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Selective Peroxygenase-Catalysed Oxidation of Toluene Derivates to Benzaldehydes

Yutong Wang,[a, b] Niklas Teetz,[c] Dirk Holtmann,[c] Miguel Alcalde,[d] Jacob M. A. van Hengst,[b] Xiaoxiao Liu,[e] Mengfan Wang,*[a] Wei Qi,*[a] Wuyuan Zhang,*[e] and Frank Hollmann*[b]

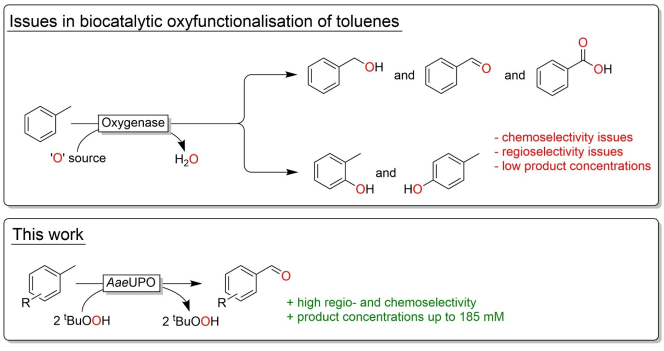
Biocatalytic oxidation reactions of toluene derivates to the corresponding aldehydes are typically challenged by regio- and chemoselectivity issues. In this contribution we address both challenges by a combined reactant- and reaction engineering approach. We demonstrate that the peroxygenase-catalysed transformation of ring-substituted toluenes proceeds highly regioselectively in benzylic position. Furthermore, neat reaction conditions not only enable attractive product concentrations (up to 185 mM) but also result in highly chemoselective oxidations to the aldehyde level.

Introduction

The selective oxyfunctionalisation of C–H-bonds still represents a challenge for organic chemistry. For example, the seemingly simple benzylic hydroxylation of toluene (derivates) is not trivial in terms of selectivity. Industrially, the gas- or liquid phase oxidation of toluene(s) is run to only low conversions in order to maintain an acceptable selectivity of the reaction.[1] Generally, mixtures of the different benzylic oxidation products alcohols, aldehydes and acids are formed.[2] As a consequence, lower yields are obtained and additional product-isolation and –purification efforts are necessary. Besides, even some of the recently developed catalytic systems still necessitate rather high (co)catalysts loadings with 10 mol-% being the rule rather than the exception.

Biocatalytic alternatives have been investigated with P450 monooxygenases being in the centre of attention.[3] Selectivity issues, however, still impair this approach (Scheme 1),[4] which could so far only be partially alleviated by enzyme engineering.[4g,5] Moreover, the preparative attractiveness of the majority of biocatalytic oxidation reactions suffer from low reagent loadings due to the preferred aqueous conditions.

Also the benzylic oxyfunctionalisation catalysed by so-called unspecific peroxygenases (UPOs)[6] is plagued by selectivity issues. Using the archetypal UPO from *Agrocybe aegerita* (*Aae*UPO)[7] the oxyfunctionalisation of toluene yielded in a complex mixture of aromatic and benzylic hydroxylation products. In contrast, *Aae*UPO-catalysed hydroxylations of the toluene homologue ethyl benzene proceed highly selectively with (*R*)-1-phenyl ethanol as the sole product.[8] Apparently, the selectivity of *Aae*UPO-catalysed reactions is governed by the positioning of the substrates relative to compound I (Cpd I, the activated oxyferryl heme species within the active site performing the oxyfunctionalisation reaction) in the active site.



Scheme 1. Biocatalytic oxyfunctionalisation reactions of toluene (derivates) are frequently challenged by selectivity issues and low product concentrations. The combined substrate- and reaction engineering approach used in this contribution addresses these issues yielding highly selective transformations at promising product concentrations.

[a] *Y. Wang, Prof. Dr. M. Wang, Prof. Dr. W. Qi*
School of Chemical Engineering and Technology
Tianjin University
Tianjin 300350 (P. R. China)
E-mail: mwang@tju.edu.cn
qiwei@tju.edu.cn
[b] *Y. Wang, J. M. A. van Hengst, Prof. Dr. F. Hollmann*
Department of Biotechnology
Delft University of Technology
2629 HZ Delft (The Netherlands)
E-mail: f.hollmann@tudelft.nl
[c] *N. Teetz, Prof. Dr. D. Holtmann*
Institute of Bioprocess Engineering and Pharmaceutical Technology
University of Applied Sciences Mittelhessen
Gießen 35390 (Germany)
[d] *Prof. Dr. M. Alcalde*
Department of Biocatalysis
Institute of Catalysis,
CSIC
28049 Madrid (Spain)
[e] *X. Liu, Prof. Dr. W. Zhang*
Tianjin Institute of Industrial Biotechnology
Chinese Academy of Sciences
Tianjin 300308 (P. R. China)
E-mail: zhangwy@tib.cas.cn

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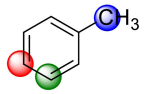
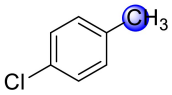
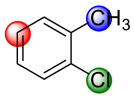
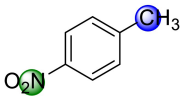
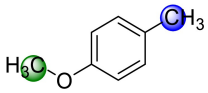
Results and Discussion

We therefore hypothesised that modifying the substitution pattern of the aromatic ring may influence the binding selectivity of the substrates relative to Cpd I and thereby influence the selectivity of the *Aae*UPO-catalysed oxyfunctionalisation reaction.

In silico docking studies (Table 1, see SI for further details) confirmed that toluene binds to the active site in various orientations to the active site (3 conformational clusters were identified presenting both the aromatic ring as well as the benzylic CH_3 group in van der Waals distance to Fe–O, Figure 1).[9] Qualitatively, this is in line with the poor regioselectivity of *Aae*UPO-catalysed oxyfunctionalisation of toluene yielding both ring- and side-chain hydroxylation products (Figure 1). In contrast, docking *p*-chloro toluene and *p*-nitro toluene revealed only one productive binding conformation, being the benzylic CH_3 group and therefore suggesting high regioselectivity for the oxyfunctionalisation of these starting materials. In case of *o*-chloro toluene and *p*-methoxy toluene, the model suggested lower regioselectivities (Table 1).

Table 1. Frequency of different binding clusters of some toluene derivates to CpdII of *Aae*UPO using Autodock.

Substrate	Conformation cluster	Occurrence [%]
	CH_3 presented C3 presented C4 presented	3 19 77
	CH_3 presented	100
	CH_3 presented Cl presented[a] C5 presented	2 96 2
	CH_3 presented NO_2 presented[a]	6 94
	CH_3 presented OCH_3 presented[b]	58 42

[a] unproductive pose, [b] would explain O-demethoxylation.

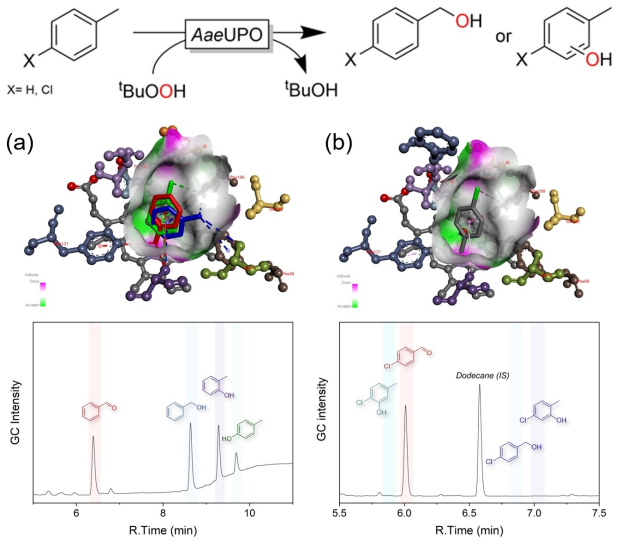


Figure 1. Comparison of the docking of toluene (a) and *p*-chloro toluene (b) to the active site of *Aae*UPO. In case of toluene the three different binding modes are overlaid. Below are the GC-chromatograms of biotransformation solutions showing the products detected. The identity peaks for possible products including benzyl alcohol, benzaldehyde or ring-hydroxylation products was confirmed with authentic standards (their retention times were labelled with corresponding colour bands in the chromatograms).

To test the influence of ring-substitution on the regioselectivity of *Aae*UPO-catalysed transformations, we performed biotransformations under non-aqueous reaction conditions using immobilised *Aae*UPO (Imm-*Aae*UPO). For the biocatalyst immobilisation we followed the protocol previously reported by Kara and co-workers (see SI for further details).[10] ^{t}BuOOH served as hydrophobic replacement for H_2O_2 as stoichiometric oxidant. Control reactions in the absence of the biocatalyst did not result in detectable product formation.

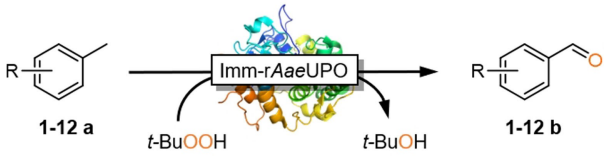
Indeed, when using *p*-chloro-toluene (**1a**) as starting material, only *p*-chloro-benzaldehyde (**1b**) was observed as sole product. Whereas, both ring- and side chain hydroxylation products were observed when using toluene as substrate under identical conditions (Figure 1).

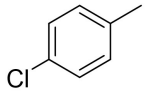
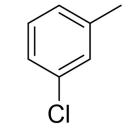
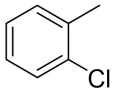
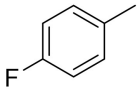
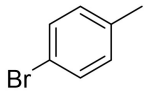
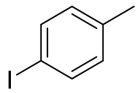
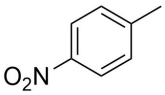
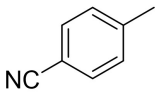
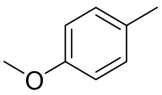
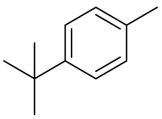
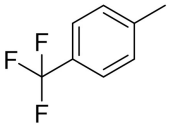
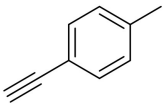
To investigate if this dramatic change in selectivity was unique to *p*-chloro toluene or rather a general phenomenon, we further evaluated a range of ring-substituted toluene derivates as substrates for the *Aae*UPO-catalysed oxyfunctionalisation reaction (Table 2). With the exemption of *p*-ethynyl toluene (**12a**, for which no product conversion was observed) all ring-substituted toluenes tested were converted into the corresponding benzaldehyde products in higher than 92% selectivity (generally >96% selectivity, see SI for chromatograms of the individual reactions). In contrast to the docking predictions, no ring hydroxylation for *o*-chloro toluene nor O-demethoxylation for *p*-methoxy toluene[11] occurred and only the corresponding benzaldehyde products were observed.

Next to the exclusive regioselectivity of the hydroxylation reaction we also observed a very high chemoselectivity for the aldehyde (double oxidation) product. The intermediate alcohols never accumulated to more than (2 mM) and benzoic acid derivates were never observed. These findings are in stark contrast to previously reported *Aae*UPO-catalysed oxyfunctionalisation of (*p*-Cl) toluene.[11] We assume that both observations may be explained by the non-aqueous reaction conditions used in this study. For the 'through oxidation' of aldehydes to carboxylic acids the aldehyde *gem*-diol has been proposed as the actual substrate for *Aae*UPO.[12] The lack of excess water in the present reaction conditions impedes the *gem*-diol formation and thereby the overoxidation of the aldehyde product to the acid.

Considering the apparent much faster oxidation of the intermediate benzyl alcohols to their aldehyde derivates we hypothesise that the relative affinity of the toluene starting materials and the benzyl alcohols for the *Aae*UPO active site differs with the polarity of the reaction medium. In a highly

Table 2. Substrate scope of the *Aae*UPO-catalysed, selective benzylic oxyfunctionalisation of ring-substituted toluene derivates.



Substrate		Initial velocity ($mM\,h^{-1}$)	[Benzaldehyde][a] (mM)	Selectivity[b] (%)	Time[c] (h)	TON
	**1a**	4.7 ± 0.02	114 ± 3	97	48	11323
	**2a**	2.3 ± 0.5	84 ± 20	98	34	8343
	**3a**	4.3 ± 0.4	99 ± 9	98	37	9833
	**4a**	3.0 ± 0.04	61 ± 0.5	96	34	6059
	**5a**	4.6 ± 0.8	109 ± 12	97	34	10826
	**6a**	3.3 ± 0.03	83 ± 11	96	34	8244
	**7a**	9.5 ± 0.02	185 ± 29	97	35	18375
	**8a**	2.6 ± 0.03	44 ± 0.7	96	34	4370
	**9a**	4.0 ± 0.06	70 ± 2	92	37	6953
	**10a**	4.3 ± 0.3	94 ± 5	97	30	9337
	**11a**	5.5 ± 0.03	98 ± 2	98	18	9734
	**12a**	N.A.	N.A.	N.A.	N.A.	N.A.

[a] The product concentration was corrected from potential volume change of the reactant solution. [b] All the non-target peaks were integrated as side products, and the concentration of side products were estimated based on the GC response factor of internal standard *n*-dodecane (5 mM). A further characterisation (e.g. via GC-MS) was not possible due to the very low intensity of the unidentified peaks. [c] Active reaction time was recorded when reaching the highest product concentration. General conditions: Neat organic reactions were conducted in 1.5 mL glass GC vials with 100 mg imm-*Aae*UPO (5 nmol UPO immobilised) and 0.5 mL substrate. Specially, when the substrate was solid under 30 °C (*p*-nitro-toluene and *p*-iodo-toluene), saturate substrate solution was prepared in acetone. ^{t}BuOOH (in decane) of 800 mM was fed under 10 $\mu L\,h^{-1}$ using syringe pump. The reaction was performed as technical duplicates at 30 °C under 800 rpm stirring in thermo-shaker. N.A.: no activity, i.e. no product formation observed.

polar medium such as water the more hydrophobic toluene starting material (logP ca. 2.7)[16] may have a higher affinity for the hydrophobic enzyme active site than the more polar benzyl alcohol (logP ca. 1.1) resulting in a lower K_M value for toluenes compared to the corresponding benzyl alcohols. In the current experiments (in hydrophobic media) this affinity effect may be less pronounced, and the relative reaction rate is dominated by the lower C–H activation energy of the alcohols compared to the toluenes. Hence the high chemoselectivity for the aldehyde product can be explained by the non-aqueous reaction conditions.

Interestingly enough, no obvious correlation between the substitution pattern (electron-donating- or -withdrawing substituents) and the conversion rate could be identified (Figure S4). This may indicate both, electronic effects and substrate binding (steric effects) determining the rate of the *Aae*UPO-catalysed oxyfunctionalisation. A more in-depth kinetic investigation will further shed light on the interplay of both factors on the conversion efficiency of *Aae*UPO.

As shown in Table 1, on average 100 mM of the desired aldehyde products were obtained corresponding to more than 10 g L^{-1} (up to 18 g L^{-1} in case of **7**) with *Aae*UPO performing 4300–18000) catalytic cycles. Though these values are already encouraging, we further investigated factors influencing to robustness of the overall reaction. Particularly, we investigated the influence of the addition rate of ^{t}BuOOH (Figure 2). Quite expectedly, there was a direct correlation of the initial reaction rate on the ^{t}BuOOH addition rate with an almost stoichiometric correlation between peroxide feed rate with product formation rate. The robustness of the overall reaction, however, decreased at elevated ^{t}BuOOH addition rates with a maximum between 4 and 8 mM h^{-1}. As a result, a maximal product formation was observed for ^{t}BuOOH addition rates between 8 and 20 mM h^{-1}.

Encouraged by these promising results, we finally performed a semi-preparative scale transformation of *p*-chloro toluene (**1 a**) in a rotating bed reactor (RBR). From 120 mL **1 a** after 20 h of reaction time approx. 8 mmol of the desired *p*-chloro benzaldehyde (**1 b**) product were formed and isolated for the reaction mixture using the bisulfite adduct method.[13] Overall 0.6 g of pure **1 b** were isolated (corresponding to 45 % of the total product formed). Obviously, future preparative applications will have to either optimise the bisulfite adduct method or rely on alternative product isolation methods (e.g. chromatographic purification).

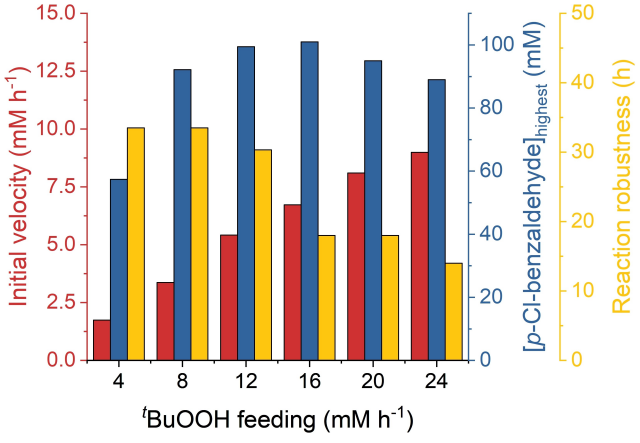


Figure 2. Initial reaction velocity, highest product concentration and reaction robustness of the imm-*Aae*UPO catalysed oxyfunctionalisation of *p*-chloro-toluene (**1 a**) to *p*-chloro-benzaldehyde (**1 b**) in a neat reaction system. The robustness was indicated by the time when highest product concentration was achieved. General conditions: [imm-*Aae*UPO] = 0.2 g × mL^{-1}, [*Aae*UPO] = 10.1 μM T = 30 °C, 800 rpm, initial reaction volume = 0.5 mL; 0.2–1.2 M ^{t}BuOOH in *n*-Decane was continuously fed at 10 μL × h^{-1}.

Conclusions

Overall, in the current contribution we have utilised substrate engineering[14] to improve the selectivity of the *Aae*UPO-catalysed benzylic oxidation of toluenes. The docking analysis will put the basis for future rational *Aae*UPO engineering to improve the regioselectivity of the transformation of toluene itself. This contribution demonstrates the power of reaction engineering to gain control over the chemoselectivity of a multi-step oxidation reaction.[15] Simply by moving from traditional aqueous reaction media to non-aqueous conditions dramatically improved the aldehyde selectivity. Furthermore, the product concentrations enabled by the non-conventional (non)solvent encourage us to further explore the preparative potential of the proposed system.

Obviously, the immobilised enzyme also offers the possibility to re-use it in further reactions, which remains to be demonstrated. Also the overall conversion of the neat starting material is rather low calling for further optimisation.

Experimental section

***In silico* docking studies**: For each substrate, 50 runs of semi-flexible docking via Autodock 4.2 was performed with a genetic algorithm. Substrates were docked against the structure of *Aae*UPO-PaDaI (PDB entry: 5OXU). Free water molecules in the structure were deleted beforehand, Kollmann charges were calculated, polar hydrogens added, and histidine charges were calculated. Structures for the substrates were downloaded from the PubChem database, Gasteiger charges were calculated, and torsion bonds defined. For each docking run, starting population was 150 and 27000 generations with 2.5 million evaluations, mutation rate of 0.02 and crossover rate of 0.8 were conducted.

Enzyme immobilisation: Amino-functionalised resin carrier LXTE-700S was utilised for the enzyme immobilisation, and the immobilisation procedure followed the product instruction with slight modifications: Firstly, the resin carrier was washed three times using sodium phosphate buffer (pH 8.0, 50 mM) and the amino-resin was then activated for 1 h by 2% glutaraldehyde with gentle shaking. Secondly, the activated carrier was mixed with enzyme solution (generally 21 μM) and incubated for 3 h under 25 °C with gentle shaking. The mixture was then set still at 4 °C for another hour followed by washing and filtration. The prepared imm-*Aae*UPO was stored at 4 °C. The supernatant after immobilisation and washing buffers were collected for further determination of immobilisation efficiency (Table S1). Between each step, the carrier was washed three times with sodium phosphate buffer (pH 8.0, 50 mM), and the free solution was removed by vacuum filtration. The ratio of carrier to solution was 1 : 4 (m/v) in all steps.

Biocatalytic oxyfunctionalisation reactions: Reactions were conducted in 1.5 mL glass GC vials with 100 mg imm-*Aae*UPO and 500 μL substrate solution (the solid starting materials **6a** and **7a** were dissolved in acetone), and ^{t}BuOOH (in decane) was fed under 10 μL h^{-1} using syringe pump. The reaction was performed at 30 °C under 800 rpm stirring in thermo-shaker.

Semi-preparative scale reaction: For the scale-up reaction, model substrate *p*-Cl-toluene was used as starting material, and rotating bed reactor (RBR) was utilised to conduct the reaction. Specifically, the reaction was conducted in a SpinChem Vessel S2 (200 mL) using a SpinChem RBR S2 rotating bed (SpinChem AB, Sweden). The rotating bed was loaded with 18 g imm-*Aae*UPO, and the reactor was filled with 120 mL *p*-Cl-toluene. The rotating bed was adjusted to be 1 cm above the reactor bottom, and the reaction was run at 400 rpm and 30 °C. ^{t}BuOOH (600 mM in *n*-decane) was fed with syringe pump 2 mL h^{-1}.

Acknowledgements

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: Biocatalytic oxidation · Selective oxyfunctionalisation · Peroxygenase · Solvent-free biocatalysis · Benzaldehydes

[1] F. Brühne, E. Wright, in *Ullmann's Encyclopedia of Industrial Chemistry*, **2011**, DOI: 10.1002/14356007.a03_463.pub2.
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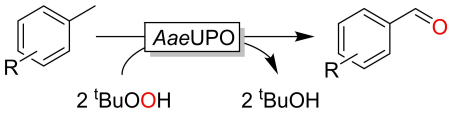

- no-aqueous reactions
- 11 examples
- up to 180 mM of product
- aldehyde selectivity > 92%

The peroxygenase from *Agrocybe aegerita* highly selectively converts substituted toluene derivates into the corresponding aldehydes. Using the immobilised enzyme enables non-aqueous reaction conditions and high product titres.

Y. Wang, N. Teetz, Prof. Dr. D. Holtmann, Prof. Dr. M. Alcalde, J. M. A. van Hengst, X. Liu, Prof. Dr. M. Wang, Prof. Dr. W. Qi*, Prof. Dr. W. Zhang*, Prof. Dr. F. Hollmann**

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Selective Peroxygenase-Catalysed Oxidation of Toluene Derivates to Benzaldehydes