Biocatalytic Aromaticity-Breaking Epoxidation of Naphthalene and Nucleophilic Ring-Opening Reactions

Wuyuan Zhang,* Huanhuan Li, Sabry H. H. Younes, Patricia Gómez de Santos, Florian Tieves, Gideon Grogan, Martin Pabst, Miguel Alcalde, Adrian C. Whitwood, and Frank Hollmann*

Cite This: ACS Catal. 2021, 11, 2644−2649

ABSTRACT: Aromatic hydroxylation reactions catalyzed by heme-thiolate enzymes proceed via an epoxide intermediate. These aromatic epoxides could be valuable building blocks for organic synthesis giving access to a range of chiral trans-disubstituted cyclohexadiene synthons. Here, we show that naphthalene epoxides generated by fungal peroxygenases can be subjected to nucleophilic ring opening, yielding non-racemic trans-disubstituted cyclohexadiene derivatives, which in turn can be used for further chemical transformations. This approach may represent a promising shortcut for the synthesis of natural products and APIs.

KEYWORDS: arene epoxides, arene oxyfunctionalization, biocatalysis, chemoenzymatic reactions, naphthalene epoxides, oxidation, peroxygenase

Aromatic systems are thermodynamically and kinetically very inert. Reactions breaking aromaticity are rare but interesting from a preparative point of view.1−3 Even less common are enzymatic aromaticity-breaking reactions. Non-heme Fe-dioxygenases have been reported for stereoselective cis-dihydroxylation of arenes.4−5 More recently, enzymatic Birch-type reductions6−8 and dearomatizing arene oxidations9−10 have also been reported.

Aromatic hydroxylation reactions catalyzed by heme-dependent enzymes such as P450 monoxygenases10 and related peroxygenases11−15 are well known to proceed via an intermediate, short-lived arene oxide prone to rapid, spontaneous rearrangement into the corresponding phenol products (known as NIH-shift, Scheme 1b).16 Interestingly, capturing the intermediate arene oxides with nucleophiles has so far not been considered. This is unfortunate as arene oxides are potentially very useful building blocks for chemical synthesis. Nucleophilic opening of the reactive epoxide ring leads to trans-disubstituted cyclohexadiene derivatives, which can serve as starting materials for various syntheses.16−20 Their synthetic relevance today, however, is limited due to tedious, multistep synthesis protocols (Scheme 1a).21−23 Utilizing heme-enzyme-derived aromatic epoxides, which are formed directly from the corresponding arenes under mild conditions, would give a more straightforward access to such trans-disubstituted cyclohexadiene derivatives.

We therefore set out to investigate whether trans-disubstituted cyclohexadiene derivatives can be obtained in a chemoenzymatic reaction comprising enzymatic formation of arene oxides followed by nucleophilic epoxide opening (Scheme 1c). As a starting point, we chose the recombinant, evolved peroxygenase from Agrocybe aergerita (rAaeUPO) as a catalyst; more specifically, we first used a previously evolved variant (PaDa-I).24 Naphthalene was used as a starting material.25 Upon addition of PaDa-I (0.2 μMfinal) to a buffered solution of naphthalene and H2O2 (2 mM each), we could detect (and quantify) naphthalene-1,2-epoxide (2) via its characteristic absorption band at 266 nm.25 2 was relatively stable and rearranged into the corresponding 1-naphthol within several minutes (Figures S30 and S31). The epoxide formation rate correlated with the concentration of the biocatalyst (Table 1, entries 1−3). Increasing the H2O2 concentration above 2 mM resulted in a decreased concentration of 2 (Table 1, entries 2, 4, and 5), which is readily explained by the oxidative inactivation of heme enzymes by excess H2O2.26 Further investigations will focus on determining the kinetic parameters of the enzyme-catalyzed epoxidation reaction. No epoxide formation was observed in the absence of PaDa-I, naphthalene, or H2O2. Isotope labeling experiments using H18O2 confirmed that the oxygen atom incorporated originated from H2O2 (Figure S27).

The decay of the intermediate epoxide accelerated upon addition of azide, which we interpreted to be the result of a nucleophilic ring opening. Next, we investigated the factors influencing the overall yield in the desired addition product.
Scheme 1. Synthesis of Arene Oxides*

*(a) The established, multistep synthesis of arene oxides at the example of benzene oxide. (b) Heme-enzyme-catalyzed oxyfunctionalisation of aromatic compounds (e.g., benzene) proceeds via an intermediate arene epoxide spontaneously rearranging into the corresponding phenol; (c) In this work, we demonstrate that the intermediate arene oxides (e.g., obtained from peroxygenase-catalyzed transformation of naphthalene) can be reacted with nucleophiles, yielding chiral trans-disubstituted cyclohexadiene derivates.

(4a) (Table 1). Increasing the nucleophile concentration significantly shifted the ratio of the desired (4a) to the undesired (3) (Table 1, entries 6–9). Prolonging the reaction time allocated to the enzymatic epoxidation had no significant influence on the overall conversion of the naphthalene starting material (ranging between 80 and 85%, Table 1, entries 10–13) but increased the yield in the undesired rearrangement product 1-naphthol (3). Adding NaN₃ from the beginning of the reaction resulted in the complete recovery of the naphthalene starting material, which we attribute to the N₃⁻-related inactivation of the heme enzyme. It is worth mentioning that the azide attacked the epoxide selectively at the C1 position (yielding the 1-azido-2-ol product). This selectivity was observed only with epoxide 2 and we are currently lacking a plausible explanation for this peculiarity.

From a semi-preparative reaction of 1-bromonaphthalene, approximately 39 mg of essentially pure (1S,2S)-2-azido-5-bromo-1,2-dihydrornaphthalen-1-ol was isolated (73% isolated yield), crystallized, and analyzed via X-ray crystallography (see Supporting Information for further details). The structure of the ring-opened product 4b showed an excellent Flack parameter [−0.02(3)], thus allowing the determination of the absolute configuration ((1S,2S)-2-azido-5-bromo-1,2-dihydrornaphthalen-1-ol). This corresponds well with the predicted stereoselectivity of the PaDa-I-catalyzed epoxidation of naphthalene (Figure 1B). Also, the crystallization of 4a was successful; however, probably due to the lack of a heavy atom in the structure, the crystallized product 4a showed a less convincing configuration (Flack) parameter [−0.3(3)]. As mentioned above, the ring opening occurred via a nucleophilic attack in the C2 position.

To further explore the substrate scope of the proposed reaction sequence, a range of naphthalene derivates were evaluated (Figure 2). Using azide as a nucleophile, the yields ranged between 19 and 75%; products 4a, 4b, 4c, 4d, and 4e were prepared on a semi-synthetic (0.2 mmol) scale (for full experimental details as well as ¹H NMR, ¹³C NMR, ¹H–¹H COSY, and HMQC-analysis of the products, see Figures S1–22). The isolated yields ranged between 20 and 73% giving access to 20–50 mg of the products, thereby correlating with the above-determined NMR-yields. So far, only the crystal structure of 4b is available, and we compared the circular dichroism spectra of some representative products (i.e., 4d, 4g, and 4i) with the CD spectrum of 4b (Figures S32 and S33). The congruence of the optical rotatory dispersion curves suggests an identical (1S, 2S) configuration of these products as well.

In an attempt to also broaden the nucleophile scope of the reaction, we tested formate as an alternative nucleophile (Figure 2, 4j and 4k). The isolated yields, however, were significantly lower (9–15%) than observed using azide, which most likely can be attributed to the poorer nucleophilicity of formate compared to azide under the reaction conditions.

Aiming at extending the arene scope of the proposed chemoenzymatic reaction sequence, we also evaluated benzene as the starting material. PaDa-I, the rAaeUPO variant used so far, exhibited poor activity with benzene (Figure 2, 4m, blue). We therefore also evaluated the SoLo variant (engineered from PaDa-I) for the transformation of benzene (Figure 2, 4m, green). The significantly higher activity of SoLo on benzene (48% yield) compared to PaDa-I (traces) can be rationalized by its modified active site geometry: SoLo carries two mutations at the heme access channel (F191S and G241D, Figure 3). Possibly, the G241D substitution induces a displacement in the α-helix hosting the catalytic acid–base pair (R189-E196). This conformational change may favor the positioning of smaller arenes (see docking simulations, Figure S34) and therefore facilitate the conversion of benzene.

Finally, we explored the synthetic possibilities of 4a. Rearomatization proved to be astonishingly difficult as only concentrated perchloric acid enabled the full conversion of the intermediate epoxide (2) into the aromatic azide (5) (Figure 4 and see also Table S1). In contrast, catalytic hydrogenation over Pd/C to form a compound (7, Figures S23 and S24) or
Table 1. Influence of Reaction Time and Nucleophile Concentration on the Proposed Epoxidation Ring-Opening Reaction

<table>
<thead>
<tr>
<th>Entry</th>
<th>c(PaDa-I) [mM]</th>
<th>c(H2O2) [mM]</th>
<th>c(NaNO2) [mM]</th>
<th>c(4a) [mM]</th>
<th>c(3) [mM]</th>
<th>TON (PaDa-I)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.100</td>
<td>2.000</td>
<td>1.270</td>
<td>0.320</td>
<td>6350</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.200</td>
<td>2.000</td>
<td>1.340</td>
<td>0.300</td>
<td>6700</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.400</td>
<td>2.000</td>
<td>1.430</td>
<td>0.260</td>
<td>7250</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.200</td>
<td>1.000</td>
<td>1.480</td>
<td>0.730</td>
<td>3650</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.200</td>
<td>4.000</td>
<td>1.480</td>
<td>0.910</td>
<td>4500</td>
<td></td>
</tr>
</tbody>
</table>

Reaction conditions: [naphthalene] = 2 mM, [H2O2] = 1 mM, and [NaNO2] = 100 mM in NaPi buffer pH 7.0 (30% CH3CN), 30 °C. The spectrum was recorded after diluting the solutions 100 fold by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 12% gels stained with Coomassie Brilliant Blue R-250 (Figure S34). Chemical reactions. A typical epoxidation reaction was performed at a 1 mL scale in NaPi buffer (100 mM, pH 7, containing 30% v/v acetonitrile as a cosolvent) at 25 °C. The reaction mixture contained an aromatic starting material (2 mM) and rAaeUPO (PaDa-I or SoLo 200 mM). Reactions were started by addition of H2O2 (2 mM final) and gently stirred for 2.5 min after which the nucleophile was added from a concentrated stock solution. After the completion of the reaction, the reaction mixture was extracted with an aliquot dichloromethane. The organic phase was dried over MgSO4 and evaporated under reduced pressure. The crude product was purified over a silica column using 10% ethyl acetate in pentane/heptane as the eluent.

Crystal Structures of 4a and 4b. Diffraction data were collected at 110 K on an Oxford Diffraction SuperNova diffractometer with Cu Kα radiation (λ = 1.54184 Å) using an Eos CCD camera. The crystal was cooled with an Oxford Instruments Cryosystems. Diffractometer control, data collection, initial unit cell determination, frame integration, and unit-cell refinement was carried out with “Crysalis”.29 Face-indexed absorption corrections were applied using spherical harmonics, implemented in the SCALE3 ABSPACK scaling algorithm.20 OLEX2 was used for the overall structure solution, refinement, and preparation of computer graphics and

In this study, we have demonstrated that peroxygenases provide a convenient access to naphthalene epoxide and possibly further arene epoxides for the synthesis of chiral trans-disubstituted cyclohexadiene derivatives.

EXPERIMENTAL SECTION

Biocatalyst Preparation. The expression of the rAaeUPO variants PaDa-I and SoLo was performed in recombinant Pichia pastoris following a previously described procedure.28 The culture broth of P. pastoris cells containing the peroxygenase in the supernatant was centrifuged at 8000 rpm for 2 h at 4 °C. The supernatant was filtered through a 20 μm filter and kept at −80 °C until further use. A detailed description of the cultivation procedure can be found in the Supporting Information. The enzymes used in the study were essentially pure as confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 12% gels stained with Coomassie Brilliant Blue R-250 (Figure S34).

Chemoenzymatic Reactions. A typical epoxidation reaction was performed at a 1 mL scale in NaPi buffer (100 mM, pH 7, containing 30% v/v acetonitrile as a cosolvent) at 25 °C. The reaction mixture contained an aromatic starting material (2 mM) and rAaeUPO (PaDa-I or SoLo 200 mM). Reactions were started by addition of H2O2 (2 mM final) and gently stirred for 2.5 min after which the nucleophile was added from a concentrated stock solution. After the completion of the reaction, the reaction mixture was extracted with an aliquot dichloromethane. The organic phase was dried over MgSO4 and evaporated under reduced pressure. The crude product was purified over a silica column using 10% ethyl acetate in pentane/heptane as the eluent.

Crystal Structures of 4a and 4b. Diffraction data were collected at 110 K on an Oxford Diffraction SuperNova diffractometer with Cu Kα radiation (λ = 1.54184 Å) using an Eos CCD camera. The crystal was cooled with an Oxford Instruments Cryosystems. Diffractometer control, data collection, initial unit cell determination, frame integration, and unit-cell refinement was carried out with “Crysalis”.29 Face-indexed absorption corrections were applied using spherical harmonics, implemented in the SCALE3 ABSPACK scaling algorithm.20 OLEX2 was used for the overall structure solution, refinement, and preparation of computer graphics and

CuI-catalyzed 1,3-dipolar cycloaddition with terminal alkynes (yielding products 8a and 8b, Figures S25 and S26) proved to be straightforward (Figure 4). S could be readily reduced into aromatic amine (6, Figure S28).

Figure 1. Crystal structure of (1S,2S)-2-azido-5-bromo-1,2-dihydronaphthalen-1-ol (A) obtained from the PaDa-I-catalyzed epoxidation of 1-Br-naphthalene followed by nucleophilic ring opening with NaN3. (B) Active site model of PaDa-I in complex with naphthalene presenting the pro-(R)-face compound I. Naphthalene is placed between Phe121 and Phe199 in a T-packing mode with strong hydrophobic interaction with Phe69 that maintains a highly conserved orientation. Dark blue: Phe triad orienting the substrate, yellow: acid–base pair, and light blue: Phe pair involved in the guidance of the substrate to the active site.
Within OLEX2, the algorithm used for the structure solution was dual-space within ShelXT.\textsuperscript{32} Refinement by full-matrix least-squares used the SHELXL\textsuperscript{33} algorithm within OLEX2. All non-hydrogen atoms were refined anisotropically. Hydrogen atoms were placed by a difference map and refined.

Molecular docking simulations were performed using the Autodock VINA algorithm\textsuperscript{34} included in YASARA Structure software.\textsuperscript{35,36} Docking computations were performed at the level of the YASARA force field by running a number of 100 docking trials. Models were visualized with PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.

Figure 2. Preliminary product scope of the rAaeUPO variants PaDa-I and SoLo. Reaction conditions: [substrate] = 2 mM, [H$_2$O$_2$] = 2 mM, and [peroxygenase] = 200 nM in NaPi buffer pH 7.0 (30\% CH$_3$CN), [NaN$_3$] = 125 mM, T = 30 °C. The conversion of benzene derivatives was determined by $^1$H NMR. n.d.: not detected. a: isolated yield and structure confirmed by the crystal structure or 2D NMR; b: NMR-yield; characteristic peaks after ring opening (between 6.2 and 6.8 ppm) were used for the integration of the substrate, phenol as the side product, and diene as the product.

Figure 3. Active site models of the rAaeUPO variants used (PaDa-I, left and SoLo, right). The mutations in SoLo with respect to PaDa-I (F191S, G241D) are highlighted in pink.
Figure 4. Exploring the synthetic potential of 4. Reaction conditions: (a) Following the epoxidation and ring-opening reactions, the pH of the solution was then adjusted to approximately 9.0 using NaOH (5 M). The Pd/C catalyst (10 mol % final concentration) was added. N₂ gas was bubbled through the solution for 10 min, and a balloon of pure H₂ was applied to initiate the reduction. The reduction was carried out for 6 h. (b) Perchloric acid (3 M) was added after the epoxidation and ring-opening reactions and the mixture was stirred for 5 h. (c) Isolated azido alcohol 7 and alkyne (e.g., 1.5 equiv of phenylacetylene) were dissolved in H₂O and tert-butyl alcohol (2:1, v/v). CuSO₄·5H₂O (5 mol %) and sodium ascorbate (10 mol %) were added. The reaction mixture was stirred for 8 h at 30 °C. (d) Sequence of reactions (b) and (a).

**ASSOCIATED CONTENT**

*Supporting Information*

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscatal.0c05588.

Experimental procedures; analytical data; methods and synthetic procedures; X-ray crystallography; and determination of the absolute configuration (PDF)

**AUTHOR INFORMATION**

**Corresponding Authors**

Wuyuan Zhang — Department of Biotechnology, Delft University of Technology, 2629HZ Delft, The Netherlands; Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China; orcid.org/0000-0002-3182-5107; Email: zhangwy@tib.cas.cn

Frank Hollmann — Department of Biotechnology, Delft University of Technology, 2629HZ Delft, The Netherlands; orcid.org/0000-0003-4821-756X; Email: f.hollmann@tudelft.nl

**Authors**

Huanhuan Li — School of Chemical Engineering and Technology, Xi’an Jiaotong University, Xi’an 710049, China

Sabry H. H. Younes — Department of Biotechnology, Delft University of Technology, 2629HZ Delft, The Netherlands; Department of Chemistry, Faculty of Sciences, Sohag University, Sohag 82524, Egypt

Patricia Gómez de Santos — Department of Biocatalysis, Institute of Catalysis, CSIC, 28049 Madrid, Spain

Florian Tieves — Department of Biotechnology, Delft University of Technology, 2629HZ Delft, The Netherlands

Gideon Grogan — York Structural Biology Laboratory, Department of Chemistry, University of York, YO10 SDD York, U.K.; orcid.org/0000-0003-1383-7056

Martin Pabst — Department of Biotechnology, Delft University of Technology, 2629HZ Delft, The Netherlands

**Miguel Alcalde** — Department of Biocatalysis, Institute of Catalysis, CSIC, 28049 Madrid, Spain; orcid.org/0000-0001-6780-7616

**Adrian C. Whitwood** — Department of Chemistry, University of York, YO10 SDD York, U.K.; orcid.org/0000-0002-5132-5468

Complete contact information is available at: https://pubs.acs.org/doi/10.1021/acscatal.0c05588

**Funding**

Financial support by the National Key Research and Development Program of China (No. 2019YFA0905100), the European Research Council (ERC Consolidator Grant No. 648026), and the Netherlands Organization for Scientific Research (VICI grant, No. 724.014.003) is gratefully acknowledged. The authors also thank the Ministry of Science, Innovation and Universities (Spain) for the FPI contract (BES-2017-080040, PGS) and the Comunidad de Madrid Synergy CAM Project Y2018/BIO-4738-EVOCHIMERA-CM.

**Notes**

The authors declare no competing financial interest.

**REFERENCES**


