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Optimization of and Mechanistic Considerations for the Enantioselective Dihydroxylation of Styrene Catalyzed by Osmate-Laccase-Poly(2-Methyloxazoline) in Organic Solvents

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The Sharpless dihydroxylation of styrene with the artificial metalloenzyme osmate-laccase-poly(2-methyloxazoline) was investigated to find reaction conditions that allow this unique catalyst to reveal its full potential. After changing the co-oxidizing agent to *tert*-butyl hydroperoxide and optimizing the osmate/enzyme ratio, the turnover frequency and the turnover number could be increased by an order of magnitude, showing that the catalyst can compete with classical organometallic catalysts. Varying the metal in the active center showed that osmate is by far the most active catalytic center, but the reaction can also be realized with permanganate and iron(II) salts.

Introduction

Pure enantiomeric compounds are essential for the synthesis of pharmaceuticals and other fine chemicals.[1] In many cases, the use of an enantioselective catalyst, for example, an organometallic one, is the most economical way to gain this. Enzymatic systems are being currently considered as alternatives to organometallic catalysts.[2] In addition to their natural catalytic activity, enzymes possess a defined chiral matrix, especially at their active site, which offers the possibility to use them as chiral ligands for catalytically active (transition) metal complexes. This merging of proteins and metal catalysts results in so called artificial metalloenzymes, which have the molecular recognition ability of proteins and the broad reactivity scope of small molecule catalysts.[3]

Artificial metalloenzymes, generated by different anchoring strategies, have been applied in various types of chemical reactions, for example, sulfoxidation,[4] hydrogenation,[5] epoxidation,[6] Diels–Alder reactions,[7] or diastereoselective coenzyme reduction.[8]

There are three generally applied strategies for the incorporation of a metal complex within the biomolecule host; dative anchoring, supramolecular anchoring, and covalent anchoring.[9]

The dative anchoring strategy, which is used in this work, is based on direct non-covalent interaction between the amino acids of the enzyme and the metal ion or metal complex. One way to achieve this, is to utilize natural metalloenzymes where the metal ion in the active center is substituted by another. Kaiser and co-workers were the first to generate such a hybrid catalyst by replacing the natural zinc of carboxypeptidase A by copper and they used the resulting artificial metalloenzyme in oxidation reactions.[10]

Dihydroxylations of alkenes by using proteins as hosts are the focus of much current research. For example, the use of bovine serum albumin (BSA) (77%) and streptavidin (95%) as ligands for osmate results in good enantiomeric excess (*ee*) for the dihydroxylation of α-methylstyrene in aqueous media.[11]

Owing to the solubility and natural environment of proteins and enzymes, most of the reactions catalyzed by artificial metalloenzymes are performed in aqueous media.[12] A few examples, which involve large amounts of organic co-solvents, have been reported.[13]

In general, enzymes are used in organic solvents by immobilizing them on designed carriers.[14] In our group, this approach has been particularly successful for enzymes in amphiphilic polymer co-networks (APCNs), which activate these biocatalysts by several orders of magnitude in membranes[15] and particles[16] in organic solvents and even in supercritical CO_2.[17] Chiral APCNs allow control of the enantioselectivity of enzymes in organic solvents.[18]

To overcome the limitations of artificial metalloenzymes in aqueous systems, organo-soluble polymer enzyme conjugates (PECs) have been investigated regarding their potential as artificial metalloenzymes for alkene dihydroxylation. Such PECs based on conjugation of enzymes with poly(2-methyloxazoline) (PMOx) show enzymatic activity in aqueous and organic solvents.[19] Their structures offer the possibility to apply them as amphiphilic polymer nanocontainers[20] for dissolving inorganic salts in organic solvents, forcing the salt to attach to the protein, which represents the hydrophilic core of the nanocontainer.

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An artificial enzyme composed of a laccase-PMOx conjugate with an osmate at the active site acts as a chiral catalyst for the asymmetric dihydroxylation of alkenes in chloroform, leading to highly enantioselective product formation (up to 99.4% *ee* for 1-phenyl-1,2-ethanediol for styrene dihydroxylation) that even exceeds the classical Sharpless catalysts (97% *ee*).[21,22] Thus, we could transfer the concept of artificial metalloenzymes from water to organic solvents with even higher selectivities.

The drawback of all artificial metalloenzyme approaches, and particularly for osmate-catalyzed dihydroxylation, is the small turnover number (TON) and turnover frequency (TOF) with regard to the amount of osmate catalyst used. Koehler et al. reported a maximum TON of 27 when streptavidin is used as host for osmate in an aqueous medium.[11] Laccase-PMOx as the host for osmate in chloroform afforded a TON of 42.[22] In the present manuscript, the latter enzyme conjugates were explored to find their full potential with respect to reaction rate and turnover number.

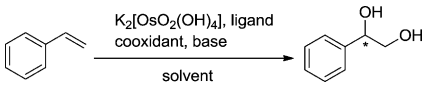
Results and Discussion

The basis of the organo-soluble osmate-laccase conjugates is the modification of the enzyme with poly(2-methyloxazoline) (PMOx)[23] and the subsequent exchange of copper with osmate.[22] As shown previously, these conjugates show excellent enantioselectivity in the dihydroxylation of styrene in chloroform.

So far, the highest selectivity of *ee* (*R*) = 99.4% could only be obtained after relatively short reactions times of three days. When running the reaction for seven days, the best result of 98.4% *ee* for the *R* enantiomer was obtained with laccase-PMOx pretreated with aqueous ethylenediaminetetraacetic acid (EDTA) solution to remove the copper of the enzyme and subsequent addition of the potassium osmate in a molar osmate/enzyme ratio (OER) = 2 (Scheme 1).

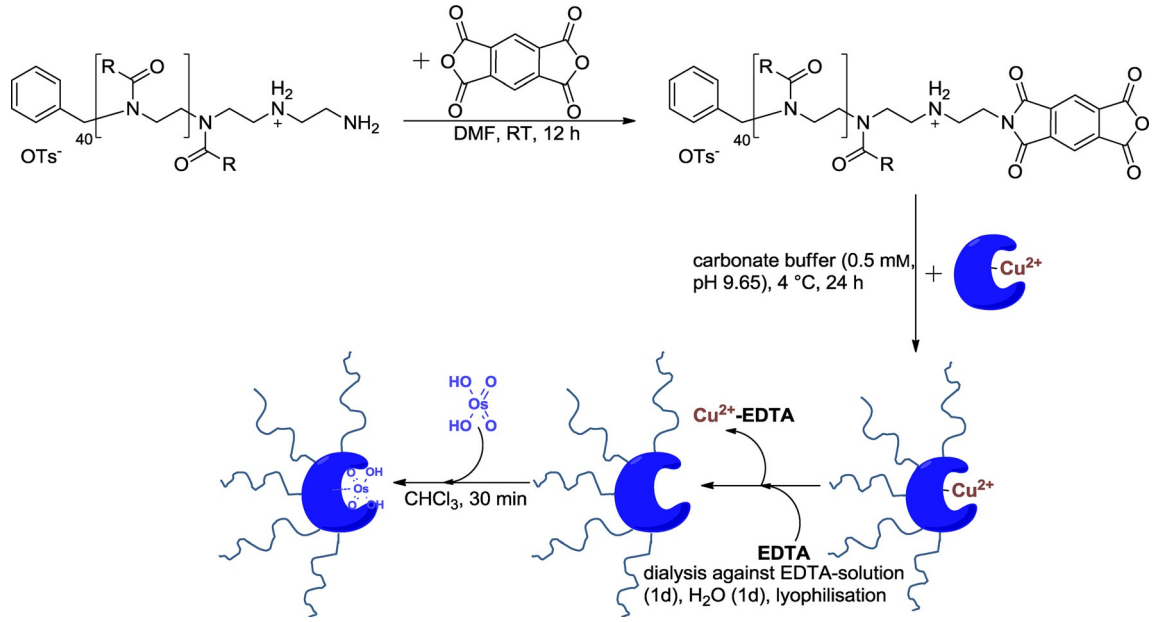
This conjugate was found to convert styrene to 1-phenyl-1,2-ethanediol in only 1%, which is calculated to a TOF of 0.25 h^{-1} and a TON of 42 (Table 1). This is about 300 times lower TOF value compared with a commercial Sharpless catalyst[24] with a somewhat lower selectivity (97% *ee*[21]).

The turnover might be limited as a result of the insolubility of the co-oxidizing agent, $K_3[Fe(CN)]_6$, in organic solvents, which restricts the accessibility of this reagent for the catalyst. Another limiting factor might be the low solubility of the OH^- source (water) in chloroform (Scheme 2).



Scheme 2. The asymmetric-osmate-catalyzed dihydroxylation of styrene.

To overcome these limitations, the co-oxidizing agent was changed from the organo-insoluble $K_3[Fe(CN)]_6/K_2CO_3$ system to *tert*-butyl hydroperoxide. As seen in Figure 1, this alteration results in a homogeneous reaction solution, which affords a six-fold increase in the TON without a significant change in *ee* (Table 1). To further raise the turnover, an additional OH^- source (methanolic KOH) was added to the reaction mixture, resulting in an increase in the TON from 258 to 328. Lowering the OER to 1 gives a turnover number of 501 with an increased *ee* value of 99.4. This value represents the detection limit of the used HPLC analytics. In summary, a 12-fold increase in the turnover number could be achieved by changing the co-oxidiz-



Scheme 1. Strategy for enzyme modification with PMOx and pyromellitic acid dianhydride and the suggested copper type 1 exchange with osmate by treatment with EDTA according to Ref. [22].

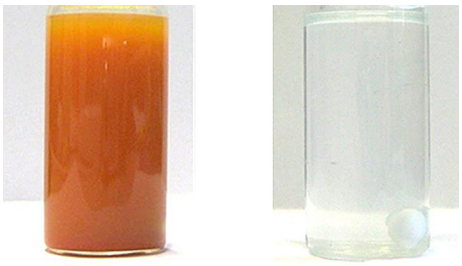


Figure 1. Photographs of reaction mixtures for the dihydroxylation of styrene with $K_3[Fe(CN)]_6/K_2CO_3$ as the co-oxidizing system (left) and *tert*-butyl hydroperoxide/KOH as the co-oxidizing system (right).

Table 1. Dependency of the *ee*, TON, TOF, and yield on the used co-oxidizing system for the dihydroxylation of styrene in chloroform by using osmate-laccase-PMOx. The reaction was run at 0 °C for seven days.

OER	Co-oxidizing agent	Base	TON[a]	TOF[b] [h^{-1}]	Yield [%]	*ee* (*R*) [%]
2	$K_3[Fe(CN)]_6$[c,d]	K_2CO_3	42	0.25	1.0	98.4
2	*t*BuOOH[c]	–	258	1.54	6.5	97.2
2	*t*BuOOH[c,e]	K_2CO_3	232	1.38	6.0	97.8
2	*t*BuOOH[c,f]	KOH/MeOH	328	1.95	8.5	98.6
1	*t*BuOOH[c,g]	KOH/MeOH	501	2.98	6.6	99.4

[a] The TON is defined as µmol product per µmol $K_2OsO_2(OH)_4$. [b] The TOF is defined as µmol product per µmol $K_2OsO_2(OH)_4$ per h. [c] Standard reaction conditions: 0.013 µmol mL^{-1} laccase-PMOx, 0.026 µmol mL^{-1} $K_2OsO_2(OH)_4$, 110 µmol mL^{-1} *t*BuOOH, and 100 µmol mL^{-1} styrene unless otherwise stated. [d] 300 µmol mL^{-1} $K_3Fe(CN)_6$ used instead of *t*BuOOH, plus 300 µmol mL^{-1} K_2CO_3. [e] Standard conditions plus 300 µmol K_2CO_3. [f] Standard conditions plus 10 µL mL^{-1} saturated methanolic KOH. [g] Standard conditions except 0.013 µmol mL^{-1} $K_2OsO_2(OH)_4$ and plus 10 µL mL^{-1} saturated methanolic KOH.

Table 2. TON, TOF, yield, and *ee* values for the dihydroxylation[a] of styrene by using different solvents and laccase-PMOx as ligand for osmate with an OER of 1.

Solvent	TON[b]	TOF[c] [h^{-1}]	Yield [%]	*ee* (*R*) [%]
chloroform	501	2.98	6.6	99.4
CH_2Cl_2	600	3.57	7.8	94.1
MeOH	315	1.87	4.1	0.0
EtOH	266	1.58	3.5	0.0
DMF	38	0.23	0.5	0.0
DMSO	10	0.06	0.1	75.7
MeCN	49	0.29	0.6	0.0

[a] Reaction performed with 0.013 µmol mL^{-1} laccase-PMOx, 0.013 µmol mL^{-1} $K_2OsO_2(OH)_4$, 110 µmol mL^{-1} *t*BuOOH, 10 µL mL^{-1} saturated methanolic KOH, and 100 µmol mL^{-1} styrene at 0 °C for seven days. [b] The TON is defined as µmol product per µmol $K_2OsO_2(OH)_4$. [c] The TOF is defined as µmol product per µmol $K_2OsO_2(OH)_4$ per h.

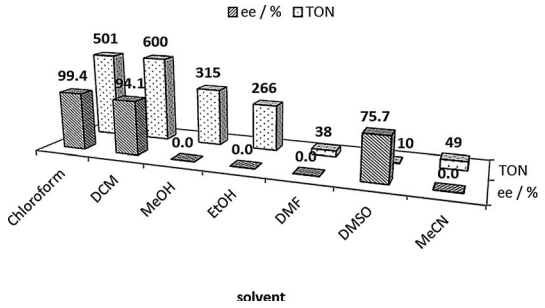


Figure 2. Turnover number (TON) and enantiomeric excess (*ee*) of the asymmetric dihydroxylation in different solvents catalyzed by osmate-laccase-PMOx with an OER of 1. The reaction was performed with 0.013 µmol mL^{-1} laccase-PMOx, 0.013 µmol mL^{-1} $K_2OsO_2(OH)_4$, 110 µmol mL^{-1} *t*BuOOH, 10 µL mL^{-1} saturated methanolic KOH, and 100 µmol mL^{-1} styrene at 0 °C for seven days. The TON is defined as µmol product per µmol $K_2OsO_2(OH)_4$.

ing reagent and providing methanolic KOH as an OH^- source. This shows the great potential of the artificial metalloenzyme.

It is known in the literature that the activity of enzymes and the enantioselectivity of enzymatic reactions is influenced by the solvent, an effect called medium engineering.[25] Following this approach, a selection of solvents that are capable of dissolving the polymer-laccase conjugate were explored under similar reaction conditions. The results clearly show the strong dependence of the TON and enantioselectivity on the nature of the solvent (Table 2, Figure 2).

The highest activity for the catalyst was found in dichloromethane, which exceeds that in chloroform by 20%. However, the enantioselectivity of the reaction was 94.1% *ee*, which is somewhat lower than that in chloroform (99.4% *ee*). In both cases, significant conversion was obtained. The protic, polar solvents methanol and ethanol also afforded a good TON, but with complete loss of enantioselectivity in both cases. DMF and MeCN afford a drastically lower activity and no enantioselectivity at all. The lowest activity of osmate-laccase-PMOx was found in DMSO, but the product formation is somewhat enantioselective (75% *ee*).

The loss of enantioselectivity of the diol formation in methanol and ethanol might be due to the fact that potassium osmate is soluble in these media. This lowers the driving force of the laccase-PMOx to bind osmate by acting as an amphiphilic polymer nanocontainer. Therefore, the specific binding position within the active site of the laccase may not be favored anymore, which leads to a less selective product formation. Control experiments under the same conditions, but without adding the PEC, showed significant conversion of styrene in methanol and ethanol, also resulting in racemic product mixtures. This indicates that the osmate/solvent complex is catalytically active. The same control reaction in the other solvents did not result in styrene conversion, indicating that the complexation of osmate by laccase-PMOx is essential for the catalytic activity in those solvents. Clearly, the complex formation in DMF, DMSO, and MeCN cannot be efficient judged by the low activity. Further, only DMSO seems to direct the osmate, at least in part, to the active site of the enzyme. Generally, TON, TOF, and selectivity can be controlled by the choice of solvent.

Considering both the enantiomeric excess and the turnover number, chloroform is the solvent of choice for further investigations on the osmate-catalyzed dihydroxylation with laccase-PMOx as a chiral ligand.

So far, all reactions had been performed at 0 °C because it was found previously that the dihydroxylation of alkenes with

different PECs as ligand is subject to a dramatic temperature dependency with regard to *ee* and TON.[22]

To explore if the TON can be increased at higher temperature without losing selectivity, the dihydroxylation of styrene was performed at 22 and 4 °C by using different OERs. It was expected that at some point the osmate only binds to the active site, which should have the greatest complexation constant according to previous experiments. It was further presumed that the selective substrate conversion at the osmate-enzyme complex is faster than the unspecific conversion at the free or non-specifically bound osmate owing to ligand acceleration (and thus lower activation energy). Based on this, we expected that suppressing the formation of non-specifically bound osmate might afford more enantioselective product formation even at higher temperatures, and thus achieve greater TONs. The results shown in Table 3 clearly prove that this concept is valid. It was found that the *ee* (*R*) values increase dramatically when the osmate/enzyme ratio (OER) is reduced from 2 to 1 (for example, from 11.3 to 67.8% *ee* (*R*) at 22 °C, Figure 3). Further lowering of the OER to 0.5 increased the enantiomeric excess of the *R* enantiomer to 73.6%. A similar picture is found for a reaction temperature of 4 °C. Although the *ee* value increased from 48 to 84% when decreasing the OER from 2 to 1, further decreasing the OER does not result in higher *ee* values. The same relationship was found for a reaction temperature of 0 °C. These results indicate that only one osmate is bound to the enantioselectivity controlling region in the active site of the laccase, because an OER of 2 affords dramatically reduced selectivity and an OER of 0.5 does not lead to significantly higher selectivity. The TONs with respect to osmate (protein concentration is kept constant) are not strongly dependent on the OER, that is, nearly all osmate molecules are catalytically active. This indicates that the non-specifically bound osmate somewhere within the protein is catalytically active, but not affording selective product formation. Thus, considering the enantiomeric excess of the reaction, lower temperatures are recommended for highly selective product formation even at an OER of 1 or lower.

Table 3. Dependency of the *ee*, TON, and yield on the temperature of the dihydroxylation of styrene[a] in chloroform by using osmate-laccase-PMOx with different OERs.

OER	TON[b]	Yield [%]	*ee* (*R*) [%]
Reaction temperature: 22 °C			
2	724	19.0	11.3
1	842	11.1	67.8
0.5	579	3.8	73.6
Reaction temperature: 4 °C			
2	680	17.9	48.8
1	636	8.4	85.8
0.5	415	2.7	84.4
Reaction temperature: 0 °C			
2	328	8.5	98.6
1	501	6.6	99.4
0.5	–	–	99.4

[a] Reaction performed with 110 μmol mL^{-1} *t*BuOOH, 10 μL mL^{-1} saturated methanolic KOH, and 100 μmol mL^{-1} styrene for seven days. [b] The TON is defined as μmol product per μmol $K_2OsO_2(OH)_4$.

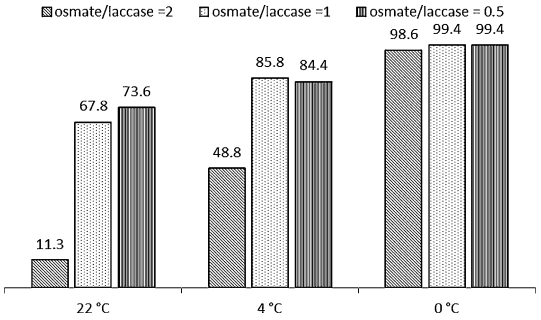


Figure 3. Enantiomeric access (*ee* (*R*) [%]) of the asymmetric dihydroxylation with osmate-laccase-PMOx at different reaction temperatures. The reaction was performed with different OERs, 110 μmol mL^{-1} *t*BuOOH, 10 μL mL^{-1} saturated methanolic KOH, and 100 μmol mL^{-1} styrene for seven days.

The turnover number relative to the osmate after seven days, and thus TOF, as expected, rises with increasing reaction temperature (Table 3). However, the elevation (1.7-fold) in TON, and thus TOF, when increasing the temperature by 22 K is not as high as expected according to the Q_{10} temperature coefficient. According to the latter, it should be a four- to nine-fold increase. This is typically an indication that the reaction is somewhat influenced within its course.

To investigate this, the reaction kinetics were followed for seven days by taking a sample every 24 h. The data are presented in a normalized form in Figure 4. As can be seen, the normalized TON increases linearly at 0 °C over the entire reaction time. When increasing the reaction temperature to 4 °C, the slope of the curve is steeper, but no further product is formed after five days. The TON increases again at 22 °C, but the product formation stops after three days. Comparing the increase in absolute TON values in the linear region shows that

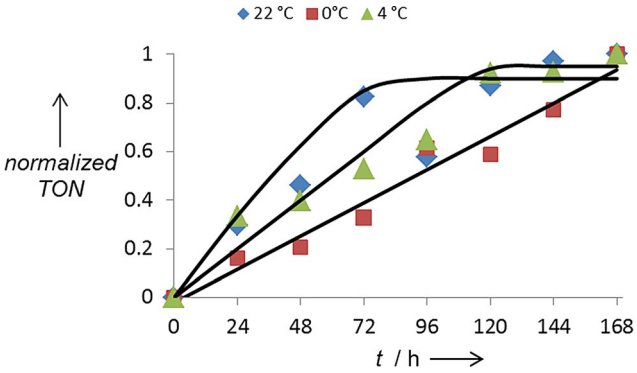


Figure 4. Normalized turnover number (TON/TON(168 h)) versus time plot of the asymmetric dihydroxylation with laccase-PMOx as ligand. The reaction was performed at different temperatures and with 0.013 μmol mL^{-1} laccase-PMOx, 0.013 μmol mL^{-1} $K_2OsO_2(OH)_4$, 110 μmol mL^{-1} *t*BuOOH, 10 μL mL^{-1} saturated methanolic KOH, and 100 μmol mL^{-1} styrene. The TON is defined as μmol product per μmol $K_2OsO_2(OH)_4$.

the TOF increases from 2.98 h^{-1} at 0 °C to 3.74 h^{-1} at 4 °C and 8.60 h^{-1} at 22 °C. This means that the TOF, which is proportional to the reaction rate in this case, increases three-fold when raising the reaction temperature from 0 to 22 °C. This is closer to the typical temperature/reaction rate relationship. Clearly, there is a temperature-dependent loss of catalytic activity of the osmate enzyme complex. This indicates that the active site of the protein is somehow altered during the course of the reaction, probably by the co-oxidizing reagent. Another possibility would be inactivation by the potassium osmate itself.

Another aspect of enzymatic reactions is their typical reaction rate dependency on the substrate concentration according to Michaelis–Menten (MM) kinetics.[26] According to this, the substrate affinity to the active site is the controlling parameter for reaching the maximal reaction rate, that is, low substrate affinity requires higher concentrations for maximal reaction rates. To examine this, the reaction was performed at different styrene concentrations between 12.5 and 300 μmol mL^{-1} at two different temperatures (0 and 22 °C) for 24 h (Table S1 in the Supporting Information).

A reaction time of 24 h was chosen to make sure that the enzyme catalyst is not denatured during the course of the reaction. It was found that the TOFs (proportional to reaction rates) follow a Michaelis–Menten-like course and, thus, trend towards a limit at high styrene concentrations.

It was observed that the TOF at 22 °C increases from 0.72 to 11 h^{-1} when increasing the styrene concentration from 12.5 to 150 μmol mL^{-1} and after that no significant increase in TOF at higher substrate concentrations occurs (Figure 5). This means that the TOF is near the maximal turnover frequency when using a styrene concentration of 150 μmol mL^{-1}. The apparent Michaelis constant K_m was calculated to be 35 μmol mL^{-1} by fitting the data with the MM kinetics.

A different picture is found for the reaction temperature of 0 °C. K_m was determined to be 115 μmol mL^{-1}, indicating that the substrate has a lower affinity to the active site of the enzyme catalyst at 0 °C compared with at 22 °C. This might be due to the fact that the protein folding at the active site changes at this temperature. This is supported by the fact that the enantioselectivity is much higher at the lower temperature, indicating that the temperature-dependent enantioselectivity of the reaction might not only be due to non-specific binding of osmate, but also to refolding of the enzyme. The consequence of this refolding is that the TOF can be increased by up to 30% by increasing the styrene concentration. Thus, we achieved another 1.4-fold increase in the turnover frequency by optimizing the substrate concentration.

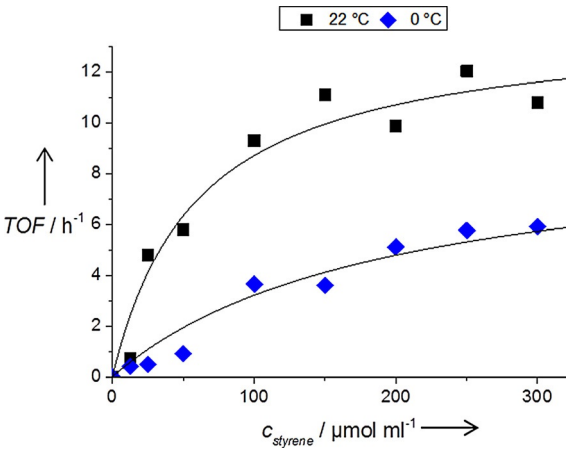


Figure 5. Turnover frequency (TOF) versus concentration of styrene ($c_{styrene}$) plot of the asymmetric dihydroxylation with osmate-laccase-PMOx. The reaction was performed with 0.013 μmol mL^{-1} laccase-PMOx, 0.013 μmol mL^{-1} $K_2OsO_2(OH)_4$, 110 μmol mL^{-1} *t*BuOOH, 10 μL mL^{-1} saturated methanolic KOH, and different concentrations of styrene at different temperatures for 24 h. The TOF is defined as μmol product per μmol $K_2OsO_2(OH)_4$ per h.

After optimizing the reaction conditions for the dihydroxylation of styrene with osmate-laccase-PMOx, we wondered if the reaction can be influenced by variation of the metal in the active site of the laccase. To explore this, potassium permanganate and iron(II) chloride were added to the laccase-PMOx conjugate with a molar metal/enzyme ratio of 1 and tested for their catalyzing potential for the dihydroxylation of styrene (Table 4). In comparison with potassium osmate, potassium permanganate led to a dramatic decrease in *ee* (*R*) from 99.4 to 25.6% and a ten-fold lower TON (501 to 51) after seven days. This might be due to the fact that $KMnO_4$ is a stronger oxidizing reagent than $K_2OsO_2(OH)_4$, which might result in faster protein denaturation. Another class of literature-known reagents for dihydroxylation of alkenes are certain iron complexes.[27] The Fe^{II}-laccase-PMOx conjugate catalyzes the dihydroxylation of styrene with an *ee* of only 11% for the *R* enantiomer and a turnover number of 20 after seven days. In contrast to $K_2OsO_2(OH)_4$, this is a rather low product formation, but the turnover still exceeds that of a literature-known system (TON 9.4 for $[Fe^{II}(4\text{-MeO-}C_6H_4\text{-DPAH})_2](OTf)_2$, no investigations on enantiomeric excess[27b]). Nevertheless, the use of potassium osmate gives the best results with respect to *ee*, TON, and TOF.

Table 4. TON, TOF, yield, and *ee* values of the dihydroxylation[a] of styrene by using different metal species and laccase-PMOx as ligand (ratio 1:1) at 0 °C.

Metal species	TON[b]	TOF[c] [h^{-1}]	Yield [%]	*ee* [%]
$K_2OsO_2(OH)_4$	501	2.98	6.6	99.4 *R*
$KMnO_4$	51	0.30	0.6	25.6 *R*
$FeCl_2$	20	0.07	0.2	11.0 *R*

[a] Reaction performed with 0.013 μmol mL^{-1} laccase-PMOx, 0.013 μmol mL^{-1} of the metal species, 110 μmol mL^{-1} *t*BuOOH, 10 μL mL^{-1} saturated methanolic KOH, and 100 μmol mL^{-1} styrene for seven days. [b] The TON is defined as μmol product per μmol $K_2OsO_2(OH)_4$. [c] The TOF is defined as μmol product per μmol $K_2OsO_2(OH)_4$ per h.

Conclusions

The dihydroxylation of styrene by using an organo-soluble osmate-laccase-PMOx artificial metalloenzyme was explored in detail to find the limitations of this reaction. It was found that exchanging the oxidizing agent from organo-insoluble

$K_3[Fe(CN)]_6$ to *tert*-butyl hydroperoxide results in a homogeneous reaction mixture that affords a 12-fold increase in reaction rate. Investigations on the best-suited solvent showed that chloroform is the most favorable solvent with respect to TOF, TON, and *ee*. Changing the reaction temperature for optimized molar metal/laccase ratios revealed that the enantioselectivity is strongly temperature dependent. Investing the reaction kinetics showed that the affinity of the substrate styrene to the active site of laccase is also temperature dependent. We hypothesize that this is due to a temperature-dependent refolding of the protein, which might also be the reason for the temperature-dependent enantioselectivity. Unfortunately, the reaction must be performed at 0 °C to avoid catalyst deactivation. Exchanging the metal in the active site did not improve the reaction rate or selectivity, but did reveal that, in principle, permanganate and iron(II) salts can also be used to create artificial metalloenzymes with catalytic activity in dihydroxylation reactions. Altogether, the increased reaction rate of TOF = 12 h^{-1} at 22 °C shows that this catalyst is capable of performing the dihydroxylation of styrene in the range of the literature-known Sharpless catalysts also with comparable *ee* values. The aspect of using activating additives such as organic sulfonamides[21b] to accelerate the reaction will be considered in future work. Further, conformational studies on the artificial metalloenzyme to get a deeper insight into the folding of the osmate-laccase complex at different temperatures will be performed.

Experimental Section

Materials

All chemicals were purchased from Sigma–Aldrich, Applichem, Merck, ABCR, Acros, Armar Chemicals, or Carl Roth and were of analytical grade or purer and, with exception of laccase, used without further modification.

Measurements

The turnover number of the dihydroxylation of styrene was determined quantitatively by high performance liquid chromatography (HPLC) measurements (LaChrom Elite, Hitachi) equipped with a Purospher RP-18 column (Merck, LiChroCART 125–4.6, 5 μm). As eluent, a mixture of water and acetonitrile at a flow rate of 1 mL min^{-1} was applied. Thereby, the following eluent gradient was utilized: 10 vol% acetonitrile for 5 min, up to 90 vol% acetonitrile until 10 min, holding for 5 min, down to 10 vol% acetonitrile in 5 min. A diode array detector at 210 nm was applied.
The enantiomeric excess was determined by using a HPLC system (LaChrom Elite, Hitachi) equipped with a chiral column (Chiralpack IC, Daicel Chemical Industries). As eluent, a mixture of *n*-heptane and isopropanol in a ratio of 9:1 was used. A flow rate of 0.5 mL min^{-1} and a diode array detector at 216 nm for 1-phenyl-1,2-ethanediol was applied.

Enzyme concentration

The laccase from *trametes versicolor* (Sigma Aldrich) was concentrated with a fast protein liquid chromatography system (Äkta Start, GE Healthcare) equipped with a HiPrep 16/60 Sephacryl S-100 HR gel filtration column with phosphate buffer (50 mM, 150 mM NaCl, pH 7) as eluent followed by subsequent desalting and lyophilization. The laccase activity of the resulting solid (protein content 30 wt%) was measured to 12370 U mg^{-1} with 2,6-dimethoxyphenol as substrate (25 °C, pH 4.5, acetate buffer, 1 U is defined as absorbance increase of 0.001 per min).

Sharpless dihydroxylation of styrene with poly(2-methyloxazoline) laccase conjugates as ligand (general procedure)

The laccase-PMOx (10 mg, 0.13 μmol) was dissolved in 10 mL of the respective solvent. Afterwards, $K_2OsO_2(OH)_4$ (48 μg, 0.13 μmol for an OER = 1), $KMnO_4$ (21 μg, 0.13 μmol) or $FeCl_2$ (16 μg, 0.13 μmol) was added to the reaction mixture and stirred for 30 min. After that, styrene (115 μL, 1 mmol) and saturated methanolic KOH (100 μL) were added. Finally, a *t*BuOOH solution in decane (5.5 M, 200 μL, 1100 μmol) was added and the reaction mixture was vigorously stirred at different temperatures (0, 4, or 22 °C). The reaction was stopped by extraction with an aqueous sodium thiosulfate solution (1 M, 2×10 mL). Afterwards, the phases were separated and the aqueous phase was extracted five times with chloroform (25 mL) and the organic phases were combined. After removal of the solvent, the obtained solid was characterized by HPLC.
The polymer synthesis and enzyme modification were performed according to previously published work on dihydroxylation with osmate-PECs.[22] Experimental details can be found in the Supporting Information.

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Keywords: asymmetric catalysis · asymmetric dihydroxylation · metalloenzymes · organic solvents · polymer enzyme conjugates

[1] S. C. Stinson, *Chem. Eng. News* **1999**, *77*, 57–65.
[2] a) T. R. Ward, *Chem. Eur. J.* **2005**, *11*, 3798–3804; b) U. T. Bornscheuer, G. W. Huisman, R. J. Kazlauskas, S. Lutz, J. C. Moore, K. Robins, *Nature* **2012**, *485*, 185–194.
[3] a) P. J. Deuss, R. den Heeten, W. Laan, P. C. J. Kamer, *Chem. Eur. J.* **2011**, *17*, 4680–4698; b) F. Yu, V. M. Cangelosi, M. L. Zastrow, M. Tegoni, J. S. Plegaria, A. G. Tebo, C. S. Mocny, L. Ruckthong, H. Qayyum, V. L. Pecoraro, *Chem. Rev.* **2014**, *114*, 3495–3578; c) J. Bos, G. Roelfes, *Curr. Opin. Chem. Biol.* **2014**, *19*, 135–143; d) O. Pàmies, M. Diéguez, J.-E. Bäckvall, *Adv. Synth. Catal.* **2015**, *357*, 1567–1586; e) C. Marchi-Delapierre, L. Rondot, C. Cavazza, S. Ménage, *Israel J. Chem.* **2015**, *55*, 61–75.
[4] a) P. K. Das, J. M. M. Caaveiro, S. Luque, A. M. Klibanov, *Biotechnol. Bioeng.* **2002**, *78*, 104–109; b) C. Esmieu, M. I. V. Cherrier, P. Amara, E. Girgenti, C. Marchi-Delapierre, F. Oddon, M. Iannello, A. Jorge-Robin, C. Cavazza, M. Ménage, *Angew. Chem. Int. Ed.* **2013**, *52*, 3922; *Angew. Chem.* **2013**, *125*, 4014–3925; c) K. Ogura, M. Fujita, H. Iida, *Tetrahedron Lett.* **1980**, *21*, 2233–2239.
[5] a) J. Collot, J. Gradinaru, N. Humbert, M. Skander, A. Zocchi, T. R. Ward, *J. Am. Chem. Soc.* **2003**, *125*, 9030–9031; b) M. T. Reetz, J. J.-P. Peyeralans, A. Maichele, Y. Fu, M. Maywald, *Chem. Commun.* **2006**, 4318–4320; c) M. E. Wilson, G. M. Whitesides, *J. Am. Chem. Soc.* **1978**, *100*, 306–307.
[6] a) A. Fernández-Gacio, A. Codina, J. Fastrez, O. Riant, P. Soumillion, *ChemBioChem* **2006**, *7*, 1013–1016; b) K. Okrasa, R. J. Kazlauskas, *Chem. Eur. J.* **2006**, *12*, 1587–1596.
[7] a) J. Podtetenieff, A. Taglieber, E. Bill, E. J. Reijerse, M. T. T. Reetz, *Angew. Chem. Int. Ed.* **2010**, *49*, 5151–5155; *Angew. Chem.* **2010**, *122*, 5277–

5281; b) M. T. Reetz, N. Jiao, *Angew. Chem. Int. Ed.* **2006**, *45*, 2416 – 2419; *Angew. Chem.* **2006**, *118*, 2476 – 2479.
[8] T. Quinto, D. Haussinger, V. Kohler, T. R. Ward, *Org. Biomol. Chem.* **2015**, *13*, 357 – 360.
[9] a) C. Letondor, T. R. Ward, *ChemBioChem* **2006**, *7*, 1845 – 1852; b) J. Steinreiber, T. R. Ward, *Coord. Chem. Rev.* **2008**, *252*, 751 – 766.
[10] K. Yamamura, E. T. Kaiser, *J. Chem. Soc. Chem. Commun.* **1976**, 830 – 831.
[11] V. Köhler, J. Mao, T. Heinisch, A. Pordea, A. Sardo, Y. M. Wilson, L. Knoerr, M. Creus, J.-C. Prost, T. Schirmer, T. R. Ward, *Angew. Chem. Int. Ed.* **2011**, *50*, 10863 – 10866; *Angew. Chem.* **2011**, *123*, 11055 – 11058.
[12] C. M. Thomas, T. R. Ward, *Chem. Soc. Rev.* **2005**, *34*, 337 – 346.
[13] a) M. Allard, C. Dupont, V. M. Robles, N. Doucet, A. Lledós, J.-D. Maréchal, A. Urvoas, J.-P. Mahy, R. Ricoux, *ChemBioChem* **2012**, *13*, 240 – 251; b) S. Colonna, A. Manfredi, M. Spadoni, *Tetrahedron Lett.* **1987**, *28*, 1577 – 1580; c) W. Laan, B. K. Munoz, R. den Heeten, P. C. J. Kamer, *ChemBioChem* **2010**, *11*, 1236 – 1239.
[14] a) H. R. Luckarift, B. S. Ku, J. S. Dordick, J. C. Spain, *Biotechnol. Bioeng.* **2007**, *98*, 701 – 705; b) P. Tielmann, H. Kierkels, A. Zonta, A. Ilie, M. T. Reetz, *Nanoscale* **2014**, *6*, 6220 – 6228; c) P. Adlercreutz, *Chem. Soc. Rev.* **2013**, *42*, 6406 – 6436.
[15] a) S. Dech, V. Wruk, C. P. Fik, J. C. Tiller, *Polymer* **2012**, *53*, 701 – 707; b) I. Sittko, K. Kremser, M. Roth, S. Kuehne, S. Stuhr, J. C. Tiller, *Polymer* **2015**, *64*, 122 – 129.
[16] a) G. Savin, N. Bruns, Y. Thomann, J. C. Tiller, *Macromolecules* **2005**, *38*, 7536 – 7539; b) I. Schoenfeld, S. Dech, B. Ryabenky, B. Daniel, B. Glowacki, R. Ladisch, J. C. Tiller, *Biotechnol. Bioeng.* **2013**, *110*, 2333 – 2342.
[17] a) N. Bruns, W. Bannwarth, J. C. Tiller, *Biotechnol. Bioeng.* **2008**, *101*, 19 – 26; b) N. Bruns, J. C. Tiller, *Macromolecules* **2006**, *39*, 4386 – 4394.
[18] a) J. Tobis, L. Boch, Y. Thomann, J. C. Tiller, *J. Membr. Sci.* **2011**, *372*, 219 – 227; b) J. Tobis, Y. Thomann, J. C. Tiller, *Polymer* **2010**, *51*, 35 – 45; c) J. Tobis, J. Tiller, *Biotechnol. Lett.* **2014**, *36*, 1661 – 1667.
[19] S. Konieczny, C. Krumm, D. Doert, K. Neufeld, J. C. Tiller, *J. Biotechnol.* **2014**, *181*, 55 – 63.
[20] a) N. Kapil, A. Singh, D. Das, *Angew. Chem.* **2015**, *127*, 6592 – 6595; b) C. H. Ho, M. Thiel, S. Celik, E. K. Odermatt, I. Berndt, R. Thomann, J. C. Tiller, *Polymer* **2012**, *53*, 4623 – 4630.
[21] a) H. C. Kolb, M. S. VanNieuwenhze, K. B. Sharpless, *Chem. Rev.* **1994**, *94*, 2483 – 2547; b) K. B. Sharpless, W. Amberg, Y. L. Bennani, G. A. Crispino, J. Hartung, K. S. Jeong, H. L. Kwong, K. Morikawa, Z. M. Wang, D. Xu, X. L. Zhang, *J. Org. Chem.* **1992**, *57*, 2768 – 2771.
[22] S. Konieczny, M. Leurs, J. C. Tiller, *ChemBioChem* **2015**, *16*, 83 – 90.
[23] S. Konieczny, C. P. Fik, N. J. H. Averesch, J. C. Tiller, *J. Biotechnol.* **2012**, *159*, 195 – 203.
[24] H. Becker, K. B. Sharpless, *Angew. Chem. Int. Ed. Engl.* **1996**, *35*, 448 – 451; *Angew. Chem.* **1996**, *108*, 447 – 449.
[25] A. M. Klibanov, *Nature* **2001**, *409*, 241 – 246.
[26] L. Michaelis, M. L. Menten, *Biochem. Z.* **1913**, *49*, 333 – 369.
[27] a) M. Fujita, M. Costas, L. Que, *J. Am. Chem. Soc.* **2003**, *125*, 9912 – 9913; b) P. D. Oldenburg, Y. Feng, I. Pryjomska-Ray, D. Ness, L. Que, *J. Am. Chem. Soc.* **2010**, *132*, 17713 – 17723; c) P. D. Oldenburg, A. A. Shteinman, L. Que, *J. Am. Chem. Soc.* **2005**, *127*, 15672 – 15673.

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