linker. However, papain showed very little decrease in reactivity after one extra night and a comparable decrease in activity was observed also for papain incubated at room temperature for two nights even without the presence of the inhibitor \(5.47a\). The process was definitely too slow and did not guarantee completion, whereas, the poor solubility of \(5.47a\) in phosphate buffer did not allow to reach higher concentrations, which anyway did not show to improve the results even when using \(5.58b\).

Prolonged incubation times and higher temperatures seemed to hamper the intrinsic activity of papain and were not particularly beneficial for the alkylation process. Furthermore, shorter reaction times would also preserve the stability of the phosphite cofactor \(5.47a\) itself. In order to achieve a more efficient alkylation protocol both the reactivity of the labeling moiety and the solubility of the cofactor had to be improved, which made it necessary to redesign the phosphite cofactor.

### 5.13 Improvements in the cofactor design

In order to improve the solubility of the cofactor in buffered solutions, the bisphenol skeleton was equipped with two triethylene glycol tails (Figure 5.24). Polyether-substituted phosphorus ligands have been used on a few occasions in Rh-catalyzed hydrogenation\(^{129}\) and hydroformylation\(^{130}\) reactions in order to improve
their water solubility as an alternative to the more commonly used amino, carboxylic acid, hydroxy and sulfonate groups.\textsuperscript{131}

![Figure 5.24 Improved design for the new cofactor 5.47c](image)

The labeling benzyl bromide moiety was also abandoned and it was substituted with a potentially more reactive bromomethyl ketone. As shown in Scheme 5.34, there are two possible pathways for the mechanism of inhibition of cysteine proteases whilst using halomethyl ketones.\textsuperscript{65}

![Scheme 5.34 Mechanism of inhibition using halomethyl ketones 5.38a](image)

According to pathway A, Cys-25 would react directly with the carbon of the halomethyl group of 5.38a in a nucleophilic substitution fashion directly providing the thioether 5.39. The same reaction is expected whilst using the benzyl bromide 5.58b and the corresponding phosphite cofactor 5.47a. Instead of following pathway B, Cys-25 would first attack the carbonyl group of 5.38a with the subsequent formation of a tetrahedral intermediate 5.69 which is trapped as a sulfonium ion intermediate 5.70, finally affording the identical product 5.39. Following the classification previously provided about inhibitors (Figure 5.14) halomethyl ketones 5.38a should then be better defined as mechanism based inhibitors than affinity labels, since the formation of a tetrahedral intermediate is part of the proteases catalytic pathway. According to the literature, pathway B seems to be the most accepted, however, experimental proof for the existence of
adducts like 5.69 has been only provided for trypsin which is a serine and not a cysteine protease. In this respect, it should have also been considered that in the case of serine proteases the irreversible inhibition is the result of the alkylation of the catalytic histidine and not of the serine, which makes the comparison not consistent. This does not exclude nucleophilic substitution on a benzyl bromide; however, the use of a halomethyl ketone might result in a faster reaction, especially if the carbonyl moiety is also involved in hydrogen bonding with some of the protein residues as shown in Figure 5.11.

The new affinity label 3-hydroxy-phenacyl bromide (5.72) was conveniently prepared following a literature procedure as depicted in Scheme 5.35.133

![Scheme 5.35](image)

**Scheme 5.35 Synthesis of 3-hydroxy-phenacyl bromide (5.72)**

The heterogeneous reaction consisted in the bromination of 2-hydroxy acetophenone (5.71) with CuBr$_2$ and was very convenient as the by-products of the reaction were either volatile (HBr) or could be filtered at the end of the reaction (CuBr). The procedure was selective for one α-bromination, as the presence of over brominated products was not observed by $^1$H-NMR. The α-bromo ketone 5.72 was obtained in a good 85% isolated yield after column chromatography. The lachrymatory compound 5.72 was preferably stored at 4 °C.134

Unlike the bisphenols 5.62a-b previously prepared, in this case the required starting material was not commercially available. Phenol 5.60c was obtained by monoalkylation of 2-t-butyl-hydroquinone (5.75) with the chlorinated derivative of triethyleneglycol 5.74 as depicted in Scheme 5.36.

![Scheme 5.36](image)

**Scheme 5.36 Synthesis of 5.60c by monoalkylation of 2-t-butyl-hydroquinone (5.75) with 1-[2-(2-chloro-ethoxy)-ethoxy]-2-methoxy-ethane (5.74)**
The chlorination of methoxy triethylene glycol 5.73 was achieved by reaction with thionyl chloride in the presence of pyridine. The desired product 5.74 was obtained in 93% yield after work up and was used without any further purification. A modification of a literature procedure reported by Stoddard and coworkers for the preparation of polyether macrocycles was used for the monoalkylation step. In the original procedure to achieve monoalkylation a huge excess of a hydroxy phenol was reacted in acetonitrile in the presence of K$_2$CO$_3$ as base. As depicted in Scheme 5.36, in this case the chlorinated triethylene glycol derivative 5.74 was reacted with a small excess (1.2 equivalents) of 2-t-butyldihydroquinone (5.75) and Cs$_2$CO$_3$ was used as inorganic base. The expectation was that, due to the bulkiness of the t-butyldihydroquinone, reaction at the less bulky hydroxy group would be preferred. It was pleasing to see that the assumption proved to be correct and the desired product 5.60c was obtained in 80% yield. At this point, bisphenol 5.62c was prepared using one of the previously described oxidative dimerization procedures involving the use of a stoichiometric amount of Fe(III) and it was obtained in 60% yield as a thick yellowish oil (Scheme 5.37).

Finally, bisphenol 5.62c and PCl$_3$ were reacted in dry toluene in the presence of NEt$_3$ for 3 hours. Quick filtration of the salts after addition of dry ether and removal of the solvents afforded the crude chloro phosphite 5.66c that without further purification was used in the following step (Scheme 5.38). Similarly to the first step of the synthesis, chloro phosphite 5.66c was reacted with 5.72 in the presence of NEt$_3$ using toluene as solvent. The progress of the reaction was followed by $^{31}$P-NMR and at the disappearance of the phosphorus chloride signal (172 ppm) the reaction was stopped. Phosphite 5.47c was directly purified and obtained in 72% yield after column chromatography and was stored at -12 °C.

The stability of phosphite cofactor 5.47c in the presence of water was checked by $^{31}$P-NMR (137.6 ppm). No degradation of the ligand was detectable even after 1 week in a 1:1 mixture D$_2$O/acetone.
5.14 Alkylation of papain using linker 5.72 and cofactor 5.47c

In two separate experiments, activated papain was incubated for one hour in the presence of the labeling compound 5.72 or the phosphite cofactor 5.47c, respectively. The only difference with the protocols previously used was the presence of only 5% 1,4-dioxane. The residual activity of the enzyme was assessed following the catalyzed hydrolysis of 5.67 by UV, as previously described and the hydrolysis profiles are depicted in Figure 5.25.

![Scheme 5.38 Synthesis of phosphite 5.47c](image)

Figure 5.25 Hydrolysis of 5.67 catalyzed by papain followed in time by monitoring the release of p-nitrophenolate (5.68) by UV-Vis absorption spectroscopy (λ= 404 nm) after 1h incubation: without 1,4-dioxane (★); with 5% 1,4-dioxane (●); with 50 μM 5.72 (▲); with 50 μM 5.47c (●).
It was pleasing to see that papain showed almost no activity after only one hour incubation in both experiments with the affinity label 5.72 and the phosphite cofactor 5.47c using just 5% 1,4-dioxane and a final concentration of both alkylating agents of 50 μM. Although the solution containing 5.47c was slightly turbid, the alkylation of papain seemed to be very efficient and definitely much faster than when using cofactor 5.47a. Both changes in the structure of the cofactor seemed to have played an important role as not only the cofactor 5.47c was more soluble than 5.47a but the labeling compound 5.72 was more reactive than 5.58b as it can be seen in Figure 5.26, which shows the result of one hour incubation of papain in the presence of 5.72 and 5.58b using the same final concentration (50 μM) and the same amount of 1,4-dioxane (5%). The residual activity monitored was comparable to the spontaneous hydrolysis of the substrate 5.67 in the absence of the enzyme, also present in Figure 5.26.

![Figure 5.26](image)

**Figure 5.26** Hydrolysis of 5.67 followed in time by monitoring the release of p-nitrophenolate (5.68) by UV-Vis absorption spectroscopy (λ= 404 nm): without papain (■); without affinity label (♦); after 1h incubation with 50 μM 5.72 (▲); after 1h incubation with 50 μM 5.58b (●).

Although the lack of activity shown by papain was an important first indication that the alkylation had been successful, it was still indirect evidence. ESI-MS analysis was considered to be probably the most straightforward option to actually identify the modified enzyme.

### 5.15 ESI-MS analysis of the modified papain adduct PapPhos

Surprisingly, there is still some confusion about the actual mass of papain, the reason for the necessary activation procedure and the state of the Cys-25 in the commercially available enzyme. Although such information does not seem to be
essential whilst using papain simply as a hydrolytic enzyme, it was important for this study and it deserves a small digression.

According to the data sheet provided by Sigma-Aldrich the mass of papain should be 23,406 Da with reference to the sequence reported by Smith and coworkers (1970),\textsuperscript{137} which was said to be in agreement with the one reported by Drenth and coworkers determined by crystallographic methods (1968).\textsuperscript{70a} However, this mass is wrong as indicated by a more careful literature search. Several other studies on papain reported both the wrong sequence and/or the wrong mass (1965-1971).\textsuperscript{138} Kamphuis and coworkers did eventually present a corrected sequence reporting a wrong mass of 23,350 Da (1984).\textsuperscript{70b} Just a couple of years later (1986) Cohen and coworkers reported the cloning and sequencing of papain-encoding cDNA which analysis furnished a slightly different sequence of papain.\textsuperscript{139} Several sequences are stored in the data banks and they are all available without making any comment on the fact that they are wrong or without removing them.\textsuperscript{140} As recently as 2005 a report was published in which papain was shown to have a mass of 23,429 Da by ESI-MS, however, the authors justified their finding by assuming that an atom of Na\textsuperscript{+} must be bound to the enzyme again referring to Smith and coworkers (!).\textsuperscript{141} The mass of papain used in our study (23,428 Da) is also the most recently reported one by Vernet and coworkers (1989) who prepared a synthetic gene encoding for papain and the sequence obtained was in agreement also with the one connected with the study of Cohen and coworkers.\textsuperscript{78}

The mass related to a given sequence can be calculated by using biopolymers calculators or sequencing simulator programs. All these programs provide a protein mass corresponding to a structure in which all the cysteine residues present are in a reduced state. Therefore, as papain possesses 6 cysteine residues beside Cys-25 and they are all involved in disulfide bonds, the mass used as reference during the ESI-MS was 23,422 Da. The use of DTT or other reducing agents for a prolonged time is known to be able to affect the stability of disulfide bonds present in a protein.\textsuperscript{137} In consideration of the fact that DTT was used only during the activation and alkylation steps and afterwards removed, this was not considered to be possible.

The preparation of the samples for ESI-MS analysis was less straightforward than expected. As previously described, the entire alkylation procedure was performed in phosphate buffer (100 mM, pH 7); however, this buffer is not suitable for ESI-MS as it is non volatile. Therefore, an exchange of buffer to ammonium carbonate was performed using a pre-packed desalting column. Fractions of 1 mL were collected and checked for the presence of protein at 280 nm. Unfortunately, the large excess of cofactor 5.47c used, which also absorbed at 280 nm, appeared to have been also collected with consequent failure of the ESI-MS analysis. Purification after the alkylation via dialysis was considered a better way of removing the excess of cofactor 5.47c and the phosphate buffer. For the dialysis to be effective the amount of 1,4-dioxane used during the alkylation step was increased to 10% to help the cofactor 5.47c to completely solubilize. The solutions containing protein
modified with the linker 5.72, the complete cofactor 5.47c and the sample without any treatment were centrifuged five times against pure water with 1% of formic acid. Formic acid was added as it helps the protonation of the protein with consequent better results during the analysis. The use of centrifugal filters allowed also the solutions to first be concentrated and then further diluted to a volume of 200 µL using a solution made up from 70% methanol, 29% water and 1% formic acid and kept at 4 °C overnight. The results of the analysis are presented in Table 5.5.

Table 5.5  Results of the ESI-MS analysis

<table>
<thead>
<tr>
<th>entry</th>
<th>papain modified with linker 5.72 (5.76)</th>
<th>modified with ligand 5.47c (5.77)</th>
</tr>
</thead>
<tbody>
<tr>
<td>expected (Da)</td>
<td>23,422</td>
<td>23,556</td>
</tr>
<tr>
<td>found (Da)</td>
<td>23,454</td>
<td>23,557</td>
</tr>
</tbody>
</table>

The first analysis was performed on the commercial papain without activation. The observed mass was 23,454 Da. This was a puzzling result as this mass at first could be only assigned to papain in which the free Cys-25 is oxidized to the corresponding sulfinic acid (+32 Da) (Scheme 5.39).

Scheme 5.39  Thiol, disulfide, sulfenic and sulfinic acids functionalized papain

Sulfenic and sulfenic acids, which are difficult to detect in normal organic reactions due to their reactivity, have been detected in cysteine-containing enzymes. However, the formation of the sulfenic acid from the sulfenic acid is not reversible, in contrast to the formation of the sulfenic acid from the thiol. Nevertheless, as shown before, upon activation by incubation with DTT papain was active. This gave only few possibilities: 1) papain was commercially available as free thiol oxidized to sulfinic acid during the ESI-MS analysis or during sample preparation due to the absence of DTT; 2) papain was sold as sulfenic acid and therefore unstable; 3) the active Cys-25 was protected as some kind of disulfide which can be reduced (Scheme 5.39); 4) the sulfenic acid could be reduced by DTT. It is reported in the literature that the state of Cys-25 and therefore the role of DTT as reducing agent depends on the way in which papain is extracted and prepared.

When directly questioned, Sigma-Aldrich again could not provide any answer.
disulfide by dimerization of papain itself is also not possible as no reducing agent was used during the analysis.

The second sample analyzed was the one containing papain treated with 3-hydroxy-phenacyl bromide (5.72), used as a test reagent for the modification protocol. This adduct 5.76 would also be present in case of hydrolysis of the phosphite. The mass found for 5.76 was 23,557 Da which corresponds to the expected one if an initial mass of 23,422 Da is considered (Table 5.5). The analysis also showed the presence of a small peak with a mass of 23,454 Da, which at this point is still not clear if it corresponds to oxidized papain or to some kind of residual disulfide adduct.

Surprisingly, the analysis of the third sample showed mainly the adduct corresponding to the hydrolyzed cofactor 5.76 (23,557 Da). This alarming degradation of PapPhos (5.77) was explained as caused by the presence of 1% formic acid in the sample solution kept at 4°C overnight and final 0.1% during the analysis which might have hydrolyzed the phosphite. A new sample of PapPhos (5.77) was prepared and during its purification by dialysis only water was used. Moreover, during the ESI-MS analysis the amount of formic acid was reduced to 0.01%.

![Figure 5.27](image)

**Figure 5.27** ESI-MS analysis of PapPhos 5.77 resulting from the modification of papain with phosphite 5.47c

As shown in Figure 5.27, the ESI-MS analysis demonstrated the clear presence of a papain adduct with a mass of 24,207 Da which fitted the expected mass of PapPhos (5.77). This confirmed the successful monoalkylation of papain with cofactor 5.47c as expected in consideration of the high reactivity of Cys-25.
compared to other residues. The use of 0.01% formic acid significantly reduced the presence of the hydrolysis adduct 5.76. It was not clear whether the residual presence of 5.76 was still caused by the analysis conditions or if it was already present in the sample. As it was noticed during the analysis of adduct 5.76, that sample also contained residual not modified papain probably present as sulfinic acid (23,454 Da).

5.16 Digestion of PapPhos (5.77) and tandem mass spectrometry of peptide Asn\textsuperscript{18}-Lys\textsuperscript{39} containing Cys-25.

ESI-MS results confirmed that monoalkylation of papain with cofactor 5.47c was successfully achieved. The lack of native hydrolytic activity and the rather fast inhibition strongly suggested that Cys-25 was the residue involved in the chemical modification, also due to its much higher reactivity (vide supra). In order to confirm that the modification indeed occurred at Cys-25 and not at a close-by residue with consequent conformational distress, trypsin-mediated digestion was performed on native papain and modified 5.77.\textsuperscript{144}

Due to the preference of trypsin to hydrolyze proteins at the C-terminus of arginine and lysine\textsuperscript{145} papain digestion would provide up to 20 specific peptides, one of which (Asn\textsuperscript{18}-Lys\textsuperscript{39}) contains the cysteine residues Cys-25 and also Cys-22.\textsuperscript{146} The in-gel digestion allows isolating the peptide of interest which can be analyzed by mass spectrometry. To avoid analysis complications due to thiol oxidation, cysteine residues need to be protected, for example by reaction with iodoacetamide after reduction of all the disulfide bonds present.

Upon activation and treatment with iodoacetamide, the tryptic digestion of native papain, used as reference, will provide peptide Asn\textsuperscript{18}-Lys\textsuperscript{39} (2270 Da) with an increased mass of 2384 Da due to the carbamidomethylation (2 x 57 Da) of both Cys-22 and Cys-25. In the case of modified papain 5.77 the expected mass of the same peptide Asn\textsuperscript{18}-Lys\textsuperscript{39} should be 3114 Da, as the reaction of the protein with iodoacetamide (57 Da) would follow the incubation with 5.47c (787 Da). The results are shown in Table 5.6.

**Table 5.6 Digestion of papain with trypsin and analysis of peptide Asn\textsuperscript{18}-Lys\textsuperscript{39}a**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Native papain reacted with iodoacetamide</th>
<th>PapPhos 5.77 reacted with iodoacetamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expected</td>
<td>2384 Da</td>
<td>3114 Da</td>
</tr>
<tr>
<td>Found</td>
<td>2384 Da</td>
<td>2463 Da</td>
</tr>
</tbody>
</table>

\textsuperscript{a}For the in-gel digestion, the native and modified papain protein bands were electrophoresed in SDS-PAGE gel and then cut out of the gel.

During the mass analysis of peptide Asn\textsuperscript{18}-Lys\textsuperscript{39} of native papain, an ion at 1192.7 m/z was found corresponding to the doubly protonated expected ion of peptide Asn\textsuperscript{18}-Lys\textsuperscript{19} (2384 Da). However, during the analysis of the same peptide obtained
from PapPhos 5.77 the expected mass (3114 Da) was not observed. Instead, an ion at 1232.8 m/z was found, corresponding to a doubly protonated peptide Asn\textsuperscript{18}-Lys\textsuperscript{39} with mass 2463 Da. The mass obtained would have been expected from the analysis of 5.76, therefore after alkylation of papain with linker 5.72 (136 Da). The result clearly showed that the expected peptide of 5.77 had been chemically modified with 5.47c; however, hydrolysis of the phosphite moiety must have happened during the procedure.

In order to determine which of the cysteine residues, Cys-22 or Cys-25, of peptide\textsuperscript{18}NQGSCSGCWAFSAVVTIEGIK\textsuperscript{39} from 5.77 was modified with what was remaining of the cofactor, the peptide was further fragmented and analyzed by electrospray/tandem mass spectrometry. Fragmentation of peptides during MS/MS analysis provides very characteristic (so called b-type) ions as shown in Scheme 5.40.\textsuperscript{147}

\begin{equation}
\text{Scheme 5.40 } b\text{-Type ions obtained from electrospray / tandem mass spectrometry}
\end{equation}

The different level of fragmentation of the parent ion and the fact that each ion is unequivocally characterized by its side chain allow the identification of mass changes for a specific fragment. The expected b-type fragments were calculated for peptide Asn\textsuperscript{18}-Lys\textsuperscript{39} modified with iodoacetamide at both cysteine residues or with linker 5.72 ligand at either Cys-22 or Cys-25. The results were compared with the fragmentation spectra experimentally obtained (Table 5.7). The product ion scan of the parent ion (1192.8 m/z) of the peptide from native papain displayed most of the possible b-framents including fragments b5 (Cys-22, 548.17 m/z) and b8 (Cys-25, 852.23 m/z) both showing the iodoacetamide adducts (57 m/z). The fragmentation of the parent ion (1232.8 m/z) of the modified peptide Asn\textsuperscript{18}-Lys\textsuperscript{39} from 5.77 showed the expected increase of ion b5 due to carbamidomethylation. A mass increase of 134 m/z starting at b8 was instead attributed to the adduct of what was remaining of 5.47c at Cys-25. Once more, also this analysis confirmed that the chemical modification using cofactor 5.47c had been indeed achieved on Cys-25, excluding a possible alkylation of Cys-22.
Table 5.7  
Electrospray/tandem mass spectrometry of peptide Asn18-Lys39 of 5.77

<table>
<thead>
<tr>
<th>Fragments ion (M+2H)²⁺</th>
<th>Native papain²</th>
<th>Modified at Cys-25b</th>
<th>Modified at Cys-22c (calculated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>b1: N18</td>
<td>115.05</td>
<td>115.05</td>
<td>115.05</td>
</tr>
<tr>
<td>b2: NQ19</td>
<td>243.11</td>
<td>243.11</td>
<td>243.11</td>
</tr>
<tr>
<td>b3: NQG20</td>
<td>300.13</td>
<td>300.13</td>
<td>300.13</td>
</tr>
<tr>
<td>b4: NQGS21</td>
<td>387.16</td>
<td>387.16</td>
<td>387.16</td>
</tr>
<tr>
<td>b5: NQGSC22</td>
<td>548.17 (+57)</td>
<td>548.17 (+57)</td>
<td>625.17 (+134)</td>
</tr>
<tr>
<td>b6: NQGSCG23</td>
<td>605.19</td>
<td>605.19</td>
<td>683.19</td>
</tr>
<tr>
<td>b7: NQGSCGS24</td>
<td>692.23</td>
<td>692.23</td>
<td>770.23</td>
</tr>
<tr>
<td>b8: NQGSCGSC25</td>
<td>852.23 (+57)</td>
<td>930.23 (+134)</td>
<td>930.23 (+57)</td>
</tr>
<tr>
<td>b9: NQGSCGSCW26</td>
<td>1038.31</td>
<td>1115.31</td>
<td>1115.31</td>
</tr>
<tr>
<td>b10: NQGSCGSCW27</td>
<td>1110.35</td>
<td>1186.35</td>
<td>1186.35</td>
</tr>
<tr>
<td>b11: NQGSCGSCWAF28</td>
<td>1255.42</td>
<td>1333.42</td>
<td>1333.42</td>
</tr>
</tbody>
</table>

²Fragment masses found for native papain modified with iodoacetamide at both Cys-22 and Cys-25. ³Fragment masses found for peptide derived from 5.77. ⁴Fragment masses calculated for modification with 5.47c at Cys-22.

5.17 Kinetic parameters for the alkylation process

The rate of the irreversible inhibition of papain was determined by incubation of the enzyme for 45 min. in the presence of different concentrations of 5.47c. Samples of these solutions were tested for residual hydrolytic activity using Z-Gly-p-NPE (5.67) as substrate at regular intervals.

Inhibition reactions are generally expressed in agreement with the Michaelis-Menten mechanism (Scheme 5.16) which involves the initial formation of a reversible bound enzyme-inhibitor complex followed by covalent modification, in this case irreversible. The kinetic data were processed to fit a minimal kinetic scheme in agreement with an expected irreversible alkylation as depicted in Scheme 5.41.¹⁴⁸

\[ E + I \xrightarrow{K_i} EI \xrightarrow{k_i} E-I \]

\[ E^0 = [E] + [E \cdot I] + E-I \]

\[ E^0 = \text{initial enzyme concentration} \]

\[ E-I = \text{alkylated enzyme} \]

\[ \varepsilon = [E] + [E \cdot I] \]

\[ [E \cdot I] = \text{enzyme-inhibitor complex} \]

Scheme 5.41  
Inhibition process treated according to a minimal kinetic scheme

---

221
According to Scheme 5.41, $K_I$ is the apparent dissociation constant of the enzyme-inhibitor complex and $k_i$ is the first-order rate constant of inactivation following the formation of the assumed enzyme-inhibitor complex. Therefore, these constants are expressed as:

$$\frac{[I][E]}{[E] \cdot [I]} = K_I \quad \text{and} \quad -\frac{d[I]}{dt} = k_i [E \cdot I]$$

**Equation** No text of specified style in document..1 *Definition of $K_I$ and $k_i$ according to Scheme 5.41*

The amount of initial active enzyme ($E^0$) and residual active enzyme ($\varepsilon$) can be expressed in terms of residual hydrolytic activity ($A_0$ and $A$, respectively). For $[I] \gg E^0$ the observed rate constant (defined as $k_{app}$) and the solution of the equation become:

A) $\ln \frac{A}{A_0} = - \frac{k_i \cdot t}{1 + K_I / [I]} = k_{app} \cdot t$; with B) $\frac{1}{k_{app}} = \frac{1}{k_i} + \frac{1}{K_I}$ and C) $k_2 = \frac{k_i}{K_I}$

**Equation** No text of specified style in document..2 *Definition of $K_I$ and $k_i$ as function of the hydrolytic activity, providing the apparent second order rate constant $k_2$*

The relative $A/A_0$ values listed in Table 5.8 were obtained from the experimental residual hydrolytic activity of papain (0.12 $\mu$M) at different moments of the incubation with different concentrations of 5.47c.

**Table 5.8** Values of relative hydrolytic activity ($A/A_0$) observed during the incubation time for each concentration of 5.47c

<table>
<thead>
<tr>
<th>time (min)</th>
<th>$A/A_0$ (1.16 $\mu$M)</th>
<th>$A/A_0$ (2.31 $\mu$M)</th>
<th>$A/A_0$ (3.47 $\mu$M)</th>
<th>$A/A_0$ (4.62 $\mu$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>0.377167</td>
<td>0.317993</td>
<td>0.227045</td>
<td>0.227314</td>
</tr>
<tr>
<td>30</td>
<td>0.217293</td>
<td>0.143941</td>
<td>0.077458</td>
<td>0.07003</td>
</tr>
<tr>
<td>45</td>
<td>0.137463</td>
<td>0.065612</td>
<td>0.056617</td>
<td>0.033704</td>
</tr>
</tbody>
</table>
Merging Homogeneous Catalysis with Biocatalysis: Papain as Hydrogenation Catalyst

Figure 5.28  Time dependence of the inactivation-alkylation of papain (0.12 μM) at different concentrations of 5.47c plotted as Ln(A/A₀) vs. incubation time. Concentrations of 5.47c used: 1.2 μM (■), 2.3 μM(●), 3.5 μM(▲), 4.6 μM(▼).

Figure 5.28 shows the interpolation of the data obtained from the hydrolytic activity of papain with different amounts of 5.47c as function of the incubation time, which provided a set of k_{app} according to Equation 5.2A (Table 5.9).

Table 5.9  Values of k_{app} determined for each concentration of 5.47c

<table>
<thead>
<tr>
<th>5.47c (mM)</th>
<th>k_{app} (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.16</td>
<td>0.0434</td>
</tr>
<tr>
<td>2.31</td>
<td>0.0598</td>
</tr>
<tr>
<td>3.47</td>
<td>0.0646</td>
</tr>
<tr>
<td>4.62</td>
<td>0.0757</td>
</tr>
</tbody>
</table>

The Kitz-Wilson plot of this values (1/k_{app}) as function of the concentration of 5.47c (1/[I]) is shown in Figure 5.29.
The interpolation of the data allowed to calculate $K_I$ and $k_i$ and consequently $k_2$
according to Equations 5.2B-C and the results are listed in Table 5.10.

**Table 5.10 Kinetic parameters for the alkylation of papain by 5.47c**

<table>
<thead>
<tr>
<th>$k_i$ (min⁻¹)</th>
<th>$K_I$ (μM)</th>
<th>$k_i/K_I = k_2$ (M⁻¹s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.47c</td>
<td>0.095</td>
<td>1.385</td>
</tr>
</tbody>
</table>

The apparent second-order rate constant $k_2$ is a value with which inhibitors are
often compared in the literature and can be defined as a measure of the efficiency
of the inhibition. For example, $k_2$ for chloroketone TLCK is 750 M⁻¹s⁻¹ and $k_2$ for the
natural inhibitor (L) E-64 is 638,000 M⁻¹s⁻¹.

**5.18 Scaling up the preparation of PapPhos (5.77)**

It was first necessary to scale up the amount of protein that could be obtained from
the alkylation step, in order to be able to perform any kind of catalysis using 5.77
as ligand for a suitable Rh(I) precursor.

**5.18.1 Alkylation protocol**

Therefore a new protocol had to be developed. As shown in Table 5.11, 5.47c was
directly added to the activation solution A instead of performing the alkylation step
in the more diluted solution B (Table 5.3 and Table 5.4). However, to reach this
higher concentration of protein it was necessary to add 5.47c with a higher final
concentration and the cofactor started to be not completely soluble, as a turbid solution was obtained.

**Table 5.11  New protocol for the scaled up alkylation step**

<table>
<thead>
<tr>
<th></th>
<th>volume (mL)</th>
<th>final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-buffer (100 mM, pH 7)</td>
<td>9.8</td>
<td>100 mM</td>
</tr>
<tr>
<td>papain (1.1 mM)</td>
<td>0.1</td>
<td>9.2 μM</td>
</tr>
<tr>
<td>DTT (0.1 M)</td>
<td>0.1</td>
<td>10 mM</td>
</tr>
<tr>
<td>5.47c (2mM, 1,4-dioxane)</td>
<td>2.0</td>
<td>0.33 mM</td>
</tr>
</tbody>
</table>

Due to the much higher concentration of enzyme, a dilution solution B was prepared to obtain the right concentration of enzyme for the activity test (solution C). Therefore, 50 μL of solution A were diluted to 1 mL (solution B). As before, 90 μL of solution B were added to 890 μL of phosphate buffer (solution C), the solution was placed in a UV cuvette and the substrate 5.67 (20 μL) was added to it as last.

The alkylation step performed according to this new protocol was slower (2-3 h) than the previous one and not always complete. It was not clear whether this was caused by the turbidity of the solution or the lower cofactor 5.47c to papain ratio (36:1, compared to the previous 91:1). It was tried to dilute the buffer to 50 mM, but this did not improve the results. When 5 mL of cofactor 5.47c were used, increasing the ratio back to 91:1, no real improvement was noticed. Moreover, the higher amount of final solution (15 mL) became problematic for the following work-up. Not even maintaining the ratio 5.47c to papain at 36:1 but using more 1,4-dioxane (final 5 mL) helped. Finally a good compromise was found by adding the cofactor 5.47c in six portions of 0.5 mL (final 3 mL) every 30 minutes.

**5.18.2  Purification of PapPhos 5.77**

As previously described, whilst preparing the samples for the ESI-MS (page 215) it was established that a good way of removing the excess of ligand and concentrating the solutions was dialysis by ultracentrifugation. The centrifugal filters initially used had a 10K cut-off, which means that any molecule smaller than 10,000 NMWL (nominal molecular weight limit) is removed. However, the filters previously used allowed the centrifugation of only 2.5 mL at the time, making the work-up an extremely long process. Other filters adopted (Amicon® Ultra-15) still had a 10K cut-off but an improved capacity of 12 mL whilst using a fixed angle rotor. The presence of a vertical membrane allowed also faster concentration during the centrifugation (from 13 mL to 150 μL in 20 min.). Moreover, the filter seemed to better tolerate the presence of 1,4-dioxane. The solutions at the end of the alkylation step were rather turbid and even dialysis against pure water or in the presence of 50% organic solvent (MeOH, EtOH) did not help eliminating the excess of 5.47c. As a solution, a different kind of filters was used before the
centrifugation (Minisart SRP 15, 0.45 µm). These filters for syringes allowed to retain the precipitate and clear solutions of 5.77 were obtained, which could be dialyzed against distilled water (3 × 15 mL) and concentrated (final 150 µL) as before. With this improved and reliable purification step in hand, it was decided to test the efficiency of PapPhos in catalysis using Rh(I) precursors. To ensure a maximum degree of reproducibility, adduct 5.77 was always prepared the same day at which the catalysis experiments were performed.

5.19 Rh-catalyzed hydroformylation in aqueous media

Among the possible Rh-catalyzed reactions, hydroformylation (Scheme 5.42) seemed to be an interesting transformation to challenge the newly obtained modified papain 5.77 with, as it cannot be performed by existing enzymes.8

\[
\begin{align*}
R^' & \quad [\text{Rh}] \quad H_2/\text{CO} \\
\text{water/organic solvent} & \quad \rightarrow \\
\text{5.48} & \quad \rightarrow R^'\text{CHO} + R^'\text{CHO} \\
\text{5.49a: branched} & \quad \text{5.49b: linear}
\end{align*}
\]

Scheme 5.42  Generic hydroformylation scheme

This organometallic transformation has been previously performed in aqueous media, which is an important requirement.150 Biphasic conditions are often inevitable as the alkenes 5.48 used as starting material are generally not water-miscible liquids, therefore, they can be used also as cosolvent or in combination with another organic solvent as, for example, toluene.

Hydroformylation is an important industrial transformation. This means that particular interest has been devoted to solve problems such as separation of the product from the catalyst and catalyst recycling. Performing the reaction in a biphasic media appears to be one possible solution to these issues151 and the continuous biphasic Rh-catalyzed process developed by Rhône Poulenc and Ruhrchemie AG for the hydroformylation of propene is a successful example.152 The sulfonated triphenylphosphine 5.78 used is probably still the most well-known water soluble phosphorus based ligand (Figure 5.30). Biphasic hydroformylation reactions are generally performed using temperatures of 40-80 °C and under pressures of 50-80 bar of syngas. Cosolvents often used are toluene, n-hexane, n-heptane and cyclohexane.

\[
\begin{align*}
\text{Na}_3\text{SO}_4 & \quad \text{Na}_3\text{SO}_4 \\
\text{TPPTS} & \quad 5.78 \\
\end{align*}
\]

Figure 5.30  TPPTS (5.78), a successful ligand in biphasic hydroformylation
However, very few chiral water soluble ligands have been used in Rh-catalyzed hydroformylation.\textsuperscript{153} Some of the results obtained in the hydroformylation of styrene are depicted in Scheme 5.43.

\begin{center}
\begin{tikzpicture}
\node (a) at (0,0) {$\text{5.79}$};
\node (b) at (1,0) {$\text{Rh(I) / } L^* \rightarrow \text{H}_2 / \text{CO}$};
\node (c) at (2,0) {$\text{5.80a}$};
\node (d) at (3,0) {$\text{5.80b}$};
\end{tikzpicture}
\end{center}

\begin{itemize}
\item Rh(acac)(CO)\textsubscript{2}-\textbf{5.81a}: 92\% conv., \textbf{5.80a} 95\%, 18\% ee (toluene, MeOH, H\textsubscript{2}O)
\item [Rh(\(\mu\)-OMe)(cod)]-\textbf{5.81b}: 67\% conv., \textbf{5.80a} 76\%, 17\% ee (MeOH, H\textsubscript{2}O)
\item [Rh(\(\mu\)-OMe)(cod)]-\textbf{5.81c}: 4\% conv., \textbf{5.80a} 90\%, 14\% ee (MeOH, H\textsubscript{2}O)
\item Rh(COD)\textsubscript{2}BF\textsubscript{4}-\textbf{5.81d}: 71\% conv., \textbf{5.80a} 76\%, 17\% ee (H\textsubscript{2}O)
\item Rh(COD)\textsubscript{2}BF\textsubscript{4}-\textbf{5.81e}: 99\% conv., \textbf{5.80a} 83\%, 25\% ee (H\textsubscript{2}O)
\end{itemize}

\textbf{Scheme 5.43} \textit{Rh-catalyzed hydroformylation of styrene \textbf{5.79} using chiral water soluble phosphorus ligands \textbf{5.81a-e}}

The first use of sulfonated BINOL based bidentate phosphorus ligand \textbf{5.81a}\textsuperscript{154a} was followed by the report of two examples of chiral alkyl sulfonated diarylphosphines.\textsuperscript{154b} Polyether-phosphite ligands \textbf{5.81d-e} are instead examples of interesting water-soluble non-ionic monodentate and bidentate phosphite ligands used in thermo-regulated phase transfer conditions.\textsuperscript{154c} In all cases, the enantioselectivities obtained for the branched product \textbf{5.80a} were rather low and this seems to be a general feature of hydroformylation reactions performed in aqueous media.

\subsection*{5.19.1 Rh-catalyzed hydroformylation reactions using Pap-Phos \textbf{5.77} as chiral ligand}

The metal precursor Rh(acac)(CO)\textsubscript{2} was usually added in considerable excess as a solid to an aqueous solution of \textbf{5.77}. The heterogeneous solution was stirred for 30 min. and the excess Rh(I) was filtered away using syringe filters (0.45 \(\mu\)m). A yellowish solution was obtained. The expectation was that only the Rh(I) solubilized by the presence of the protein would remain in solution.
Subsequently, styrene (5.79) and the required cosolvent were added and the reaction was performed overnight. Results and reaction conditions are depicted in Table 5.12.

**Table 5.12** Biphasic hydroformylation of styrene (5.79) using H₂O / toluene

<table>
<thead>
<tr>
<th>entry</th>
<th>ligand</th>
<th>H₂O : toluene (mL)</th>
<th>conv. (%)</th>
<th>5.80a (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.77</td>
<td>2:2</td>
<td>100</td>
<td>92</td>
</tr>
<tr>
<td>2</td>
<td>papain</td>
<td>2:2</td>
<td>100</td>
<td>85</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>2:2</td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td>4</td>
<td>5.77</td>
<td>3:1</td>
<td>78</td>
<td>92</td>
</tr>
<tr>
<td>5</td>
<td>5.77</td>
<td>3:0</td>
<td>8</td>
<td>89</td>
</tr>
</tbody>
</table>

*aReactions performed in 4 mL of solvent with 2.17 mmol of substrate (250 μL) and 0.11 μmol protein for 16 h. Conversions determined by ¹H NMR. Enantioselectivity was determined by HPLC after reduction of the aldehyde to the corresponding alcohol when 5.77 was used. †An average of 120 equiv. of Rh(acac)(CO)₂ were used. All the solutions were filtered before the addition of toluene and styrene, unless otherwise stated. ‡In this case the excess of Rh(I) was not removed by filtration, Rh(I) to protein ratio 74:1 and a substrate to Rh(I) ratio of 293:1.

The reaction seemed at first to be very efficient (entry 1) as full conversion was obtained with a substrate to protein molar ratio of ≈20,000:1. However, the same result with just slightly lower regioselectivity (85%) was obtained when only papain was used (entry 2). It is possible that the reaction is catalyzed by Rh(I) non specifically bound to the protein structure, although in this case the excess of not solubilized rhodium was not removed. However, when no protein was used full conversion was still obtained (entry 3) pointing at the fact that the reaction could also have been catalyzed by RhH(CO)₄ known to be an efficient catalyst. Moreover, the presence of toluene (2:2) as cosolvent could have helped the extraction of the metal from the aqueous layer to the organic one, where the reaction was actually taking place. This possibility was confirmed by a progressive reduction of activity observed when using 5.77 as ligand in the presence of a reduced amount of toluene (entry 4) or without any cosolvent (entry 5). In all cases using papain or 5.77, product 5.80a was obtained as a racemic mixture.

It was decided to change the cosolvent as it had been reported that the use of n-heptane helps reducing the leaching of rhodium from the aqueous layer. The results are shown in Table 5.13.
Table 5.13 Biphasic hydroformylation of styrene (5.79) using H₂O / n-heptane

<table>
<thead>
<tr>
<th>entry</th>
<th>ligand</th>
<th>H₂O : heptane (mL)</th>
<th>conv. (%)</th>
<th>5.80a (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.77</td>
<td>2:2</td>
<td>100</td>
<td>79</td>
</tr>
<tr>
<td>2</td>
<td>papain</td>
<td>2:2</td>
<td>100</td>
<td>80</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>2:2</td>
<td>80</td>
<td>79</td>
</tr>
<tr>
<td>4</td>
<td>5.77</td>
<td>3:1</td>
<td>98</td>
<td>79</td>
</tr>
<tr>
<td>5</td>
<td>5.77</td>
<td>3:0</td>
<td>59</td>
<td>76</td>
</tr>
</tbody>
</table>

Reactions performed in 4 mL of solvent with 2.17 mmol of substrate (250 μL) and 0.11 μmol protein for 16 h. Conversions determined by ¹H NMR. Enantioselectivity was determined by HPLC after reduction of the aldehyde to the corresponding alcohol when 5.77 was used. An average of 29 equiv. of Rh(acac)(CO)₂ were used. All the solutions were filtered before the addition of n-heptane and styrene.

At the end of the reaction the organic layer was colorless, contrary to the yellow toluene layers that were previously observed and the regioselectivity decreased considerably. Again full conversion was obtained using both 5.77 and papain (entries 1 and 2) and an equal amount of solvents. High but not full conversion was achieved also without the presence of the protein (entry 3). Surprisingly, in this case reducing the amount of cosolvent (entries 4 and 5) resulted in a higher conversion to 5.80a than previously observed (Table 5.12, entries 4 and 5). These results gave the idea that the metal center might have been more active in the aqueous layer, however the result of entry 3 did not allow to assume that this was due to the presence of the protein. As once more no enantioselectivity was detected for 5.80a it was also not possible to say if any specific binding was present between the metal center and the phosphite group.

In order to suppress the reaction catalyzed by rhodium not bound to the enzyme and to understand if the phosphite had any influence in the catalysis, another set of experiments was performed in which the syngas pressure was reduced to 30 bar (Table 5.14).
Chapter 5

Table 5.14 Biphasic hydroformylation of styrene (5.79) using H₂O / n-heptane

<table>
<thead>
<tr>
<th>entry</th>
<th>ligand</th>
<th>H₂O : heptane (mL)</th>
<th>conv. (%)</th>
<th>5.80a (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.77</td>
<td>2:2</td>
<td>2.8</td>
<td>64</td>
</tr>
<tr>
<td>2</td>
<td>5.77</td>
<td>2:2c</td>
<td>4.3</td>
<td>64</td>
</tr>
<tr>
<td>3</td>
<td>papain</td>
<td>2:2</td>
<td>1.8</td>
<td>66</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>2:2</td>
<td>1.4</td>
<td>63</td>
</tr>
</tbody>
</table>

*aReactions performed in 4 mL of solvent with 2.17 mmol of substrate (250 μL), 0.11 μmol protein for 16 h, unless otherwise stated. Conversions determined by 1H NMR. Enantioselectivity checked by HPLC after reduction of the aldehyde to alcohol when 5.77 was used. An average of 56 equiv. of Rh(acac)(CO)₂ were used. All the solutions were filtered before the addition of n-heptane and styrene. A buffered aqueous solution was used (P-buffer 50 mM, pH 7).

As expected, at this lower pressure, the conversion decreased drastically. However, 5.80a was still obtained as a racemic mixture with a lower regioselectivity. A higher conversion was obtained when using a buffered aqueous solution instead of water (entry 2). This might have increased the stability of the protein. Interestingly, slightly lower conversions were obtained without 5.77 or in the presence of native papain (entries 3 and 4).

Although the substrate to protein ratio was very high (≈20,000:1) there was no clear evidence that the reaction was indeed performed by rhodium bound to the protein and even less evidence that it was bound to the phosphite group as no enantioselectivity could be induced in all cases. Furthermore, until now biphasic hydroformylation reactions using chiral ligands far more defined than 5.77 provided very low enantioselectivities (Scheme 5.43, 14-25% ee). There was also little information about how the amount of Rh(I) was actually dissolved in solution as even when a lower amount was used (Table 5.13, 19-39 equiv.) all the solutions needed to be filtered. The high temperature and pressure of syngas applied might also cause instability of the protein, although it has been reported that native papain is active and therefore stable at temperatures up to 80 °C.

Biphasic hydroformylation has been defined as a complicated gas-liquid-liquid reaction and despite the number of studies conducted on the various parameters involved, many contradictions are still present in the literature. Because of these reasons, it was decided that hydroformylation reactions were probably not a suitable choice, as a lot of parameters need to be established and understood for this catalytic reaction to be efficient and reliable in aqueous solutions.

230
5.20 Rh-catalyzed hydrogenation in aqueous media

As discussed in previous chapters, the Rh-catalyzed asymmetric hydrogenation reaction is a very well established transformation. The reaction can be efficiently performed with benchmark substrates using a large variety of both bidentate and monodentate chiral phosphorus based ligands. The advantages of using water as reaction medium are essentially the same as described for hydroformylation reactions. Jóó and coworkers were the first to report the use of sulfonated phosphines as ligands in Ru-catalyzed hydrogenation.\(^{159}\) Subsequently, a number of water-soluble chiral and achiral phosphorus-based ligands have been reported.\(^{160}\) Hydrophilic groups are generally sulfonate, phosphonate, carboxylate, hydroxy, ammonium, guanidinium, amine and polyether moieties.\(^{161}\) However, rarely good enantioselectivities were obtained in Rh-catalyzed asymmetric hydrogenation reactions. Some of the most relevant results reported in the hydrogenation of common dehydroamino acids and their methyl esters (Scheme 5.44) are shown in Table 5.15.

![Scheme 5.44](image)

**Scheme 5.44**  Rh-catalyzed hydrogenation of α-dehydroamino acids and their methyl esters, using chiral water soluble phosphorus ligands
Table 5.15  Rh-catalyzed asymmetric hydrogenation of prochiral substrates in aqueous media using water-soluble ligands 5.83-5.85

<table>
<thead>
<tr>
<th>entry</th>
<th>ligand</th>
<th>substrate</th>
<th>solvent</th>
<th>ee (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.83a</td>
<td>5.9</td>
<td>H2O</td>
<td>90</td>
</tr>
<tr>
<td>2</td>
<td>5.83b</td>
<td>5.9</td>
<td>H2O</td>
<td>94</td>
</tr>
<tr>
<td>3</td>
<td>5.84a</td>
<td>5.51</td>
<td>H2O:MeOH:EtOAc (6:4:10)</td>
<td>98</td>
</tr>
<tr>
<td>4</td>
<td>5.84a</td>
<td>5.51</td>
<td>H2O +10% SDS</td>
<td>&gt;99</td>
</tr>
<tr>
<td>5</td>
<td>5.84a</td>
<td>5.9</td>
<td>H2O:MeOH:EtOAc (6:4:10)</td>
<td>96</td>
</tr>
<tr>
<td>6</td>
<td>5.84b</td>
<td>5.81</td>
<td>H2O</td>
<td>94</td>
</tr>
<tr>
<td>7</td>
<td>5.84b</td>
<td>5.5</td>
<td>H2O</td>
<td>&gt;99</td>
</tr>
<tr>
<td>8</td>
<td>5.85</td>
<td>5.5</td>
<td>H2O:MeOH (1:1)</td>
<td>89</td>
</tr>
<tr>
<td>9</td>
<td>5.85</td>
<td>5.5</td>
<td>H2O +10% SDS</td>
<td>89</td>
</tr>
</tbody>
</table>

Quaternary ammonium diphosphine ligands 5.83a and 5.83b belong to the category of ionic ligands. Their preparation was considered a valuable alternative to sulfonation and their application in asymmetric hydrogenation for example of (Z)-2-(acetamido)cinnamic acid (5.9) provided good enantioselectivities (entries 1 and 2). Instead, both RajanBabu and Uemura and their coworkers proposed water soluble diphosphinites derived from α,α-trehalose, however, the results obtained using their Rh(I)-complexes in the asymmetric hydrogenation on the same substrates depicted in Scheme 5.44 were rather modest (49-76% ee). In the same paper, Uemura and coworkers reported also the use of the β,β-trehalose derived diphosphinite 5.84a that proved to be a very efficient ligand in the asymmetric hydrogenation of 5.9 and 5.51 (entries 3-5). Even more interest was directed towards carbohydrate based ligands with the appearance (in the same year) of the D-mannitol derived DuPhos analogue 5.84b introduced by Börner and coworkers. The Rh(I)-5.84b complex provided very good enantioselectivities in the hydrogenation of 2-(acetamido)acrylic acid and its methyl ester (5.5 and 5.51, entries 6 and 7). Very recently, a water soluble monodentate phosphoramidite 5.85 has also been presented by our research group in collaboration with the group of Hiemstra and Van Maarseveen. Water solubility was achieved using polyether groups and 89% ee was reached in the hydrogenation of 5.5 using the corresponding Rh(I)-complex (entries 8 and 9). The beneficial influence of the use of surfactants in hydrogenation reactions performed in aqueous media has been frequently reported. For example, the use of 10% SDS eliminated the necessity of cosolvents using both diphosphinite 5.84a and phosphoramidite 5.85 (entries 4 and 9). Moreover, Uemura and coworkers reported also a considerable increase in reactivity and enantioselectivity.
Zhang and coworkers reported the asymmetric hydrogenation in aqueous media of itaconic acid derivatives 5.7 and 5.86 using chiral ligand 5.84c, another water-soluble analogue of DuPhos derived from D-mannitol. The excellent enantioselectivities obtained are depicted in Scheme 5.45.

**Scheme 5.45**  
Rh-catalyzed hydrogenation of 5.7 and 5.86 using chiral water-soluble carbohydrate-based phosphorus ligand 5.84c

This overview shows that Rh(I)-catalyzed asymmetric hydrogenation in aqueous media is a much more developed field than the corresponding hydroformylation (Scheme 5.43). The good results obtained by different groups seemed to ensure a better control over the reaction parameters which would help to more easily extrapolate the influence of PapPhos 5.77 from the results. Moreover, milder conditions are used, as the reaction can be performed at room temperature and using relatively low hydrogen pressure.

### 5.20.1 Rh-catalyzed hydrogenation reactions using PapPhos 5.77 as chiral ligand

Methyl 2-(acetamido)acrylate 5.81 was chosen as test substrate due to its good water solubility which provided also easier work up and subsequent analysis. Due to the better solubility of the precursor in water, the removal of the excess Rh(COD)2BF4 after the complexation was attempted by extraction with toluene. The results of the preliminary attempts are shown in Table 5.16.

Very excitingly, full conversion and 5% ee were obtained in the hydrogenation of 5.81 (entry 1). The hybrid catalyst 5.88 was prepared by the complexation of 5.77 with Rh(COD)2BF4 added as a stock solution in toluene (15 equivalents). At the end of the complexation time (30 min.) the toluene was removed and the aqueous layer extracted with 1 mL of toluene. The aqueous solution obtained had a yellowish color and a milky appearance. However, the reaction could not be fully reproduced (entry 2) and the control experiment provided by using papain as ligand (*vide infra*) afforded 5.82 in 99% conversion. In another set of experiments the extraction with toluene was performed twice on both 5.88 and Rh(I)-papain. In this case, 5.88 lost almost all the activity but almost full conversion was still obtained for the control experiment (entries 4 and 5). In all cases, turbid gray solutions were obtained after catalysis caused by the presence of rhodium black.
When only 1 equivalent of rhodium precursor was added (from a stock solution in toluene) no conversion was obtained.

**Table 5.16 Rh-catalyzed hydrogenation of methyl 2-(acetamido)acrylate 5.81 using PapPhos 5.77 as chiral ligand after extraction with toluene**

<table>
<thead>
<tr>
<th>entry</th>
<th>ligand</th>
<th>extraction</th>
<th>conv. (%)</th>
<th>ee (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.77</td>
<td>1</td>
<td>100</td>
<td>5 (S)</td>
</tr>
<tr>
<td>2</td>
<td>5.77</td>
<td>1</td>
<td>96</td>
<td>2 (S)</td>
</tr>
<tr>
<td>3</td>
<td>papain</td>
<td>1</td>
<td>99</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>5.77</td>
<td>2</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>papain</td>
<td>2</td>
<td>97</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>5.77*</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Reactions performed in 2 mL of H₂O for 16 h, using a substrate to protein ratio of 400:1 and a Rh(COD)₂BF₄ to protein ratio of 15:1, unless otherwise stated. *Number of times the aqueous layer was extracted with toluene. *Conversions determined by ¹H NMR and GC. *Enantiomeric access determined by chiral GC. *Only one equivalent of Rh(COD)₂BF₄ was used.

Although an unreliable small degree of enantioselectivity was induced, the reaction did not seem to be particularly reproducible, papain as ligand was as efficient as 5.77 and it was not clear if toluene was indeed a good solvent for the removal of the excess of metal precursor. The use of an equimolar amount of Rh(I) was not enough to obtain an active catalyst.

A few more experiments were performed, in which the removal of the excess of Rh(I) precursor was attempted by extraction with CH₂Cl₂. The metal precursor Rh(COD)₂BF₄ was in this case added as a stock solution in CH₂Cl₂ in which it was also more soluble and after 30 min. the organic solvent was removed. The hydrogenation of 5.81 was performed in the presence of 5.88, Rh(I)-papain and without any ligand. The results are depicted in Table 5.17.

Once more, even after an additional extraction with the organic solvent, almost full conversion was obtained also when using just papain and even without the presence of any ligand (entries 3 and 4). In all cases, after the extraction with CH₂Cl₂ the aqueous solution resulted to be yellow even when only water was used. The product 5.82 obtained was found to be racemic.
Table 5.17 Rh-catalyzed hydrogenation of methyl 2-(acetamido)acrylate \(5.81\) using PapPhos \(5.77\) as chiral ligand after extraction with \(\text{CH}_2\text{Cl}_2\)\(^a\)

<table>
<thead>
<tr>
<th>entry</th>
<th>ligand</th>
<th>extraction(^b)</th>
<th>conv. (%)(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(5.77)</td>
<td>1</td>
<td>72</td>
</tr>
<tr>
<td>2</td>
<td>(5.77)</td>
<td>2</td>
<td>98</td>
</tr>
<tr>
<td>3</td>
<td>papain</td>
<td>2</td>
<td>90</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>2</td>
<td>93</td>
</tr>
</tbody>
</table>

\(^a\)Reactions performed in 2 mL of \(\text{H}_2\text{O}\) for 16 h, using a substrate to protein ratio of 400:1 and a \(\text{Rh(COD)}_2\text{BF}_4\) to protein ratio of 15:1, unless otherwise stated. \(^b\)Number of times the aqueous layer was extracted with toluene. \(^c\)Conversions determined by \(^1\)H NMR and GC. \(^d\)Enantiomeric access determined by chiral GC. \(^e\)Only one equivalent of \(\text{Rh(COD)}_2\text{BF}_4\) was used.

Although the Rh(I)-catalyzed hydrogenation seemed to be the adequate catalytic reaction to test the efficiency of \(5.77\) as chiral ligand, the purity of the catalyst remained an issue. It seemed necessary to have a closer look at the complexation step and alternative ways to purify the complex \(5.88\), in order to minimize the amount of residual rhodium precursor and guarantee the stability of the protein and the complex.

5.21 Complexation of PapPhos (5.77) with \(\text{Rh(COD)}_2\text{BF}_4\)

The introduction of cofactor \(5.47\)c in the active site of papain afforded a hybrid protein structure which has lost its original hydrolytic activity and cannot be defined as an enzyme anymore. The preferential and stable complexation of a metal precursor to the newly introduced non-proteinogenic moiety would convert the hybrid protein into a metalloprotein, which could be considered a bio-organometallic catalyst in case reliable catalysis could be performed. The experimental results previously shown, demonstrated that it was not possible to make such a statement just yet, as the catalysis was not reproducible or selective. This can be attributed to a lack of control over the complexation step and the purification of the complex obtained (Figure 5.31). As the rhodium precursor was added to the already modified protein, it is important to understand the entity of the possible interactions between the metal center and the different residues of papain.
Figure 5.31  Metal complexation: from hybrid protein to metalloprotein

It was assumed that the rhodium would preferentially bind to a phosphite rather than an amino acid side chains or the protein backbone. However, due to the excess of catalyst precursor used, unspecific binding may occur, which would lead to the presence of metallic rhodium during the hydrogenation reaction. Thus, hydrogenation with metallic rhodium would dominate the results. It was also shown that the addition of only one equivalent of metal precursor failed to provide any catalytic activity. Only one equivalent was insufficient but the use of an excess of metal precursor required a better purification protocol than what had been used so far.

5.21.1 Papain as a source of binding sites

Metalloprotein reactivity is generally tuned by the presence of different metal ions and coordination geometries and 1/3 of all the structurally characterized proteins contain metal-binding sites. The physiologically most relevant metals are in evidence in the periodic table depicted in Figure 5.32.

Figure 5.32  The metals of biology (bold text) and transition metals used in antitumoral studies (bold text and cells)

Rhodium obviously is not among these metals, but an increased interest in finding less toxic organometallic compounds with antitumoral activity has stimulated
Merging Homogeneous Catalysis with Biocatalysis: Papain as Hydrogenation Catalyst

studies on metals such as rhodium, ruthenium and iridium.\textsuperscript{170} For this reason, different dimeric \(\mu\)-acetato dimers of Rh(II), monomeric square planar Rh(I) and octahedral Rh(III) complexes have been studied and showed interesting properties,\textsuperscript{171} mostly concerning their interactions with DNA (Figure 5.33).\textsuperscript{172}

![Figure 5.33](image)

\textbf{Figure 5.33} \(\text{Rh(II)}\) carboxylates 5.89 and few of the \(\text{Rh(I)}\) complexes 5.90 used as alternative cytostatic drugs

To obtain insight into the molecular modes of action of intravenously administered antitumoral metal complexes, investigations have been conducted on the interaction of \(\text{Rh(II)}\) carboxylates 5.89 with serum proteins.\textsuperscript{173} Human serum albumin (HSA), for example, is the principal protein component of plasma; as such, it binds and transports a wide variety of substances, including metals. \(\text{Rh(II)}\) carboxylates 5.89 have shown to form relatively stable mono and bis-adducts with a variety of residues of HSA possessing donor atoms such as nitrogen, sulfur, and oxygen.\textsuperscript{174} Spectroscopic studies using a variety of amino acids indicated that only histidine, cysteine and methionine form adducts with \(\text{Rh}_2(\text{OAc})_4\), histidine being the preferred binding site.\textsuperscript{175} Tryptophan seemed to provide a weaker binding site. It was also observed that \(\alpha\)-amino acids containing free sulfhydryl groups could lead to disruption of the Rh-Rh bond with formation of monomeric chelate species.

In conclusion, according to the literature studies, histidine, methionine, cysteine and to some extent tryptophan were assumed to be suitable residues for metal binding. In consideration of the presence of such residues in papain (Figure 5.34), it was assumed that 8 equivalents of \(\text{Rh(COD)}_2\text{BF}_4\) could complex to the protein.

![Figure 5.34](image)

\textbf{Figure 5.34} Papain residues as potential metal-binding sites
None of the cysteine residues were considered available as six of them were involved in disulfides bonds and Cys-25 carried the phosphite group which counts as one binding site. Nevertheless, eight equiv. of Rh(I) could well be an excess as, for example, a couple of the indole residues are buried in the structure and might not be accessible.

5.21.2 Improvements in the purification of Rh(I)-PapPhos complex

Purification of the conjugate 5.88 obtained after the complexation of Pap-Phos 5.77 with Rh(COD)\(_2\)BF\(_4\) was pursued in a milder and more efficient way using two different prepacked desalting columns, which both follow the principal of size exclusion chromatography. One column was prepacked with Sephadex\textsuperscript{TM} G-25 (HiTrap\textsuperscript{TM}) and connected with an MPLC\textsuperscript{176} system (AKTA purifier). The second column was prepacked with polyacrylamide Bio-Gel\textsuperscript{TM} P-6DG gel (Econo-Pac\textsuperscript{TM}) and the purification was performed under atmospheric pressure. The protein was eluted first using both systems, which allowed the separation from excess or weakly bound Rh(I) and an exchange of buffer if necessary. In this respect, both systems efficiently replaced a dialysis step. In both cases, the fractions collected during the purification were checked for the presence of protein by UV at 280 nm. However, the MPLC system had the advantage of having a graphic interface which allowed in real time to monitor the elution at different wavelengths and Figure 5.35 is an example of the UV profile obtained.

![UV profile obtained during MPLC purification of the conjugate 5.88](image)

It was possible to detect at three different wavelengths: 280 nm identified the protein, 440 nm was related to the presence of metal, 600 nm was related to the presence of heterogeneous elute. During this particular purification the complexation was performed using 15 equivalents of rhodium precursor. More interestingly, the fractions containing protein also showed a band at 440 nm and the same band was overlapping the band at 600 nm, thus confirming that the
purification allowed to remove the excess of metal present in solution. However, there was no illusion that only one equivalent of Rh(I) was bound to the protein and coordinated to the phosphite group and indeed the same purification performed on not modified papain also showed the presence of a band at 440 nm eluting with the protein.

Spectroscopic studies conducted on cationic Rh(I)-diphosphine complexes in methanol reported UV-Vis absorptions between 385 and 442 nm of rather low intensity ($\varepsilon = 1.6-5.0 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$)\(^{177}\) compared to the intensity of the nearby band of papain at 280 nm ($\varepsilon = 57.5 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$). The profile of the elution confirmed the large difference in intensity, as also observed in preliminary UV studies.

5.21.3 ESI-MS analysis of Rh(I)-PapPhos complex 5.88

Once an improved purification method was established, ESI-MS was again used to identify what kind of complex had been obtained and, if possible, how many equivalents of Rh(I) had been incorporated in PapPhos and papain itself. The use of the MPLC system yielded the conjugates in good purity.

During the complexation eight equivalents of Rh(COD)$_2$BF$_4$ were added from a stock solution in dioxane to a solution in pure water of both freshly prepared PapPhos and papain. The solution was allowed to stir for one hour after which time it was purified with pure water using the MPLC system previously described. The solutions of the purified conjugates were stored overnight at 4 °C. The ESI-MS spectrum obtained for 5.88 is shown in Figure 5.36.

![Figure 5.36 ESI-MS analysis of Rh(I)-PapPhos complex 5.88](image)

Very excitingly, mass spectral analysis of 5.88 showed the disappearance of the peak at 24,207 Da (5.77) and the appearance of a clear and distinct peak at
24,434 Da (Table 5.18) corresponding to the addition of Rh(COD) (211 Da) and possibly a molecule of water. This confirmed the presence of a single rhodium which was assumed to be solely bound to the phosphite group, this being the best ligand for the metal. An additional peak (23634 Da) appears in the ESI-MS, which could be only attributed to an adduct between papain in its reduced state and one unit of Rh(COD). However, this is probably an artifact, as in all the experiments the reduced papain was reacted completely with the cofactor or was oxidized.

Table 5.18 Results of the ESI-MS analysis of conjugate 5.88

<table>
<thead>
<tr>
<th>entry</th>
<th>native papain</th>
<th>5.77</th>
<th>5.77 complexed with Rh(COD)$_2$BF$_4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>expected (Da)</td>
<td>23,422</td>
<td>24,207</td>
<td>-</td>
</tr>
<tr>
<td>found (Da)</td>
<td>23,455</td>
<td>24,207</td>
<td>24,434</td>
</tr>
</tbody>
</table>

The same analysis was performed on native papain and a complex and unclear spectrum was obtained, which is shown in Figure 5.37. None of the peaks could be identified. This does not mean that no adduct between Rh(I) and papain was present, as such complex formation was evident from the UV-Vis absorption at 440 nm detected during the purification. Instead, this result showed that if any adduct was present it was quite labile, as it could not survive the otherwise mild conditions of the analysis.

Figure 5.37 ESI-MS analysis of papain after complexation with Rh(COD)$_2$BF$_4$

The results of the ESI-MS analysis also proved that a good level of purity had been achieved after purification of the product of the reaction with Rh(COD)$_2$BF$_4$. The impact of this improved preparation on the activity of complex 5.88 was tested by performing a new set of hydrogenation reactions.
5.22 Hydrogenation of methyl 2-(acetamido)acrylate (5.81) after the new purification procedure of complex 5.88

All the hydrogenation reactions were performed after purification of the complex 5.88 using the MPLC system. Generally, the complex was recovered after purification as a 1.5 mL solution. The substrate 5.81 was added as a stock solution in the same solvent (buffer or H2O). The experimental results, which also provided new information on how to improve the procedure, are depicted in Table 5.19.

At first it was decided to perform both complexation and hydrogenation reaction in phosphate buffer to see if this would have improved the stability of the protein and would have had an influence on the results. An excess of Rh(COD)2BF4 was used (15 eq., added as a solid) and the solution was stirred for one hour before purification. The same procedure was adopted also with native papain used as a reference for undesired catalysis caused by random and not specific binding of rhodium to the protein structure.

Table 5.19 Rh-catalyzed hydrogenation of methyl 2-(acetamido)acrylate 5.81 using PapPhos 5.77 as chiral ligand

<table>
<thead>
<tr>
<th>entry</th>
<th>ligand</th>
<th>Rh(I) eq. (state)</th>
<th>complexation solvent</th>
<th>hydrogenation solvent</th>
<th>conv. (%)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.77</td>
<td>15 (solid)</td>
<td>buffer</td>
<td>buffer</td>
<td>79</td>
</tr>
<tr>
<td>2</td>
<td>papain</td>
<td>15 (solid)</td>
<td>buffer</td>
<td>buffer</td>
<td>89</td>
</tr>
<tr>
<td>3</td>
<td>5.77</td>
<td>4 (buffer)</td>
<td>buffer</td>
<td>buffer</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>papain</td>
<td>4 (buffer)</td>
<td>buffer</td>
<td>buffer</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>5.77</td>
<td>8 (1,4-dioxane)</td>
<td>H2O</td>
<td>H2O</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>papain</td>
<td>8 (1,4-dioxane)</td>
<td>H2O</td>
<td>H2O</td>
<td>3</td>
</tr>
</tbody>
</table>

*aReactions performed in 2 mL of H2O or phosphate buffer (25 mM, pH 7) for 16 h, using a substrate to protein ratio of 400:1. *bConversions determined by 1H NMR and GC. Enantiomeric access determined by chiral GC.

Unfortunately, conversion was detected in both cases (entries 1 and 2). A reason for the undesired activity was attributed to an overlap between the protein eluted and excess metal precursor during the purification step as shown in Figure 5.35. Therefore, less rhodium was used (entry 3 and 4) and for convenience it was
added to the solution containing \(5.77\) or papain from a stock solution in phosphate buffer. In this case, no excess of metal precursor eluting after both proteins was visible at 440 nm which meant that it was all bound to \(5.77\) or papain, however, no activity was detected with both systems in the hydrogenation of \(5.81\). The purification in this case proceeded efficiently and the lack of activity was attributed to a possible insufficient amount of metal precursor. Moreover, the stock solution of Rh(I) in phosphate buffer was slightly turbid.

Due to the good results obtained during ESI-MS analysis (page 239), the same conditions for the preparation of the complex \(5.88\) were applied, using eight equivalents of rhodium from a stock solution in 1,4-dioxane and performing the complexation and the hydrogenation of \(5.81\) in pure \(\text{H}_2\text{O}\) (entries 5 and 6). The combination of \(\text{H}_2\text{O}\) and 1,4-dioxane (10\%) during the complexation step afforded a clear yellowish solution. This amount of precursor allowed a good purification between the protein and the excess of Rh(I). However, no activity was detected using both \(5.77\) and papain as ligands. It was in this case assumed that the purification in the absence of buffer might have compromised the stability of the protein.

As a compromise, it was decided to perform the complexation in \(\text{H}_2\text{O}\) and exchange it to phosphate buffer during the purification of the conjugate \(5.88\) and the results are shown in Table 5.20.

**Table 5.20** Rh-catalyzed hydrogenation of methyl 2-(acetamido)acrylate \(5.81\) using PapPhos \(5.77\) as chiral ligand

<table>
<thead>
<tr>
<th>entry</th>
<th>ligand</th>
<th>conv. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(5.77)</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>papain</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>(5.77)</td>
<td>97</td>
</tr>
<tr>
<td>4</td>
<td>papain</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>(5.77^c)</td>
<td>100, 22% ee ((R))</td>
</tr>
<tr>
<td>6</td>
<td>(5.77^c)</td>
<td>100</td>
</tr>
</tbody>
</table>

\(^a\)Reactions performed in 2 mL of phosphate buffer (25 mM, pH 7) for 16 h, using a substrate to protein ratio of 400:1 and a Rh(COD)\(_2\)BF\(_4\) to protein ratio of 8:1, unless otherwise stated.
\(^b\)Conversions determined by \(^1\)H NMR and GC. Enantiomeric access analyzed by chiral GC.
\(^c\)A substrate to protein ratio of 800:1 was used.
Merging Homogeneous Catalysis with Biocatalysis: Papain as Hydrogenation Catalyst

It was pleasing to see that under these conditions full conversion in the hydrogenation of 5.81 was obtained by using 5.88 as catalyst (entry 1 and 3). The reaction showed to be reproducible and negligible activity was observed using papain as control (entries 2 and 4). This result confirmed that the phosphite-bound rhodium was responsible for the observed catalytic activity. Moreover, full conversion under 12 bar H₂ pressure was also obtained when the substrate to catalyst ratio was increased to 800:1 (entry 5 and 6). On one single occasion 5.82 was obtained with 22% enantioselectivity (entry 5), although this result could not be reproduced (entry 6).

By repeating the reaction a number of times, a progressive decrease of activity from 97% (entry 3) to eventually 17% conversion became evident. Eventually, it was discovered that this was related to the continuous use of the same prepacked Sephadex™ G-25 (HiTrap™) column for the purification of the Rh-protein conjugates. This was confirmed by a regain of activity observed when the other desalting column available was used (polyacrylamide Bio-Gel® P-6DG gel). The reason for the degradation of the column over time could not be clearly identified.

5.23 Conclusions and outlook

In conclusion, it has been shown that it is possible to convert a hydrolytic enzyme into a unique fully functional hydrogenation catalyst by attaching a single phosphorus ligand to its active site and treating it with a rhodium precursor. The hydrogenation reaction performed using 5.88 yielded the desired alanine derivative 5.82 with 100% selectivity. The protocol achieved seemed to be reliable and effective, as no activity was detected when using native papain treated with Rh(I) precursor and purified in the same way. The lack of induction of enantioselectivity points to a catalytic system in which the metal site could be too flexible or too far removed from the chiral environment of the enzyme. In order to understand what the new active site might look like some molecular modeling was attempted (Figure 5.38).

Figure 5.38 Manual docking of 5.47c into the active site of papain
Although programs and algorithms for protein molecular modeling for the minimization of modified structures are available, they are not compatible with the insertion of non-proteogenic structures or organometallic complexes. Therefore, it was only possible to obtain a manual docking of the cofactor 5.47c anchored to Cys-25 in the active site of papain.

One of the reasons for choosing papain as host for the organometallic catalyst was that its cavity was assumed to be spacious enough to accommodate ligand, metal and substrate. It was also hoped that additional binding sites for Rh(I), if necessary, could be found between the residues in the enzyme pocket, as for example His-159 positioned opposite to Cys-25. Although only indicative, Figure 5.38 showed that the cavity might be too large to guarantee enough secondary interactions necessary to ensure a more rigidly bound metal complex. In all cases multiple conformations of the complex could be present and the phosphite moiety would be free enough to flip in the cavity.

The cavity of papain could have facilitated the induction of chirality in different ways: (1) by providing an extra coordination for the unsaturated Rh-monophosphite complex; (2) by forcing the structurally fluxional phosphite backbone in a preferred conformation; (3) by favoring a specific complexation of the substrate to the catalyst due to the bulk of the protein itself. None of these interactions between papain and the catalyst or the substrate could be predicted beforehand. Therefore, although disappointing, it is not completely surprising that no induction of enantioselectivity was observed.

The important target achieved was the establishment of a rational and reliable protocol for the attachment of the ligand and the complexation of the metal precursor to the hybrid protein. At this point, the challenge would be to be able to randomly or specifically modify the protein backbone in search for the necessary secondary interactions to guarantee stereocontrol. Of course, this could also be achieved by screening completely different protein structures as scaffold. In this respect, a more stable and conformationally defined complex could also be obtained by double anchoring of the ligand to the protein structure. An interesting approach was recently reported by Lu and coworkers, who showed the beneficial effect of the two points attachment of a salen catalyst into Apo-Mb on the enantioselectivity of the sulfoxide product (Scheme 5.12).

The establishment of a covalent approach allows a wide range of possibilities in terms of choice of protein structure and cofactor design. However, it is at this point not surprising anymore that more examples have been reported using a supramolecular approach (page 174). From a practical point of view, a non-covalent approach requires less protein manipulation and purification, which, besides being time consuming, require extensive preparation and purification protocols and might hamper the stability of the protein itself.

Exciting progresses could be obtained in the field of artificial metalloproteins by modification or novel design of a metal-binding pocket with the right geometry...
between the different residues in order to accommodate different metals, as an expansion of the work of Sheldon and coworkers (Scheme 5.14). An interesting but not exploited approach is also the introduction of unnatural \( \alpha \)-amino acid derivatives in the backbone of a protein as suggested by Imperiali and Roy (Scheme 5.14). The possibility of engineering a gene that would code for the desired \( \alpha \)-amino acid which already bears a metal-binding side chain instead of using solid state synthesis would make this approach even more exciting.

Looking at the preparation of semisynthetic metalloproteins as an expansion of the combinatorial approach for the identification of new efficient catalysts with novel catalytic properties, we should ask ourselves in how much these fascinating but still tailor-design approaches can cope with the fast tempo involved in high throughput experimentation, in particular of low molecular weight catalysts. However, the growing enthusiasm and commitment shown by the scientific community in embracing a truly interdisciplinary mentality might provide for this new field exciting and fast progress in the near future.

5.24 Experimental section

General remarks
For general remarks, see Chapter 2. For synthetic purposes, solvents were reagent grade, dried and distilled before use following standard procedures. Dioxane was distilled over sodium under nitrogen or purified over neutral alumina and degassed; buffer solutions and water were degassed for 2-3 h prior to use. Papain was obtained from Sigma-Aldrich as a buffered aqueous suspension in 0.05 M sodium acetate (1.1 mM). \( N \)-\( Z \)-glycine \( p \)-nitrophenyl ester was purchased from Bachem. DTT (dithiothreitol) was obtained from Roche. Mass spectra were recorded on an AEI-MS-902 mass spectrometer. UV-Vis measurements were performed on a Hewlett-Packard HP 8453 FT spectrophotometer. Enantiomeric excesses were determined by capillary chiral GC analysis on a HP 6890 gas chromatograph equipped with a flame ionization detector.

The kinetics of papain inhibition with \( 5.47c \) and the digestion of \( 5.77 \) were performed by Jianfeng Jin (department of biochemistry). In-gel digestion was performed as previously described in the literature. All the mass analysis (ESI-MS and MS/MS) were performed in collaboration with C. M. Jeronimus-Stratingh and/or H. Permentier (faculty centre for mass spectrometry). Manual docking of \( 5.47c \) in the active site of papain was performed by Marco W. Fraaije.

3-Bromomethyl-phenol (5.58b)

**Method A.** Pyridine (2.6 mL, 32.4 mmol) in dry THF (5 mL) was added to a solution of \( \text{PBr}_3 \) (10.2 g, 37.7 mmol) also in THF (10 mL) at -5 °C. Finally, a solution of alcohol \( 5.57b \) (13.9 g, 112 mmol) in dry THF (170 mL) was added dropwise. The
reaction mixture became turbid. It was allowed to reach room temperature and
stirred overnight. The mixture was then filtered over celite that was washed with
dry THF. The solvent was removed at room temperature under reduced pressure
and a dark red oil was obtained. Toluene (100 mL) was added and the solution
obtained was kept at -20 °C for 2 h and filtered over celite. The light brown solution
obtained was stored at 4 °C. Eventually, the solvent was removed and purification
by flash column chromatography on silica gel (toluene) afforded product 5.58b
(50%). **Method B.** A solution of PBr₃ (4.4 g, 16.1 mmol) in CHCl₃ (18 mL) was
added to a suspension of alcohol 5.57b (4.0 g, 32.2 mmol) in CHCl₃ (18 mL) at 0
°C. At the end of the addition a clear solution was obtained which was allowed to
reach room temperature and was stirred for additional 2 h. The reaction was
stopped by addition of ice (36 g) and separation of the organic layer. The aqueous
layer was extracted with CHCl₃ (3 x 40 mL). The combined organic layers were
washed with brine, dried over Na₂SO₄ and the solvent removed under reduced
pressure. The product 5.58b was obtained pure as an oil (98%) and stored at 4 °C,
where it solidified. After a couple of months, 5.58b was purified by flash column
chromatography on silica gel (toluene) and recovered with 90% yield.

**Method A.** Phenol 5.60a (25.0 g, 152 mmol) was dissolved in
dry MeOH (64 mL) under a nitrogen atmosphere and CuCl₂ (0.1 g,
0.7 mmol) was added to this solution. TMEDA (250 μL, 1.6 mmol)
was added dropwise and the solution turned from yellow to dark
green. Vacuum was applied and nitrogen was replaced by pure
oxygen. The reaction mixture was stirred at room temperature and
it was stopped after 5 d. The work up consisted of addition of water
and extraction with CHCl₃. The organic layer was washed with
brine, dried over Na₂SO₄ and the solvent removed under reduced pressure.

Purification by flash column chromatography on silica gel (toluene) afforded the
desired product 5.62a as an off-white solid (20%). **Method B.** Phenol 5.60a (5.0
g, 30.4 mmol) and FeCl₃ 6H₂O (2.0 g, 7.6 mmol) were dissolved in CCl₄ (76 mL).
After a solution of t-BuOOH (3.0 mL, 5.6 M in n-decane) was added the reaction
mixture was heated at 80 °C for 3 h. The solution was washed with 10% aqueous HCl, the organic layer was dried over Na₂SO₄ and the solvent removed under reduced pressure. Purification by flash column chromatography on silica gel (toluene) afforded the desired product 5.62a (69%).

**3,3’-Di-tert-butyl-5,5’-dimethyl-biphenyl-2,2’-diol (5.62a)**

Method A. Phenol 5.60a (25.0 g, 152 mmol) was dissolved in
dry MeOH (64 mL) under a nitrogen atmosphere and CuCl₂ (0.1 g,
0.7 mmol) was added to this solution. TMEDA (250 μL, 1.6 mmol)
was added dropwise and the solution turned from yellow to dark
green. Vacuum was applied and nitrogen was replaced by pure
oxygen. The reaction mixture was stirred at room temperature and
it was stopped after 5 d. The work up consisted of addition of water
and extraction with CHCl₃. The organic layer was washed with
brine, dried over Na₂SO₄ and the solvent removed under reduced pressure.

Purification by flash column chromatography on silica gel (toluene) afforded the
desired product 5.62a as an off-white solid (20%). Method B. Phenol 5.60a (5.0
g, 30.4 mmol) and FeCl₃ 6H₂O (2.0 g, 7.6 mmol) were dissolved in CCl₄ (76 mL).
After a solution of t-BuOOH (3.0 mL, 5.6 M in n-decane) was added the reaction
mixture was heated at 80 °C for 3 h. The solution was washed with 10% aqueous HCl, the organic layer was dried over Na₂SO₄ and the solvent removed under reduced pressure. Purification by flash column chromatography on silica gel (toluene) afforded the desired product 5.62a (69%).
Merging Homogeneous Catalysis with Biocatalysis: Papain as Hydrogenation Catalyst

2C), 29.6 (q, 6C), 20.8 (q, 2C). MS, m/z (%): 326 (M⁺, 97.6%); HRMS for C₂₂H₃₀O₂, calcd: 326.225, found: 326.224.

3,3’-Di-tert-butyl-5,5’-dimethoxy-biphenyl-2,2’-diol (5.62b) ¹⁷⁹

A solution of phenol 5.60b (4.3 g, 24 mmol) in acetone (50 mL) was added to a solution of K₃[Fe(CN)₆] (8.2 g, 25 mmol) in H₂O (80 mL) to yield a yellow/orange mixture. Subsequently, an aqueous solution of NaOH (0.95 g, 23.8 mmol, 10 mL H₂O) was added and the solution turned blue/green. The reaction mixture was stirred for 2 h at room temperature, after which time it was extracted with CHCl₃ (200 mL), the organic layer was dried over Na₂SO₄ and the solvent removed under reduced pressure. The desired product 5.62b was obtained as a pinkish solid after recrystallization from MeOH (60%).

M.p. 224.7-226.0 °C. ¹H-NMR (300 MHz, CDCl₃) δ 6.96 (d, J = 3.0 Hz, 2H), 6.63 (d, J = 3.0 Hz, 2H), 5.02 (s, 2H), 3.78 (s, 6H), 1.43 (s, 18H). ¹³C-NMR (50 MHz, CDCl₃) δ 153.2 (s, 2C), 145.9 (s, 2C), 138.9 (s, 2C), 123.1 (s, 2C), 115.3 (d, 2C), 111.7 (d, 2C), 55.7 (q, 2C), 35.2 (s, 2C), 29.5 (q, 6C). MS, m/z (%): 358 (M⁺, 100%); HRMS for C₂₂H₃₀O₄, calcd: 358.214, found: 358.212.

6-(3-Bromomethyl-phenoxy)-4,8-di-tert-butyl-2,10-dimethyl-5,7-dioxa-6-phospha-dibenzo[a,c]cycloheptene (5.47a)

PCl₃ (250 μL, 2.9 mmol) was added dropwise to a solution of NEt₃ (2 mL, 12.4 mmol) in dry toluene (5 mL) at 0 °C. Bisphenol 5.62a (0.92 g, 2.8 mmol) was dried by repeated addition and removal under reduced pressure of toluene (5 mL, 3 times) and then dissolved in toluene (10 mL) and added dropwise to the reaction mixture at 0 °C. The yellow solution was allowed to reach room temperature and was stirred for 3 h, until the starting material PCl₃ was not visible by ³¹P-NMR (202 ppm). The reaction mixture was cooled to 0 °C and NEt₃ (0.8 mL, 5.8 mmol) was added. Benzyl bromide 5.58b (0.53 g, 2.8 mmol) was dried by repeated addition and removal under reduced pressure of toluene (5 mL, 3 times) and then dissolved in toluene (10 mL) and added dropwise to the reaction mixture. Stirring at room temperature was continued for 3 h, after addition of Et₂O (5 mL) the salts were filtered and the solvent was removed under reduced pressure. The desired product 5.47a (50%) was obtained after purification by flash column chromatography on silica gel as an oil (n-hexane and 0.5% NEt₃) and it was stored at -12 °C.

¹H-NMR (400 MHz, CDCl₃) δ 7.29-7.22 (m, 3H), 7.13 (d, J = 7.6 Hz, 1H), 7.09 (s, 1H), 7.05-7.01 (m, 3H), 4.41 (s, 2H), 2.39 (s, 6H), 1.49 (s, 18 H). ¹³C-NMR (100 MHz, CDCl₃) δ 152.3 (s), 145.3 (s), 140.8 (s, 2C), 139.4 (s, 2C), 133.8 (s, 2C), 132.9 (s, 2C), 130.0 (d, 2C), 129.9 (d), 128.0 (d, 2C), 124.4 (d), 121.1 (d), 120.4 (d), 35.1 (t), 32.7 (s, 2C), 31.2 (q, 3C), 31.1 (q, 3C), 21.1 (q, 2C). ³¹P-NMR (162 MHz, CDCl₃) δ 138.4. MS, m/z (%): 540 (¹⁷⁹Br M⁺, 61.9%); HRMS for C₂₉H₂₆O₃P²⁷⁹Br, calcd: 540.143, found: 540.142.
6-(3-Bromomethyl-phenoxy)-4,8-di-tert-butyl-2,10-dimethoxy-5,7-dioxa-6-phospha-dibenzo[a,c]cycloheptene (5.47b)

PCl₃ (250 μL, 2.9 mmol) was added dropwise to a solution of NEt₃ (1.6 mL, 11.4 mmol) in dry toluene (5 mL) at 0 °C. Bisphenol 5.62b (1.0 g, 2.8 mmol) was dried by repeated addition and removal under reduced pressure of toluene (5 mL, 3 times) and then dissolved in toluene (10 mL) and added dropwise to the reaction mixture at 0 °C. The dense yellow reaction mixture was allowed to reach room temperature and it was stirred for 3 h. At this point Et₂O (5 mL) was added, the salts were quickly filtered under a nitrogen atmosphere and the solution obtained was concentrated at room temperature under reduced pressure. The vacuum was then replaced by nitrogen atmosphere and the crude mixture was dissolved in toluene (5 mL). The solution was cooled to 0 °C and NEt₃ (1.6 mL, 11.4 mmol) was added. Benzy bromide 5.58b (0.52 g, 2.8 mmol) was dried by repeated addition and removal under reduced pressure of toluene (5 mL, 3 times), dissolved in toluene (10 mL) and added dropwise to the reaction mixture. Stirring at room temperature was continued for 3 h, after addition of Et₂O (5 mL) the salts were filtered and the solvent was removed under reduced pressure. The desired product 5.47b (50%) was purified by flash column chromatography on silica gel as a white foam (heptanes / ethyl acetate, 95:5). It was stored at -12 °C.

M.p. 41.5-43.8 °C. ¹H-NMR (400 MHz, CDCl₃) δ 7.29-7.24 (m, 1H), 7.12 (d, J = 8.4 Hz, 1H), 7.09 (s, 1H), 7.04-7.00 (m, 1H), 7.02 (d, J = 2.8 Hz, 2H), 6.75 (d, J = 2.8 Hz, 2H), 4.41 (s, 2H), 3.84 (s, 6H), 1.48 (s, 18H). ¹³C-NMR (100 MHz, CDCl₃) δ 155.8 (s, 2C), 152.3 (s), 142.6 (s, 2C), 141.3 (s), 139.4 (s, 2C), 133.7 (s, 2C), 130.0 (d), 124.4 (d), 121.0 (d), 120.3 (d), 114.4 (d, 2C), 112.9 (d, 2C), 55.6 (q, 2C), 35.4 (t), 32.6 (s, 2C), 31.0 (q, 6C). ³¹P-NMR (162 MHz, CDCl₃) δ 138.4. MS, m/z (%): 572 (⁷⁹Br M⁺, 79.4%); HRMS for C₂₉H₃₄O₅P₇⁷Br, calcd: 572.133, found: 572.133.

1-Chloro-2-[2-(2-methoxy-ethoxy)-ethoxy]-ethane (5.74)

Pyridine (6 mL, 74 mmol) was added to a solution of polyether alcohol 5.73 (10 mL, 62 mmol) in toluene (60 mL). The solution was warmed to 76 °C and SOCl₂ (5.4 mL, 74 mmol) was added dropwise and stirring was continued overnight. The reaction mixture was allowed to reach room temperature and it was poured in H₂O (80 mL). The organic layer was separated and the aqueous layer was extracted with toluene. The combined organic layers were washed with brine, dried over Na₂SO₄ and the solvent was removed under reduced pressure. The product 5.74 was used as such in the following step without further purification.

¹H-NMR (300 MHz, CDCl₃) δ 3.78-3.70 (m, 2H), 3.70-3.57 (m, 8H), 3.57-3.50 (m, 2H), 3.37 (s, 3H). ¹³C-NMR (50 MHz, CDCl₃) δ 71.7 (t), 71.3 (t), 70.6 (t), 70.5 (t, 2C), 59.0 (q), 42.6 (t). MS for C₇H₁₅ClO, m/z (%): 137 (M⁺, 4.4%), 91 (100%).
Merging Homogeneous Catalysis with Biocatalysis: Papain as Hydrogenation Catalyst

2-tert-Butyl-4-(2-[2-(2-methoxy-ethoxy)-ethoxy]-ethoxy)-phenol (5.60c)

Hydroquinone 5.75 (4.70 g, 0.028 mol) was added in one portion to a mixture of Cs₂CO₃ (7.7 g, 0.024 mol) in CH₃CN (80 mL) and the solution started to color from yellow to dark orange. Polyether chloride 5.74 (4.3 g, 0.024 mol) was then added dropwise. The resulting mixture was stirred under reflux overnight. After cooling the salts were filtered, the solvent was completely removed under reduced pressure and a mixture of EtOAc/H₂O (200 mL) was added. After separation of the two phases, the aqueous layer was extracted with EtOAc, the combined organic layers were washed with brine, dried over Na₂SO₄ and the solvent removed under reduced pressure. The crude mixture was purified by flash column chromatography on silica gel (ethyl acetate / heptanes, 2:8). The desired product 5.60c was obtained in 80% yield as a brownish oil.

1H-NMR (300 MHz, CDCl₃) δ 6.86 (d, J = 3.0 Hz, 1H), 6.60 (d, J = 8.4 Hz, 1H), 6.53 (dd, J = 3.0, 8.4 Hz, 1H), 5.05 (br s, 1H), 4.05-4.98 (m, 2H), 3.84-3.78 (m, 2H), 3.76-3.64 (m, 6H), 3.58-3.53 (m, 2H), 3.37 (s, 3H), 1.37 (s, 9H). 13C-NMR (50 MHz, CDCl₃) δ 152.4 (s), 148.6 (s), 137.4 (s), 116.7 (d), 114.9 (d), 111.3 (d), 71.9 (t), 70.7 (t), 70.6 (t), 70.5 (t), 69.9 (t), 67.8 (t), 59.0 (q), 34.6 (s), 29.4 (q, 3C).

MS, m/z (%): 312 (M⁺, 92.4%); HRMS for C₁₇H₂₈O₅, calcd: 312.194, found: 312.198.

3,3’-Di-tert-butyl-5,5’-bis-{2-[2-(2-methoxy-ethoxy)-ethoxy]-ethoxy}-biphenyl-2,2’-diol (5.62c)

Phenol 5.60c (2.0 g, 6.4 mmol) was dissolved in acetone (13.5 mL) and the solution obtained was added to a solution containing K₃[Fe(CN)₆] (2.1 g, 6.4 mmol) in H₂O (20.5 mL). As last, an aqueous solution of NaOH (0.26 g, 6.4 mmol, 2.7 mL H₂O) was also added. The heterogeneous reaction mixture was stirred at room temperature for 3 h, after which time CHCl₃ (20 mL) was added, the organic layer was separated and the aqueous layer was again extracted with CHCl₃ (2 x 25 mL). The combined organic layers were dried over Na₂SO₄ and the solvent removed under reduced pressure. Purification by flash column chromatography on silica gel (ethyl acetate / hexane, 1:1) afforded the desired product 5.62c (60%) as a thick yellowish oil.

M.p. 76.7-77.2 °C. 1H-NMR (300 MHz, CDCl₃) δ 6.98 (d, J = 2.4 Hz, 2H), 6.62 (d, J = 3.0 Hz, 2H), 5.09 (s, 2H), 4.11-4.14 (m, 4H), 3.87-3.81 (m, 4H), 3.76-3.61 (m, 12H), 3.56-3.51 (m, 4H), 3.36 (s, 6H), 1.42 (s, 18H). 13C-NMR (50 MHz, CDCl₃) δ 152.4 (s, 2C), 146.0 (s, 2C), 138.9 (s, 2C), 123.1 (s, 2C), 116.0 (d, 2C), 112.7 (d, 2C), 72.0 (t, 2C), 70.8 (t, 2C), 70.7 (t, 2C), 70.6 (t, 2C), 69.9 (t, 2C), 68.0 (t, 2C), 59.0 (q, 2C), 35.2 (s, 2C), 29.5 (q, 6C). MS, m/z (%): 622 (M⁺, 100%); HRMS for C₃₄H₅₄O₁₀, calcd: 622.372, found: 622.372.
2-Bromo-1-(3-hydroxy-phenyl)-ethanone (5.72)

A warm solution of 3-hydroxy-acetophenone (5.71) (8.0 g, 59 mmol) in CHCl₃ (50 mL) was added dropwise to a suspension of CuBr₂ (22.3 g, 100 mmol) in EtOAc (50 mL) at reflux. After refluxing for 2.5 h, the color of the reaction mixture changed from dark green to yellow and no evolution of HBr was visible anymore. The reaction mixture was allowed to reach room temperature and H₂O (100 mL) was added. The organic layer was separated, washed with brine, dried over Na₂SO₄ and the solvent was removed under reduced pressure. The desired product 5.72 was obtained as an oil solidifying on standing (85%) after purification by flash column chromatography on silica gel (toluene / ethyl acetate, 20:1).

M.p. 74.1-75.0 °C. ¹H-NMR (400 MHz, CDCl₃) δ 7.55-7.51 (m, 2H), 7.38 (t, J = 8.0 Hz, 1H), 7.13 (dd, J = 1.4, 8.0 Hz, 1H), 5.76 (br, 1H), 4.46 (s, 2H). ¹³C-NMR (100 MHz, CDCl₃) δ 192.1 (s), 156.4 (s), 135.1 (s), 130.2 (d), 121.7 (d), 121.4 (d), 115.3 (d), 31.2 (t). MS, m/z (%): 214 (⁷⁹Br M⁺, 14.1%); HRMS for C₈H₇O₂⁷⁹Br, calcd: 213.963, found: 213.964.

2-Bromo-1-[3-(4,8-di-tert-butyl-2,10-bis-{2-[2-(methoxy-ethoxy)-ethoxy]-5,7-dioxa-6-phopha-dibenzo[a,c]cyclohepten-6-yl}oxy]-phenyl]-ethanone (5.47c)

PCl₃ (160 µL, 1.1 eq) was added dropwise at 0 °C to a solution of dry toluene (5 mL) containing NEt₃ (0.9 mL, 4 eq). In the mean time, bisphenol 5.62c (1.0 g, 1.6 mmol, 1 eq) was dried by repeated addition and removal under reduced pressure of toluene (5 mL, 3 times) and then dissolved in toluene (10 mL) and added dropwise to the reaction mixture at 0 °C. The reaction mixture was allowed to reach room temperature and stirred for 3 h. Subsequently, dry Et₂O (5 mL) was added, the salts quickly filtered and the resulting solution was then concentrated under reduced pressure. Toluene (5 mL) was added to the syrup obtained, followed at 0 °C by NEt₃ (0.9 mL, 4 eq). A solution of 3-hydroxy-phenacyl bromide 5.72 (343.6 mg, 1.6 mmol, 1 eq) in toluene (10 mL) was then added dropwise, the reaction mixture was allowed to reach room temperature and stirring was continued for a further 3 h. After this, Et₂O (5 mL) was added and the salts quickly filtered over a plug of silica. The resulting solution was concentrated under reduced pressure and the crude mixture was purified by flash column chromatography on silica gel (ethyl acetate / heptanes, 3:2) yielding the desired product 5.47c in 72% yield as a colorless syrup, usually stored at -12 °C.

¹H-NMR (400 MHz, CDCl₃) δ 7.71 (d, J = 8.0 Hz, 1H), 7.58 (s, 1H), 7.41 (t, J = 8.0 Hz, 1H), 7.28 (d, J = 8.0 Hz, 1H), 7.04 (d, J = 3.2 Hz, 2H), 6.72 (d, J = 3.2 Hz, 2H), 4.35 (s, 2H), 4.17-4.11 (m, 4H), 3.89-3.85 (m, 4H), 3.79-3.72 (m, 4H), 3.72-3.63 (m, 8H), 3.58-3.52 (m, 4H), 3.37 (s, 6H), 1.45 (s, 18H). ¹³C-NMR (100 MHz, CDCl₃)
δ 190.4 (s), 155.2 (s, 2C), 152.5 (s), 142.6 (s, 2C), 141.2 (s), 135.3 (s, 2C), 133.5 (s, 2C), 130.1 (d), 126.0 (d), 124.3 (d), 120.6 (d), 115.3 (d, 2C), 113.6 (d, 2C), 71.9 (t, 2C), 70.8 (t, 2C), 70.64 (t, 2C), 70.56 (t, 2C), 69.7 (t, 2C), 67.8 (t, 2C), 59.0 (q, 2C), 35.4 (t), 31.0 (q, 3C), 30.9 (q, 3C), 30.8 (s, 2C). 31P-NMR (162 MHz, CDCl3) δ 137.1. MS for C₄₂H₅₈BrO₁₂P, m/z (%): 864 (M+, 0.5%), 786 (100%); HRMS (EI+), for C₄₂H₅₉O₁₂P calculated on 786 (M+-Br): 786.375, found: 786.374.

Alkylation of papain (5.77)
An aliquot (100 μL, 1.1 mM) of papain suspension was dissolved in phosphate buffer (9.8 ml, 100 mM, pH 7) containing DTT (100 μL, 100 mM) as reducing agent. The incubation was performed under nitrogen atmosphere at 25 °C for 20 min, while gently stirring. Subsequently, a solution of 3 mL in 1,4-dioxane of 5.47c (2 mM) was added in portions (6 × 0.5 mL) over a period of 3 h to ensure a high degree of modification of the protein, while gently stirring at 25 °C. The mixture was then filtered (Minisart SR P 15, PTFE-membrane, 0.45 μm) and concentrated (Amicon® Ultra-15, 10K cut off centrifugal filter device, Millipore) with distilled water (3 × 15 mL) in order to exchange solvent and to remove DTT and unreacted phosphate ligand. The resulting concentrated solution (150 μL) was diluted with distilled water to 1 mL.

Activity test
The residual activity of the enzyme was conveniently determined by monitoring the hydrolysis of Z-Gly-ONp (5.67) as test substrate. This test was performed at various moments during the modification reaction. A sample of the reaction mixture (50 μL) was first diluted in phosphate buffer (950 μL, 100 mM, pH 7). A sample of this diluted solution (90 μL) was added to a quartz cuvette containing phosphate buffer (890 μL, 100 mM, pH 7). Subsequently the substrate was added (20 μL, 2.5 mM, acetone) and its hydrolysis was followed by UV-Vis absorption spectroscopy (λ= 404 nm).

Hydroformylation of styrene (5.79)
The concentrated solution of 5.77 (150 μL, 0.73 mM), obtained after the alkylation step, was diluted with distilled water to 2 mL. The metal precursor Rh(acac)2(CO) was added and the resulting heterogeneous solution was stirred 30 min. before being filtered (Minisart SR P 15, PTFE-membrane, 0.45 μm). The desired amount of organic solvent was added, followed by styrene (250 μL, 2.17 mmol). The biphasic solution obtained was transferred to a glass vial which was placed in an autoclave. After purging with N₂ (3 × 5 bar) the system was warmed to the desired temperature and pressurized with syngas (H₂:CO, 1:1) and the reaction mixture was vigorously stirred for 16 h. The reaction was stopped by first cooling the autoclave to room temperature and then releasing the syngas pressure. The organic layer was separated and a sample (100 μL) was checked for conversion by ¹H-NMR.
2-Phenylpropanal (5.80a): $^1$H-NMR (300 MHz, CDCl$_3$) $\delta$ 9.70 (d, $J$ = 2.1 Hz, 1H), 7.43-7.12 (m, 5H), 3.64 (q, $J$ = 10.5 Hz, 1H), 1.45 (dt, $J$ = 10.5, 2.1 Hz, 3H).

3-Phenylpropanal (5.80b): $^1$H-NMR (300 MHz, CDCl$_3$) $\delta$ 9.83 (t, $J$ = 1.5 Hz, 1H), 7.35-7.10 (m, 5H), 2.96 (t, $J$ = 7.5 Hz, 2H), 2.77 (tt, $J$ = 7.5, 1.5 Hz, 2H).

**Enantiomeric excess determination.** A sample of the organic solution (300 $\mu$L) was added to EtOH (2 mL) followed by an excess of NaBH$_4$ (spatula tip) and the heterogeneous solution was stirred for 90 min. at room temperature. The reaction was quenched by adding H$_2$O (3 mL) and the mixture was extracted with EtOAc/n-hexane (1:1, 3 times). The combined organic layers were dried over Na$_2$SO$_4$ and the solvent removed. A sample of the crude mixture (100 $\mu$L) was added to a solution of n-hexane and isopropanol (90:10). A sample of the solution obtained (5-10 $\mu$L) was used for the determination of the enantiomeric excess by HPLC.

Conditions: OB-H column; heptanes/isopropanol, 975:25; 0.5 mL/min, $\lambda_{det}$ 220 nm.

Retention time: 2-phenyl-propan-1-ol (from 5.80a), 17.0/18.3 min.; 3-phenyl-propan-1-ol (from 5.80b), 20.4 min.

**Complexation of 5.77 with Rh(COD)$_2$BF$_4$ and purification of complex 5.88**

A solution of Rh(COD)$_2$BF$_4$ (8 mM, 8 eq.) in 1,4-dioxane (100 $\mu$L) was added to an aqueous solution (1 mL) containing modified papain (5.77) (0.1 mM). The resulting yellow solution was gently stirred for 1 h under nitrogen atmosphere at 25 °C. The complex was purified by size exclusion chromatography using desalting columns packed with Sephadex$^\text{TM}$ G-25 (HiTrap$^\text{TM}$) connected with an MPLC system (AKTA purifier), or with polyacrylamide Bio-Gel$^\text{TM}$ P-6DG gel (Econo-Pac$^\text{TM}$ 10DG, Biorad). The sample was eluted with phosphate buffer (pH 7, 25 mM). This step allowed to exchange of water with a buffered solution and to remove excess Rh(I) present free in solution.

**Hydrogenation of methyl 2-acetamidoacrylate (5.81)**

In a typical hydrogenation run, a glass vial was charged with degassed buffer solution (2 mL, phosphate buffer, pH 7, 25 mM) containing the artificial metalloenzyme complex 5.88 (50 $\mu$L) and methyl 2-acetamidoacrylate (5.81, 20 mM). The glass vial was placed in an autoclave and after purging with N$_2$ (3 × 5 bar) the system was pressurized with hydrogen (12 bar) and the reaction mixture was stirred at room temperature for 16 h. The reaction was stopped by release of the H$_2$ pressure. The resulting mixture was extracted with EtOAc (3 × 5 mL) and the combined organic layers were dried on Na$_2$SO$_4$. Conversion was determined by $^1$H-NMR on a sample of the organic solution. Enantiomeric excess was determined by capillary chiral GC, using the following conditions:

<table>
<thead>
<tr>
<th>Condition</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP Chirasil-L-Val column</td>
<td>25 (m x 0.25 mm) x 0.25 $\mu$m</td>
</tr>
<tr>
<td>Init. Temp.</td>
<td>110 °C, 12.5 min, 10 °C / min to 160 °C</td>
</tr>
<tr>
<td>Tdet/inlet</td>
<td>250 °C, split ratio 25:1, $t_r$ = 4.09 min, $t_5$ = 4.78 min, $t_{95}$ = 2.47 min</td>
</tr>
</tbody>
</table>
ESI-MS measurements
Electrospray mass spectrometry (ESI-MS) was performed on an API3000 mass spectrometer (Applied Biosystems/MDS-SCIEX, Toronto, Canada); a triple quadrupole mass spectrometer supplied with an atmospheric pressure ionization source and a TurboIonSpray interface. The spectra were scanned in the range between \( m/z \) 1000.0 and 2800.0. The samples were diluted with an aqueous solution of MeOH (85%) containing 0.01% HCOOH.

Kinetics of the inhibition of papain in the presence of cofactor 5.47c
Papain suspension (10 μL, 1.1 mM) and DTT (10 μL, 0.1 M) were added to a sodium phosphate buffer (0.98 mL, 20 mM, pH 6.2) containing NaCl (0.1 M) and EDTA (1 mM). Four solutions were prepared, containing an aliquot of the activation solution (10 μL, 11 μM) diluted with phosphate buffer (0.97 mL). A solution of cofactor 5.47c in 1,4-dioxane was prepared (0.2 mg/mL, 0.23 mM) and aliquots (5, 10, 15, 20 μL) were added to the solutions containing activated papain. At regular intervals (0, 15, 30 and 45 min.) substrate 5.67 was added (20 μL, 2.5 mM in acetone) and its hydrolysis followed by UV-Vis absorption spectroscopy (404 nm) to measure the residual activity of the enzyme.

5.25 References and notes

Chapter 5


(12) For an example from our group, see: (a) van der Deen, H.; Cuiper, A. D.; Hof, R. P.; van Oeveren, A.; Feringa, B. L.; Kellogg, R. M. J. Am. Chem. Soc. 1996, 118, 3801. For recent overviews, see: (b) Corma, A. Cat. Rev. 2004, 46, 369. (c) Dalby, P.; Lye, G. J.; Woodley, J. M. In Handbook of Chiral Chemicals; Ager, D. J. Ed.; CRC: Boca Raton, 2005.


(47) For the successful application of Cu(II) and DNA-intercalating based ligands in asymmetric Diels-Alder reactions, see: Roelfes, G.; Feringa, B. L. Angew. Chem. Int. Ed. 2005, 44, 3230.
(48) Roy, R. S.; Imperiali, B. Protein Eng. 1997, 10, 691.
Chapter 5

(55) A coenzyme, or cofactor, is an organic non-proteinogenic molecule that is a functional part of an enzyme such as vitamins and hemes.
(58) For enzyme nomenclature according to the catalyzed reaction, see: (a) http://www.chem.qmul.ac.uk/iubmb/enzyme. For a comprehensive information system, see:
(b) http://www.brenda.uni-koeln.de.
(60) Serine proteases and metallo-proteases are further divided into mammalian and bacterial. Many proteolytic enzymes have not been classified yet as their active site and mechanism of action are still under investigation.
(61) Relative molecular mass (Mr, pure number), or molar mass (g mol⁻¹), or molecular mass (Da), are the same number.
(64) Smith, J. J.; Conrad, D. W.; Cuneo, M. J.; Hellinga, H. W. Protein Science 2005, 14, 64.
(67) EC (Enzyme Commission) is a number that unequivocally identifies an enzyme.
(69) For an early overview, see: Lowe, G. Tetrahedron 1976, 32, 291.
(76) Lucas, E. C.; Williams, A. Biochem. 1969, 8, 5125.

256
(77) Instead, Polgár attributed the stabilization of the imidazolium to a charge-transfer interaction with Trp-177 situated spatially close to His-159 and Asn-175 and providing a less polar environment: Ref. 74.


(79) According to x-ray data: Ref. 80.


(91) Iodoacetamide is still one of the most used labels for cysteine residues in protein sequencing.

(92) TPCK: tosylphenylalanychloromethane.

(93) The main difference in inhibitory action toward the active site of serine and cysteine proteases is that chymotrypsin is alkylated at His-57 and papain at Cys-25.


(98) The reagent as much as the enzyme adduct might have a tendency to hydrolyze.

(99) Hydrogenation of some functionalized alkenes, can be achieved also by yeast mediated reduction. However, the process that is NADH-dependent is generally performed using whole cells with consequent lower productivity: see Ref. 8, section 2.2.4 and references therein.


Chapter 5

(103) The cone angle is defined as the apex angle of a cylindrical cone, centered 2.28 Å from the center of the P atom, which touches the outmost atoms of a CPK model: Tolman, C. A. Chem. Rev. 1977, 77, 313.


(123) Thymol: isopropyl cresol. It is normally used as antiseptic, local anesthetic, cooling agent and as preservative. In this case is probably used as antioxidant.

Merging Homogeneous Catalysis with Biocatalysis: Papain as Hydrogenation Catalyst

(125) Even after direct questioning, no information was provided by Sigma-Aldrich on this subject.


(134) Frequent exposure to the compound might result in sensitization.


(140) Eventually, an internet site was found were the obsolete PDB entries are archived: http://pdbobs.sdsc.edu.


(143) This would explain the presence of thymol in the buffered suspension in which papain is sold.


Chapter 5


(155) Marchetti and coworkers reported full conversion to 5.80a using HSA as non specific hosts for the rhodium precursor with substrate to protein ratio up to 500,000:1 using 60 °C and 70 bar of syngas and a substrate to Rh(I) ratio of 600:1. They stated that 30 equivalents of Rh(I) were complexed to the HSA. However, no control experiments were performed in the absence of protein. Ref. 52-52.


(157) Sinou and coworkers reported higher enantioselectivities performing the reaction at neutral pH (from 9% to 17% ee) avoiding possible racemization of the product: Ref. 154b.


(161) Whitesides and coworkers also proposed a few early examples of water soluble diphosphine ligands of which biotin derived 5.4a is the most remarkable. See: (a) Ref. 22. (b) Wilson, M. E.; Nuzzo, R. G.; Whitesides, G. M. J. *Am. Chem. Soc.* 1978, 100, 2269.


(167) Using only H2O, full conversion and 88% ee were obtained in the hydrogenation of 5.51a, instead of >99% ee in 1 hour using 10% SDS. Ref. 164b.


(169) Although, it is disputable if enantioselectivities <10% should be considered significant.
Merging Homogeneous Catalysis with Biocatalysis: Papain as Hydrogenation Catalyst

(176) MPLC: medium pressure liquid chromatography.
(178) The titre of the peroxide was not checked prior to use.
(181) Addition of a little amount of EtOAc helped to completely solubilize 5.71.
(183) Adapted from a literature procedure: Ref. 126.