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Directed evolution of enantioselective hybrid catalysts: a novel concept in asymmetric catalysis

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Dedicated to Karl Wieghardt on the occasion of his 65th birthday

Abstract—The concept of directed evolution of enantioselective hybrid catalysts was proposed in 2001/2002 and implemented experimentally for the first time in a proof-of-concept study in 2006. The idea is based on directed evolution, which comprises repeating cycles of random gene mutagenesis/expression/screening in a Darwinistic sense for the purpose of improving the catalytic profile of enzymes. In the case of hybrid catalysts, mutagenesis/expression of a protein is first performed with formation of a library of mutants, which are then modified chemically en masse with the introduction of an appropriate achiral ligand system harboring a transition metal. Screening these mutant hybrid catalysts in a given transition metal-catalyzed reaction then leads to an improved catalyst, so that the corresponding gene can be used to start another evolutionary cycle. This process can be repeated as often as needed until the desired catalytic profile has been reached, e.g., enhanced enantioselectivity.
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1. Introduction

Asymmetric catalysis continues to be an active field of research in synthetic organic chemistry. When applying this form of catalysis, chemists have several main options, namely transition metal catalysis,[1] organocatalysis[2] or biocatalysis.[3] The optimal choice will depend on a number of factors, including the nature of the immediate goal (small or large scale), cost of catalysts, catalyst activity, enantioselectivity, and stability under operating conditions as well as recyclability. When engaging in the challenging research directed toward developing new synthetic chiral catalysts, success depends upon the quality of design, but also on experience, trial-and-error, and serendipity. Along a different line, combinatorial asymmetric catalysis, based on the design and use of modular ligands, has been implemented experimentally with some degree of success[4] including the use of mixtures of monodentate P-ligands.[5]

The power of privileged synthetic catalyst systems[6] lies in the observation that the range of substrates showing high enantioselectivity (ee>95%) is fairly broad. Nevertheless, no single chiral catalyst is truly general. Indeed, this is why research in this challenging area needs to be intensified, especially with respect to the development of new concepts.

The exploitation of enzymes in synthetic organic chemistry, both in academic and industrial laboratories, has increased dramatically during the past 20 years.[3] The realization that many of them can be used in organic solvents is one of the important advances.[7] Moreover, rapid progress in biotechnological engineering and microbiology has contributed heavily to success in making biocatalysis practical, and more progress can be expected in the near future.[8] Nevertheless, the traditional problem in applied enzymology was not solved until the 1990s, namely the limited substrate scope and poor enantioselectivity that the enzymes often display. Rational design based on site-specific mutagenesis had been shown to be successful in some cases, but the process is far from general due to the structural complexity of proteins.[9]

In 1997 we proposed and implemented experimentally a fundamentally new and fairly general approach to asymmetric catalysis, namely the directed evolution of enantioselective enzymes for use in synthetic organic chemistry.[10] It is based on the appropriate combination of random gene mutagenesis, expression, and high-throughput screening, previously

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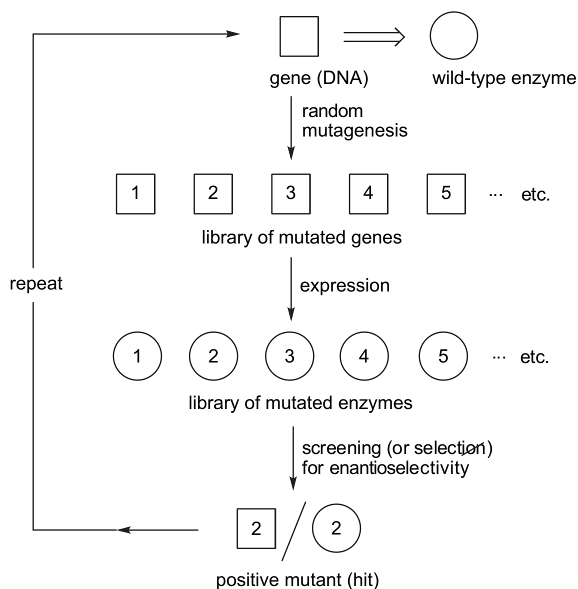


Figure 1. Strategy for directed evolution of an enantioselective enzyme.[10]

used to enhance thermostability of proteins and stability toward hostile solvents.[11] In our case we had to develop, inter alia, high-throughput ee assays,[4e,12] which turned out to be crucial for success. Moreover, it was not clear which strategies for scanning protein sequence space should be employed, and indeed, new approaches had to be developed. Using the known molecular biological methods such as error-prone polymerase chain reaction (epPCR), saturation mutagenesis, and/or DNA shuffling the hits of the initial mutant libraries (typically composed of 1000–10000 members, i.e., clones) are used as templates for another round of mutagenesis/expression/screening. The process can be repeated as many times as necessary until the desired degree of enantioselectivity has been reached (Fig. 1).[10] It is the evolutionary pressure exerted by such repeating cycles that makes directed evolution logical. In principle, knowledge of the structure or mechanism of the biocatalyst is not necessary, yet much can be learned from a theoretical analysis of enantioselective mutant enzymes evolved by directed evolution.[13]

Although our original study, in which we successfully used four rounds of epPCR in the quest to increase the enantioselectivity of a lipase, provided proof-of-principle,[10] a new challenge soon emerged. It became apparent that the efficiency in probing protein sequence space had to be improved, as in the directed evolution of other catalyst properties such as stability.[11] This required new strategies such as the appropriate application of saturation mutagenesis and DNA shuffling.[13] A more recent development is iterative saturation mutagenesis (ISM),[14] which is a symbiosis of rational design and combinatorial saturation mutagenesis. Accordingly, a Cartesian view of the protein is considered in which predetermined sites composed of one, two or three amino acid positions are chosen for saturation mutagenesis, based on rational considerations using structural information (X-ray or homology model).[14] Saturation mutagenesis is a molecular biological method with which amino acid randomization at one, two, three or more positions is induced, meaning the introduction of all 20 proteinogenic amino

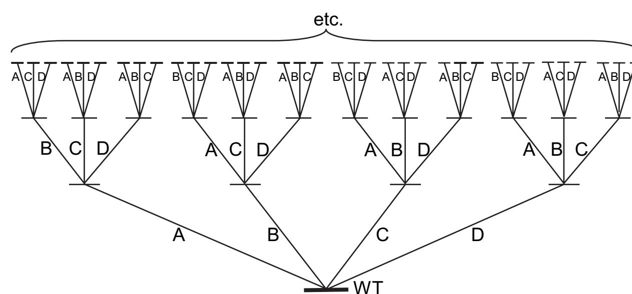


Figure 2. Schematic illustration of iterative saturation mutagenesis (ISM) involving (as an example) four randomization sites A, B, C, and D of an enzyme.[14]

acids.[11] The principle of ISM is illustrated schematically in Figure 2, in which four sites A, B, C, and D are shown (arbitrarily). Each site may be composed of one, two or three (or more) amino acid positions. Following the generation of the original saturation mutagenesis libraries, the upward climb in the fitness landscape can proceed in several pathways by using the gene of a given hit from one library as a template to perform another cycle of saturation mutagenesis at the other sites, and so on. When choosing a given upward pathway so that each site is 'visited' only once (which need not to be the case), then the scheme in Figure 2 results in convergence and a total of only 64 experiments. Of course, not all pathways need to be explored, but it is of considerable theoretical interest to see if the order of choosing the sites, i.e., the particular upward pathway, results in different mutants. The nature of the to-be-improved catalytic property determines the criterion for choosing the appropriate sites A, B, C, D, etc.

The criterion for choosing the saturation mutagenesis sites in the case of enantioselectivity or substrate scope is the so-called combinatorial active-site saturation test (CAST).[15] Accordingly, all sites having amino acids with side chains next to the binding pocket are considered, not just one or two sites as in previous studies regarding focused libraries.[11] *Thus, CASTing is the systematic generation of focused libraries around the complete binding site*. Consequently, iterative CASTing is an embodiment of ISM, which is useful for enhancing enantioselectivity and enlarging the substrate scope of enzymes.[14a] In the case of increasing thermostability of a given enzyme by ISM, the criterion for choosing sensitive sites is different.[14b,c] Accordingly, the B-factors, which correlate with the smearing of electron density in X-ray structures and thus flexibility are used to make a decision. Only the positions at which the amino acids have high average B-factors are chosen for ISM. We have shown that ISM is a fast and efficient way to perform directed evolution in the quest to enhance enantioselectivity[14a] and thermostability.[14b,c]

Directed evolution of enantioselective enzymes is now well established as a method to create biocatalysts for asymmetric organic transformations.[10,13,14a,16] Numerous academic and industrial studies have appeared. Nevertheless, this novel approach has clear limitations, the most important one being the fact that enzymes cannot catalyze numerous synthetically important transformations known to be possible by transition metal catalysis.[1,17] Some years ago we reasoned that it would be intriguing to apply the principle of

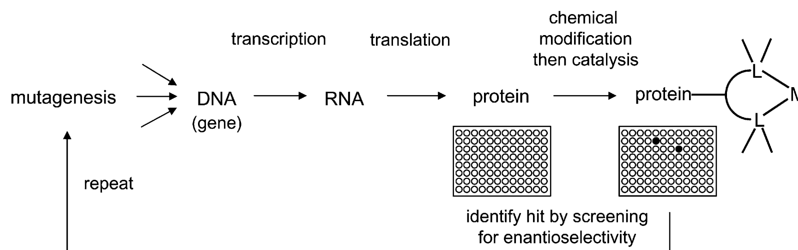


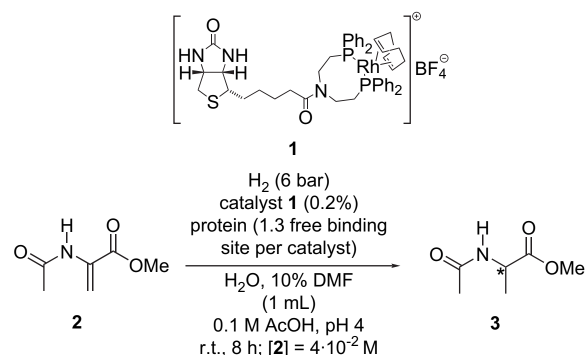
Figure 3. Concept of directed evolution of hybrid catalysts showing the flow of genetic information from the gene to transition metal hybrid catalysts.[18]

directed evolution to synthetic transition metal catalysis. We therefore proposed the notion of directed evolution of hybrid catalysts.[18] It had been known for decades that achiral ligand/metal moieties can be anchored covalently or non-covalently to proteins acting as a host.[19] Accordingly, a given system provides a single chiral catalyst, the wild-type (WT) protein providing a defined local environment around the synthetic catalytically active transition metal center. One of the milestones in this type of catalysis is the Whitesides system, in which a biotinylated diphosphine/rhodium moiety is allowed to interact non-covalently with avidin.[20] Low ee values in olefin-hydrogenation were observed (see below), but a new concept had been introduced by this seminal study. Nevertheless, in this and other systems,[19] there is no reason to believe that the particular protein environment is optimal. Therefore, the directed evolution of such hybrid catalysts provides a fascinating perspective: the possibility to tune a synthetic transition metal catalyst by an evolutionary approach (Darwinism in the test tube!).[18] The concept is shown schematically in Figure 3. The first study providing proof-of-principle was published recently[21] (see Section 2).

2. Results

As in any fundamentally new venture, proof-of-principle was the immediate goal.[21] The challenge in putting the scheme shown in Figure 3 into practice is formidable for several reasons. Firstly, unlike the process in normal directed evolution of enzymes (Fig. 1), the (over)expressed mutant protein present in each well of the microtiter plates needs to be separated from the rest of the proteins that are of no interest, but that are nevertheless present. Normally, such proteins do not affect the process of directed evolution of enzymes adversely, but in the present system chemical modification with introduction of metal/ligand entities would occur indiscriminately with formation of numerous catalysts in each well. Secondly, in classical directed evolution, only a very small amount of mutant is needed in each well due to the high activity of enzymes. In contrast, synthetic catalysts have much lower activities, which means that considerably larger amounts of a mutant protein need to be present in each well. Therefore, the expression system of the protein host has to be very efficient. Thirdly, chemical modification has to be essentially quantitative. If part of the achiral synthetic catalyst remains unbound in the presence of the desired hybrid catalyst, false information will be generated by the screening system. We originally underestimated these technical problems,[18] which is the reason why it has taken us so long to provide the proof-of-principle.[21]

One of the first examples that we proposed[18a] for the directed evolution of hybrid catalysts was the Whitesides system.[20] Accordingly, biotin is first attached covalently to an achiral diphosphine–Rh complex via a spacer to form a complex of the type **1**, which then binds with high affinity to avidin. Whitesides actually used the analogous complex in which norbornadiene takes the place of cod. The respective complex was used as a single catalyst in the Rh-catalyzed hydrogenation of α-acetamido-acrylic acid, the observed ee ranging between 33% and 44% depending upon the conditions used.[20] Ward has used chemical tuning (variation of spacer length in 1) and rational protein design to improve the enantioselectivity significantly in the same reaction using streptavidin,[22] which is fundamentally different from our Darwinistic approach. We likewise employed the biotinylated diphosphine–Rh complex **1**, but chose to use the esterified substrate **2** because this facilitates medium-throughput analysis by gas chromatography.[21] The reaction mixtures can be extracted with ethyl acetate in a parallel manner, whereas the acid of the Whitesides–Ward system is accessible efficiently only by continuous extraction.



Initially we considered avidin as the host protein,[18a] but production of eukaryotic proteins is both time consuming and low yielding. We therefore turned to streptavidin, a genetically unrelated bacterial protein, which also binds biotin with high affinity.[21] Several expression systems for streptavidin have been described,[23] and some of them were compared. Unfortunately, we ran into problems with the insufficient expression level and purification in parallel form. The best solution for our purpose turned out to be based on pET11b-sav,[24] which encodes 12 residues of T7-tag followed by Asp and Gln and residues 15–159 of the mature streptavidin. *Escherichia coli* strain BL21(DE3) transformed with this plasmid and grown in Studier's auto-induction media,[25] ZYP5052, requires less monitoring than conventional induction with IPTG at mid-log phase and thus allows

multiple unattended overnight cultures.[21] Before reaction with **1**, streptavidin mutants were purified using standard agarose/iminobiotin affinity column chromatography. Streptavidin or streptavidin/biotin themselves do not catalyze the hydrogenation reaction. Unbound complex **1** does not occur because an excess of free binding site of streptavidin was present. Per tetramer of streptavidin, 3.8–3.9 free binding sites were determined by standard titration using fluorescence quenching of biotin/4-fluorescein. About 150 ee determinations were made.

Our optimized adaptation was crucial in simplifying the screening task.[21] However, the system is still not fully suited for screening thousands of mutants since it requires a 150 mL culture scale to provide a sufficient amount of streptavidin. Typically we used 1.04×10^{-7} mol of binding site, which is equivalent to ~1.7 mg protein (based on a MW of 16.5 kDa per monomer and expecting 3.8–3.9 free binding sites per tetramer as obtained for the WT).[21]

When lower amounts of streptavidin were obtained for a given mutant, the culture scale had to be increased by up to fivefold and/or the amount of hybrid catalyst used in the reaction was decreased from 0.2% to 0.1%. Following titration of the mutant streptavidins for the purpose of determining the amount present, they were transferred into glass vessels of an in-house adapted reactor block,[26a] which was used in the Chemspeed Accelerator™ SLT 100 Synthesizer.[21] It is important to point out that traces of O_2 need to be removed from the reactors, which otherwise destroy the Rh-catalyst. Enantioselectivity was determined by conventional gas chromatographic analysis of the reaction mixtures.

It is obvious that this system really does not fulfill the technological requirements for implementing the concept of directed evolution of hybrid catalyst efficiently. Thus, we had two options: (1) invest more time in improving the (over) expression system for streptavidin, which would subsequently allow the practical formation of large libraries of mutant protein hosts (each mutant in sufficiently large quantities); or (2) use the partially optimized expression system described above, and then produce fairly small libraries. We chose the latter strategy, i.e., we settled for only a few hundred mutants in each mutagenesis experiment and proceeded with directed evolution on a 'small scale', which in fact was still labor intensive.[21]

In an initial experiment we observed that WT-streptavidin/**1** is a poor catalyst in the hydrogenation of **2**, leading to an ee of only 23% in favor of (*R*)-**3**. Rather than targeting the whole protein for amino acid substitution by error-prone PCR,[11] we applied CASTing[15] in the present hybrid catalyst system.[21] Unfortunately, an X-ray structure of the conjugate was not available. Therefore, the CAST sites for amino acid randomization were chosen on the basis of modeling the biotinylated Rh-complex **1** into the X-ray structure[27] of streptavidin/biotin using Moloc and Accelerys DS visualizer. Figure 4 shows an excerpt of the modeled structure and the amino acid sites that appeared to be appropriate for CAST experiments.[21]

Two types of sites for saturation mutagenesis were considered: the first is 'close' positions, specifically Asn49,

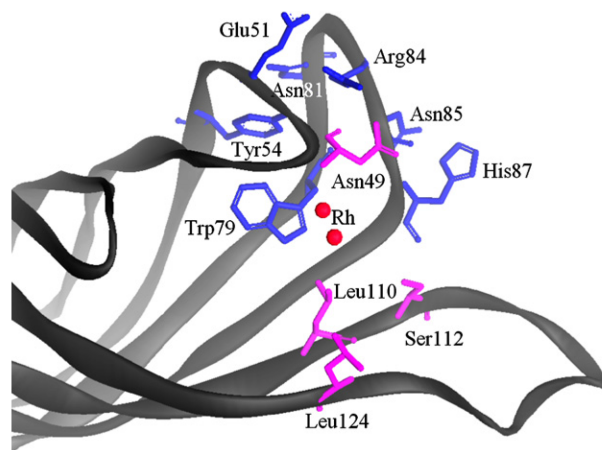


Figure 4. Selected close (purple) and distal (blue) sites from Rh(I) centers (red) of two important calculated conformers of the complex streptavidin/**1**[21] based on the X-ray structure of streptavidin/biotin.[27]

Leu110, Ser112, and Leu124. These are located about 4–6 Å away from the Rh(I) of the two calculated major conformers. We speculated that they could influence directly the conformation of the catalyst or catalyst/substrate complex.[21] The second type of sites is 'distal': Glu51, Tyr54, Trp79, Asn81, Arg84, Asn85, and His87, which are located further away from Rh(I). These second sphere CAST-positions could influence the structure of the enzyme as a whole because they are involved in hydrogen bonding between secondary elements.

Saturation mutagenesis was initiated at positions 110, 112, and 124 using the QuikChange method (Stratagene) and pET11b-sav.[24] In each saturation experiment about 200–300 clones were harvested and screened, which correspond to an oversampling of >95% coverage.[28]

Unfortunately, we observed that in some cases protein variants were formed in amounts too small for fulfilling the experimental prerequisites regarding reproducible hydrogenation. Thus, these were not considered for further study. Nevertheless, a few mutants showing enantioselectivity different from the WT were observed in a reproducible manner, the best variant **I** leading to 35% ee (*R*).[21] It is characterized by mutation Ser112Gly.

It is clear that a single round of saturation mutagenesis does not yet constitute an evolutionary process. Therefore, iterative CASTing,[14a] which is an embodiment of iterative saturation mutagenesis (ISM), was performed using the gene that encodes mutant **I** and saturating at position 49. This led to a double mutant **II** having mutations Asn49His/Ser112Gly and showing an ee value of 54% (*R*) in the model reaction (Fig. 5).[21] Finally, a third-generation saturation experiment was performed using the gene, which encodes mutant **II** and focusing once more on position 112. This experiment was designed to test whether glycine at position 112 is really the best choice when combining with histidine at position 49. This provided the improved mutant **III** leading to an ee of 65% (*R*).[21] Surprisingly, the original mutation Ser112Gly was reverted back to serine (Fig. 5). This means that the best variant is characterized by a single mutation (Asn49Val). We then proceeded to positions 51, 54, 79, 81,

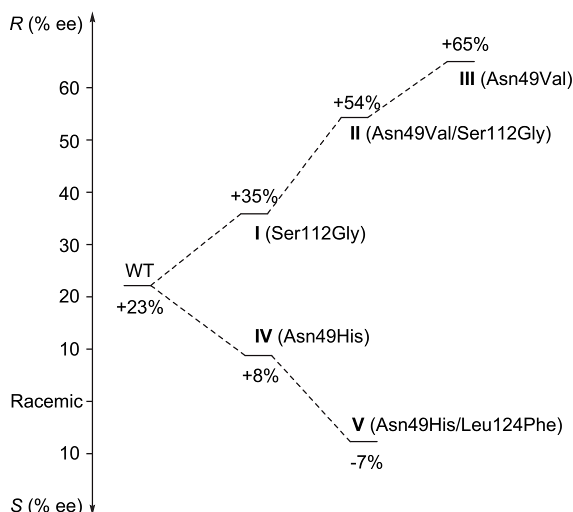


Figure 5. Directed evolution of hybrid catalysts comprising streptavidin/**1**, the Rh-catalyzed hydrogenation **2**→**3** serving as the model reaction (40–90% yield).[21]

84, 85, and 87. Unfortunately, saturation mutagenesis experiments were not successful because no soluble protein was obtained. In contrast, saturation mutagenesis at position 49 on WT template led directly to mutants **III** and **IV**. The latter is characterized by Asn49His. This variant has lower enantioselectivity than the WT (ee=8% (*R*)), suggesting the possibility of inverting stereoselectivity. Therefore, the plasmid encoding variant **IV** was utilized as a template for saturation mutagenesis in hope of inverting the sense of enantioselectivity. Indeed, upon focusing on position 124 mutant **V** (Asn49His/Leu124Phe) was identified, which is (*S*)-selective, although not by a great degree (ee=7%).[21] It would be interesting to expand the present study by applying iterative saturation mutagenesis to residues in the second shell around the close amino acid positions already considered (49, 110, 112, and 124).

This work demonstrates for the first time that it is possible to apply the methods of directed evolution to increase and/or to invert enantioselectivity of a hybrid catalyst composed of a synthetic achiral transition metal catalyst anchored to a host protein.[21] Due to the technical problems associated with the expression system, only very small mutant libraries could be generated, which in itself was labor intensive. Nevertheless, proof-of-principle has been provided for the first time.[21]

Parallel to our efforts regarding non-covalent binding based on the biotin/streptavidin system,[18,21] we also considered covalent binding (Fig. 6).[18,26]

The functional group (FG) in an appropriate binding pocket or cavity of a protein can be, for example, a thiol moiety belonging to cysteine or hydroxy originating from serine. It is conceivable that the WT of a potential protein host harbors only a single cysteine in a cavity large enough to accommodate the synthetic catalyst (and also the substrate in subsequent catalysis). This is the case with papain, a well-known cysteine protease having the only free cysteine in a relatively large cavity (Fig. 7).[29] Of course, site-specific mutagenesis can be used to place an amino acid with an appropriate functional group anywhere in the protein as desired (or remove it).

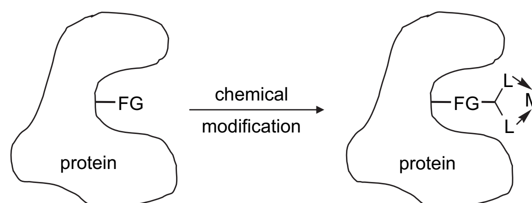
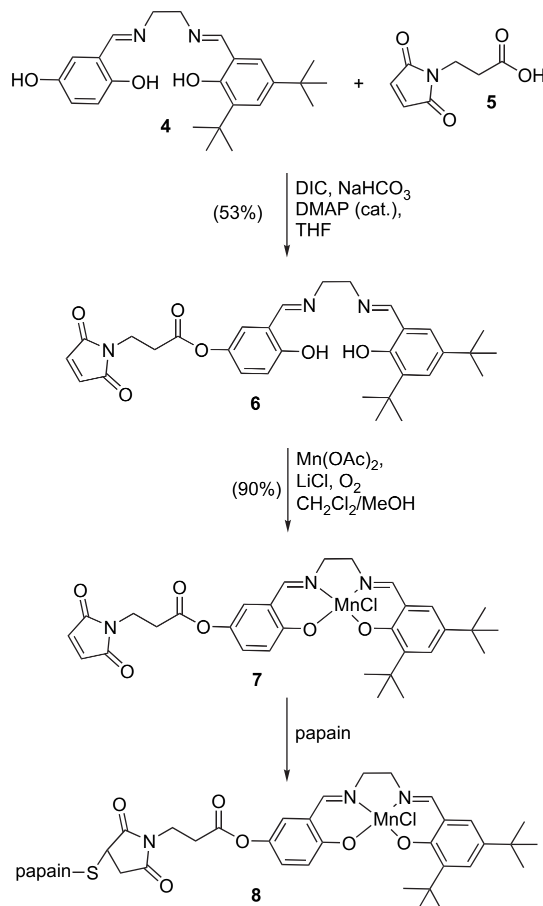


Figure 6. Implantation of ligand/metal moieties in proteins (FG=functional group).[18,26]

Indeed, these ideal characteristics contribute to the reason why the WT papain has been modified chemically in previous studies at position Cys25.[19] Either S_N2-reactions or Michael additions have been used in such endeavors, and this is exactly what we strived for in exploratory experiments.[18,26] For example, a manganese–salen complex **7** was introduced by a Michael process with formation of **8**. Dipyridyl moieties complexing copper, palladium or rhodium were also introduced in papain by appropriate reactions at Cys25.[18,26] Preliminary experiments regarding epoxidation using the hybrid catalyst containing the Mn/salen moiety and hydrogenation employing the Rh-catalyst led to ee values of up to only 10%, but this is not surprising.[18,26] Again, there is no reason to believe that an achiral synthetic catalyst in a given chiral protein environment should necessarily lead to high enantioselectivity.



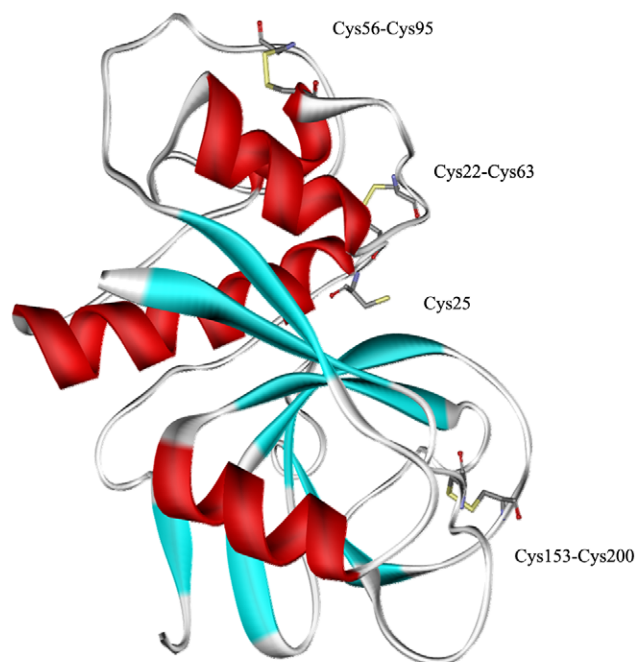
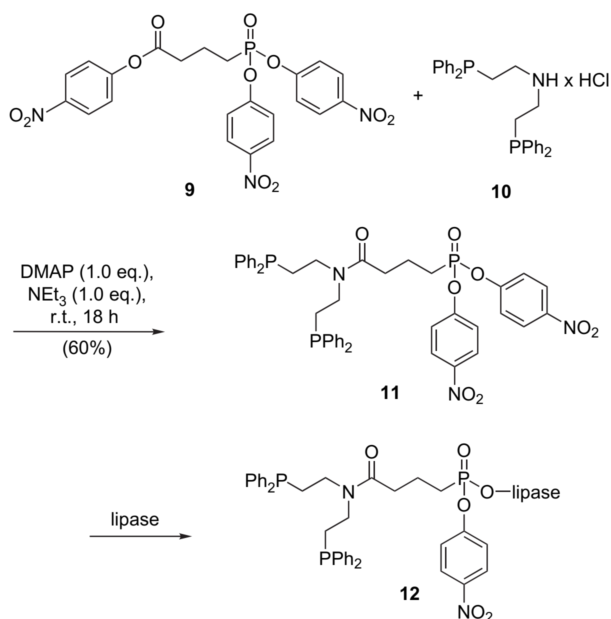


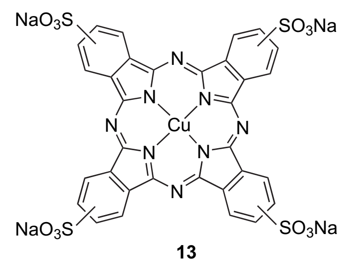
Figure 7. Globular structure of papain featuring a single free cysteine (Cys).[26b,29]

We also proposed the idea of introducing bidentate ligand systems in lipases by designing appropriate phosphonate inhibitors.[18a,26] It was well known that phosphonate inhibitors react covalently at the catalytically active serine site of a lipase selectively in the presence of other serine moieties in the enzyme. We were able to prove the incorporation of diphosphine **11** in the lipase from *Bacillus subtilis* (Lip A). However, after 24 h in water the inhibitor was hydrolytically cleaved, which is not surprising due to the presence of the second *p*-nitrophenol leaving group. It became clear that phosphonate **12** has to be replaced by an analog, which bears only one leaving group, i.e., by exchanging one of the *p*-nitrophenol groups for an alkoxy or amino moiety.[18a,26] Such work is in progress, but we also concentrated on other approaches.



In the quest to find yet another alternative protein for hosting synthetic transition metal catalysts, we turned to serum albumins.[30] These robust and easy to handle proteins are present at high concentrations in blood plasma, functioning as transport carriers for a variety of compounds such as fatty acids, bile acids, bilirubin, and hemin.[31] It was known from the X-ray work of Curry that iron–protoporphyrin dimethyl ester binds in the subdomain IB of human serum albumin (HSA) and that weak axial coordination by Tyr161 contributes to the binding.[32] Moreover, Gross had previously anchored water-soluble sulfonylated Fe^{III}– and Mn^{III}–corroles to various serum albumins and used these conjugates as catalysts in the H_2O_2-based asymmetric sulfoxidation of prochiral thioethers (up to 74% ee using WT).[33] In addition, it had been reported that the sodium salts of di-, tri-, and tetrasulfonic acid derivatives of porphyrins, phthalocyanines, and corroles bind strongly to serum albumins,[34] analogously to iron–protoporphyrin dimethyl ester.[32]

We therefore contemplated the use of the commercially available Cu^{II}–phthalocyanine complex **13**, expecting it to bind strongly to the IB subdomain of HSA or to analogous regions of other serum albumins such as bovine serum albumin (BSA), which is also a cheap and robust protein.

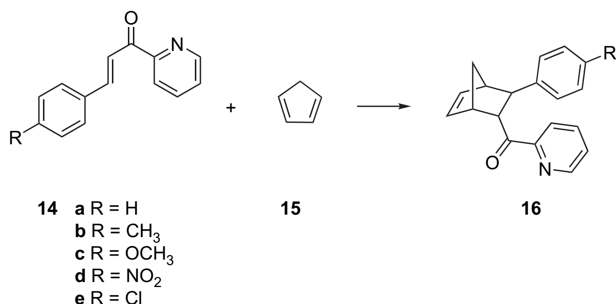


In the case of HSA we employed the X-ray data of Curry to model the desired **13**/HSA complex (Fig. 8).[30] Absolute proof that binding occurs in this manner was not obtained, i.e., Figure 8 simply presents a useful model.

As the model reaction to be mediated by hybrid catalysts comprised of **13**/serum albumins, we chose the Diels–Alder reaction of the H_2O-soluble azachalcone **14** with cyclopentadiene **15** to form adduct **16**. This reaction had been originally devised by Engberts,[35] who used Cu^{II}-complexes of amino acids in aqueous medium (ee up to 74%). Later it

Figure 8. Model of **13**/HSA[30] based on the X-ray structure of hemin/HSA.[32]

was employed by Feringa in the study of Cu^{II}-conjugates of DNA as catalysts.[36]



Surprisingly, we observed high enantioselectivities when using BSA as the host for **13** (85–93% ee) in favor of the expected *endo*-products **16**.[30] In the case of **13**/HSA (Fig. 8), the reaction of **14a** with **15** led to an ee value of 85%. BSA or HSA alone do not catalyze the reaction. Thus, since a good expression system for HSA has been reported,[37] this system could be employed in a future study regarding the directed evolution of enantioselective hybrid catalysts. Iterative CASTing based on the model in Figure 8 could then be used to increase the enantioselectivity of the present Diels–Alder cycloaddition or of other Cu^{II}-catalyzed processes. However, a procedure for en masse purification of HSA mutants needs to be developed first.

3. Discussion

In order to put the idea of directed evolution of hybrid catalysts into practice, we have considered three different approaches, namely the biotinylated Rh–diphosphine complex **1** bound non-covalently to streptavidin, transition metal complexes such as Mn/salen entities bound covalently to papain, and a Cu(II)–phthalocyanine complex anchored non-covalently to serum albumins. Proof-of-principle was achieved only in the first case, which provokes the question regarding the source of enhanced or reversed enantioselectivity. Presently, it is essentially impossible to provide a sound answer on a molecular level. It is not even clear whether the Whitesides system adheres to the Halpern (or *anti*-Halpern) rule. Thus, more work is necessary to clarify these points.

4. Conclusions

Following our original proposal regarding the directed evolution of hybrid catalysts (Fig. 3) and some exploratory experiments,[18] we were able to provide proof-of-principle in 2006.[21] This was based on the use of the Whitesides system comprising a biotinylated Rh–diphosphine complex and streptavidin as the protein host.[21] However, due to the inefficiency of the current expression systems of streptavidin, only very small mutant libraries even after intensive laboratory effort could be generated (less than 300 clones per library). Nevertheless, we applied iterative CASTing successfully in the 'mini' directed evolution study.[21] In three cycles of iterative saturation mutagenesis at selected sites around the modeled Rh-center in the hybrid catalyst the ee of Rh-catalyzed olefin-hydrogenation increased stepwise from ee=23% to ee=65%.[21] If in the future a highly improved expression system for streptavidin could be developed, then this system should be re-considered. It is very likely that much better enantioselectivities can then be achieved by iterative CASTing using considerably larger mutant libraries or by other mutagenesis methods such as DNA shuffling.

Until that goal has been reached, other systems need to be considered, such as appropriate lipase/inhibitor complexes, HSA conjugates, or alternative approaches. Another possibility is to employ thermophilic proteins as hosts, which can be purified by simple heating.[26b,c,38] Once the optimal system has been established (or systems!), a platform (or platforms) would exist from which a number of intriguing goals can be strived for, especially in view of the amazingly large number of catalytic aqueous organometallic reactions already known.[17b] No doubt, the use of molecular biology to tune a synthetic transition metal catalyst in a Darwinistic sense (Fig. 3) offers exciting perspectives for the future, including the theoretical interpretation of enhanced enantioselectivity and/or activity.[39]

5. Experimental

5.1. Directed evolution of the hybrid catalyst comprising streptavidin/1

5.1.1. Synthesis of the Rh-complex 1. To a solution of $[Rh(cod)_2]BF_4$ (80.3 mg; 197 μmol) in dry dichloromethane (7 mL) was added at 0 °C (3a*S*,4*S*,6a*R*)-5-(2,3,3a,4,6,6a-hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imida-zol-4-yl]-pentanoic acid-*N*,*N*-bis-(2-diphenyl-phosphanyl-ethyl)amide[20,40] (165 mg; 247 μmol). Dichloromethane (7 mL) was added, the cooling bath was removed, and the mixture was stirred for 3 h. After cooling to 0 °C, *n*-pentane (40 mL) was added and the mixture stirred for 0.5 h. The precipitated yellow solid was filtered, washed three times with *n*-pentane (60 mL), and dried in vacuo for 16 h. This provided compound **1** (169 mg; 89%) as a yellow solid. IR (KBr): ν=3402 cm^{-1}, 3249 (br, NH), 3054 (w, Ar-H), 2922 (m, CH_2), 1706 (s, $(HN)_2C{=}O$), 1643 (s, $CH_2C{=}O$), 1586 (w, $C_{Ar}{=}C_{Ar}$), 1572, 1480, 1435 (s, P-Ph), 1370, 1333, 1311, 1267, 1182, 1160, 1055 (br, H–C=), 998, 940, 859, 814, 746 (m, Ar-H), 698 (s, Ar-H), 518, 492. ^1H NMR (400 MHz, CD_2Cl_2) δ 1.19–1.32 (m, 3H, 3-H, 4-H), 1.34–1.51 (m, 3H, 3-H, 5-H), 1.92–1.94 (m, 2H, 1-H), 2.11–2.28 (m, 12H, COD–CH_2, $NCH_2CH_2PPh_2$), 2.59 (d, ^2J=12.8 Hz, 1H, 6′-H_b), 2.82 (dd, ^2J=12.8 Hz, ^3J=5.0 Hz, 1H, 6′-H_a), 3.08 (q, ^3J=7.7 Hz, 4′-H), 3.34 (dd, ^2J(H,P)=19.9 Hz, ^3J=4.9 Hz, 2H, *E*-$NCH_2CH_2PPh_2$), 3.72 (dd, ^2J(H,P)=21.4 Hz, ^3J=5.2 Hz, 2H, *Z*-$NCH_2CH_2PPh_2$), 4.18–4.20 (m, 1H, 3a′-H), 4.37–4.40 (m, 5H, COD–CH, 6a′-H), 5.01 (br s, 1H, 3′-H), 5.61 (br s, 1H, 1′-H), 7.03–7.46 (m, 20 H, Ar-H) ppm. ^{13}C NMR (101 MHz, CD_2Cl_2) δ 21.57 (−, CH_2), 24.04 (−, CH_2), 27.58 (−, CH_2), 29.52 (−, CH_2), 30.33 (−, CH_2), 32.61 (−, CH_2), 39.84 (−, C-4′), 45.88 (−, *E*-$NCH_2CH_2PPh_2$), 46.99 (−, *Z*-$NCH_2CH_2PPh_2$), 56.29 (+, C-3′), 60.80 (+, C-6a′), 62.55 (+, C-3a′), 99.27 (+, COD–CH), 101.58 (COD–CH), 128.26 (+, Ar-C), 128.36 (+, Ar-C), 128.64 (+, Ar-C), 130.59 (+, Ar-C), 130.72 (+, Ar-C), 131.77 (+, Ar-C), 131.86 (+, Ar-C), 132.15 (+, Ar-C),

162.58 (C_{quart}, C-2′), 173.70 (C_{quart}, C-1) ppm. ^{31}P NMR (162 MHz, CD_2Cl_2) δ 13.91 (dd, ^1J(P,Rh)=143 Hz, ^2J(P,P)=34.7 Hz, PPh_2), 22.69 (dd, ^1J(P,Rh)=142 Hz, ^2J(P,P)=34.3 Hz, PPh_2) ppm. MS (ESI/pos., CH_2Cl_2) *m/z* (%): 878 (7) [M^+−BF_4], 770 (100) [M^+−COD–BF_4]. Elemental analysis for $C_{46}BF_4H_{55}N_3O_2P_2SRh$ (965.68): C 57.28 (57.24), H 5.81 (5.74), N 4.30 (4.35), P 6.46 (6.41), Rh 10.60 (10.66) found (calculated).

5.1.2. Expression system of streptavidin and mutant library formation. The best expression system for the project, although not ideal, is based on pET11b-sav described by Gallizia,[24] which was employed in all experiments. This construct encodes 12 residues of T7-tag followed by Asp and Gln and residues 15–159 of the mature streptavidin. *E. coli* strain BL21(DE3) transformed with this plasmid and grown in Studier's auto-induction media,[25] ZYP5052, requires less monitoring than conventional induction with IPTG at mid-log phase and thus allows multiple unattended overnight cultures. This adaptation was crucial in simplifying the screening task. It requires a 150 mL culture scale to provide a sufficient amount of streptavidin. Typically 1.04×10^{-7} mol of binding site was used, which is equivalent to ~1.7 mg protein (based on a MW of 16.5 kDa per monomer and expecting 3.8–3.9 free binding sites per tetramer as obtained for the WT). However, when lower amounts of streptavidin were obtained for a given mutant, the culture scale was increased by up to fivefold and/or the amount of hybrid catalyst used in the reaction was decreased to 0.1%. Titrated mutant streptavidins were transferred into glass vessels of an in-house adapted reactor block[21,26a] for the Chemspeed Accelerator™ SLT 100 Synthesizer. Saturation mutagenesis at the defined sites in streptavidin (Fig. 1) was performed using the standard QuikChange method of Stratagene[41] and pET11b-sav.[24]

5.1.3. Enantioselective hydrogenation. All reactions were performed using the Chemspeed Accelerator™ SLT 100 Synthesizer[21] equipped with in-house adapted reactor blocks.[26a] All solvents were thoroughly degassed three times as well as redistilled in the case of dimethylformamide. Such cautions must be taken due to the high sensitivity of the reaction toward oxygen. The reaction vessels of the reactor block[26a] were evacuated at high vacuum and flushed with argon, a process that was repeated three times. The catalyst solution was prepared at room temperature by the addition of a solution of $[Rh(cod)_2]BF_4$ in dimethylformamide to a stirred solution of the ligand in dimethylformamide as well. The catalyst thus prepared has to be used immediately as it degrades readily after a few hours. The preformed Rh-catalyst (Section 5.1.1) can also be used. First, the substrate in solution in the acetate buffer was added to the protein, lyophilized in the reaction flask, and then the catalyst solution was added to the shaken solution. All conditions (H_2 (6 bar); 0.2% Rh-catalyst **1**; H_2O/10% DMF; 0.1 M AcOH/pH 4; 22 °C; 8 h) are optimal, although the catalyst amount can be lowered with some confidence to 0.01% whenever needed.

The reaction mixtures were extracted with ethyl acetate and analyzed by chiral gas chromatography using GC Hewlett Packard 6890N chromatograph equipped with DiMePe-BETA-ivadex-1 chiral column (25 m, 0.25 mm, 0.15 μm, Analysentechnik, Meerbusch, Germany), carrier (N_2), flow 1.7 mL/min, temperature profile 100 °C for 12.5 min. The uncertainty in the ee values is ±2%.

5.2. Synthesis of papain-conjugate 8

5.2.1. Preparation of salen 4. The procedure of Jacobsen[42] was used: to the mixture of 2,5-dihydroxy-benzaldehyde (61 mg; 0.44 mmol), 3,5-di-*tert*-butylsalicylaldehyde (310 mg; 1.32 mmol), and ethylene diamine (53 mg; 0.88 mmol) in 20 mL of dichloromethane was added 2 g of silica gel. After stirring for 18 h at room temperature, the solvent was removed in vacuo. The solid (SiO_2 and compounds) was brought onto a chromatography column (25 mm diameter) and filled with 20 g of silica gel. Using a 3:1 mixture of hexane/diethyl ether and an argon pressure, elution of the undesired product (non-polar yellow symmetrical salen) was performed, followed by elution of **4** using a 1:1 solvent mixture. This has to occur rapidly, avoiding high temperatures, so that undesired new formation of symmetrical salen products is avoided. The solvent is removed in vacuo, yielding **4** as an orange-yellow solid (134 mg; 77%). ^{1}H NMR (400.1 MHz, d_6-DMSO) δ 1.26 (s, 9H, CH_3), 1.37 (s, 9H, CH_3), 3.90 (m, 4H, CH_2 diiminoethane), 6.77 (d, $^3J_{H,H}$=8.6 Hz, 1H, C*H*), 6.79 (s, 1H, C*H*), 6.69 (d, $^3J_{H,H}$=8.6 Hz, 1H, C*H*), 7.24 (s, 1H, C*H*), 7.30 (s, 1H, C*H*), 8.49 (s, 1H, *H*C=N), 8.58 (s, 1H, *H*C=N), 8.93 (s, 1H, O*H*), 12.48 (s, 1H, O*H*), 13.95 (s, 1H, O*H*). ^{13}C NMR (100.6 MHz, d_6-DMSO) δ 29.6 (CH_3), 31.6 (CH_3), 34.2 (quart. C), 34.9 (quart. C), 59.1 (CH_2 diiminoethane), 59.4 (CH_2 diiminoethane), 116.8 (*C*H), 117.2 (*C*H), 118.0 (*C* imine), 119.2 (*C* imine), 120.3 (*C*H), 126.5 (*C*H), 126.6 (*C*H), 136.0 (C-*t*-butyl), 139.8 (C-*t*-butyl), 150.0 (*C*–OH), 153.1 (*C*–OH), 158.2 (*C*–OH), 167.1 (*C*=N), 168.4 (*C*=N). MS (EI) *m/z* (%): 396 (100) [M^+], 381 (30) [M^+−CH_3], 259 (14), 234 (52), 218 (19), 163 (16).

5.2.2. Preparation of ester 6. The mixture of **4** (134 mg; 0.338 mmol), acid **5**[43] (122 mg; 0.721 mmol), diisopropylcarbodiimide (170 μmol), and $NaHCO_3$ (75 mg; 0.89 mmol) in a 0.1 M solution of *N*,*N*-4-dimethylaminopyridin in THF (20 mL) was stirred for 2 h at room temperature. The solvent was removed in vacuo and the mixture extracted with ethyl acetate. Chromatography over silica gel (20 g) using a 1:1 mixture of ethyl acetate and hexane afforded **6** as a yellow solid (115 mg; 62%). IR (KBr, cm^{-1}): 2960 (s, ν[C–H], *t*-butyl), 1750 (m, ν[C=O], aryl ester), 1710 (s, ν[C=O], maleinimide), 1640 (s, ν[C=N]), 1170 (m, ν[C–O], aromatic), 830 (w, δ[=CH], aryl). ^{1}H NMR (400.1 MHz, d_4-methanol) δ 1.32 (s, 9H, CH_3), 1.44 (s, 9H, CH_3), 2.88 (t, $^3J_{H,H}$=6.8 Hz, 2H, CH_2 propionic acid), 3.94 (t, $^3J_{H,H}$=6.8 Hz, 2H, CH_2 propionic acid), 3.99 (m, 4H, CH_2 diiminoethane), 6.87 (s, 2H, C*H* maleinimide), 6.88 (dd, $^3J_{H,H}$=4.9 und 8.9 Hz, 2H, C*H* aromatic), 7.12 (s, 1H, C*H* aromatic), 7.09 (d, $^3J_{H,H}$=8.9 Hz, 1H, C*H* aromatic), 7.19 (s, 1H, C*H* aromatic), 7.41 (s, 1H, C*H* aromatic), 8.47 (s, 1H, C*H* imine), 8.48 (s, 1H, C*H* Imin). ^{13}C NMR (100.6 MHz, d_4-methanol) δ 30.0 (CH_3), 32.0 (CH_3), 34.1 (CH_2 propionic acid), 34.7 (CH_2 propionic acid), 35.0 (quart. C), 36.0 (quart. C), 60.2 (CH_2 diiminoethane), 60.5 (CH_2 diiminoethane), 118.7 (*C*H aromatic), 119.5 (*C* imino group), 119.7 (*C* imino group), 125.0 (*C*H aromatic), 127.3 (*C*H aromatic), 127.5 (*C*H aromatic), 129.9

(*C*H aromatic), 135.6 (*C*H maleinimide), 137.5 (*C*-*t*-butyl), 138.7 (*C*-*t*-butyl), 141.4 (*C*–O aromatic), 143.4 (*C*–O aromatic), 167.6 (*C*=N), 169.4 (*C*=N), 172.2 (*C*=O), 180.8 (*C*=O). MS (EI) *m*/*z* (%): 547 (100) [M$^+$], 532 (21) [M$^+$−CH$_3$], 396 (21), 259 (19), 234 (29) [C$_4$H$_9$].

5.2.3. Preparation of Mn-complex 7. The solution of ester **6** (17 mg; 31 μmol), $Mn(OAc)_2 \cdot 2H_2O$ (11 mg; 61 μmol), and LiCl (13 mg; 31 μmol) in methanol (4 mL) was stirred under air for 16 h at room temperature. The volume of the mixture was reduced in vacuo to 0.5 mL and then diluted with CH_2Cl_2. After washing with an aqueous solution of NaCl, the organic phase was separated and dried over Na_2SO_4. Removal of the solvent in vacuo afforded **7** (18 mg; 90%) as a black solid. MS (methanol, ESI pos.) *m*/*z* (%): 600 [M−Cl]$^+$, 632 [M−Cl+CH$_3$OH]$^+$. High resolution MS (ESI, methanol): calcd for $C_{31}H_{35}N_3O_6Mn$: 600.190639, found: 600.190776, difference: 0.23 ppm. IR (KBr, cm^{-1}): 2960 (s, ν[C–H], *t*-butyl), 1750 (m, ν[C=O], aryl ester), 1710 (s, ν[C=O], imide), 1620–1540 (s, ν[C=C], ν[C=N]), 1170 (m, ν[C–O], aromatic), 830 (w, δ[=CH], aryl).

5.2.4. Formation of papain conjugate 8. In an initial step papain-lyophilisate (10 mg) obtained from Sigma–Aldrich was activated by treatment with L-cysteine (10 mg) in H_2O (1 mL) for 30 min. The non-reacted cysteine was then separated by gel permeation chromatography (Sephadex G-25; 50 mM acetate buffer at pH=5.2). The protein fraction was identified by its absorption at 280 nm and its proteolytic activity tested. The fractions containing active papain (about 3 mL) were combined and kept at 4 °C for further use. This mixture (3 mL) was mixed with phosphate buffer (300 μL; 1 M) and brought to pH=7.0. It was then treated with complex **7** (0.648 mg; 1.08 μL of DMSO). After 6 h no protease activity was observed, showing that conjugation with formation of **8** had occurred.

5.3. Enantioselective Diels–Alder reactions catalyzed by the hybrid catalyst 13/BSA

5.3.1. Small scale reaction. An aqueous formate buffer (2 mL; 30 mM; pH 4.0) was prepared and 39.6 mg of BSA added. Subsequently a 12.5 μL solution of the commercially available Cu-complex **13** (Aldrich) in H_2O (40 mM) was added. This solution was stirred at room temperature for 30 min. Then, 21.2 μL solution of dienophile **14a** in CH_3CN (0.94 M) was added. The resulting mixture was cooled to 3 °C. The reaction was started with the addition of 10 μL pure cyclopentadiene (**15**) and stirred for 3 days at 3 °C, followed by extraction with diethyl ether (6 mL), removal of the ether, and measurement of the conversion and ee value by chiral HPLC (Chiralcel-OD-H column, elution with *n*-heptane/*i*-propanol=98:2; anisol as the internal standard).

5.3.2. Larger scale reaction. The solution of 792 mg BSA in 40 mL formate buffer (30 mM; pH 4.0) was treated with a 250 μL solution of **13** in H_2O (40 mM). This solution was stirred at room temperature for 30 min. Then 424 μL solution of dienophile **14a** in CH_3CN (0.94 M) was added. The resulting mixture was cooled to 3 °C. The reaction was started with the addition of 200 μL pure cyclopentadiene (**15**) and stirred for 3.5 days at 5 °C, followed by extraction with diethyl ether. Subsequently, 10 mL of methanol was added to the aqueous phase to destroy the structure of protein. Following extraction with ethyl acetate (90 mL/three times), the organic phases were combined and dried with anhydrous Na_2SO_4. After evaporation, the residue was purified through silica gel chromatography (eluent: petroleum hexane/ethyl acetate=10:1) to afford 92 mg of **16a** (85%, *endo*/*exo*=93:7, 89% ee for *endo*-**16a**). The stereochemical assignments were made by comparison with authentic samples.[35,36]

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