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COMMUNICATION

Scalable Biocatalytic Oxygenation of Aliphatic Amine Derivatives Using a Class I Unspecific Peroxygenase

Jiacheng Li,[a] Katy A. S. Cornish,[a] Jared Cartwright,[b] William P. Unsworth*[a] and Gideon Grogan*[a]

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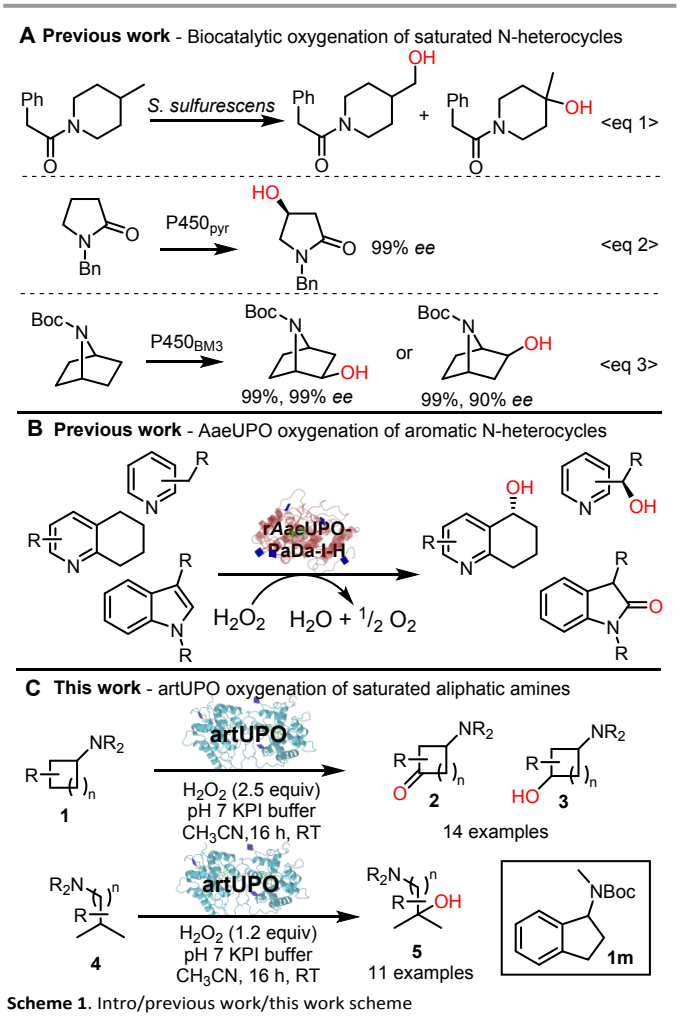
artUPO is revealed as an effective biocatalyst for the regioselective oxygenation of cycloalkylamines to form cyclic amino ketones on preparative scale. Furthermore, amino alcohols can be formed *via* the selective C-H oxygenation of isopropyl-tethered amine derivatives. Enantiomerically enriched amino alcohols are also accessible using a kinetic resolution approach.

N-Heterocycles feature prominently in biologically active compounds and FDA approved pharmaceuticals.[1-3] New methods to prepare oxygenated *N*-heterocycles *via* oxidative processes are therefore of value, both for organic synthesis and the study of metabolites. In this context, the selective oxygenation of saturated *N*-heterocycles presents a significant challenge, as they usually have several potential reaction sites with similar susceptibility to oxygenation.

Biocatalysis has thus been investigated for its utility in preparing oxygenated *N*-heterocycles *via* C–H oxygenation. Early studies at Upjohn in the late 1960s focused on the use of whole-cell preparations of filamentous fungi such as *Beauveria bassiana* (formerly *Sporotrichum sulfurescens*);[4-6] this work was further developed by the groups of Roberts/Willetts[7] and Flitsch.[8] In these cases, various *N*-heterocycles were hydroxylated at different positions, assisted by derivatisation of the N atom, which was though to assist substrate recognition by the cytochromes P450 (P450s) in the cells. However, in many cases mixtures of products formed, presumably as a consequence of the activity of multiple P450s within the organism (*e.g.* Scheme 1A, eq. 1). The heterologous expression of isolated microbial P450s has allowed greater specificity to be achieved; *e.g.* Li/Witholt and co-workers exploited the $P450_{Pyr}$ from *Sphingomonas* sp. HXN-200 for the regio- and enantio-selective hydroxylation of pyrrolidinones (*e.g.* Scheme 1A, eq. 2).[9,10] More recently, Wong and co-workers showed that variants of the well-studied P450 BM3 from *Bacillus megaterium* deliver selectively hydroxylated products of a range of *N*-heterocycles with high conversions and enantioselectivity (*e.g.* Scheme 1A, eq. 3).[11,12]



Scheme 1. Intro/previous work/this work scheme

Despite their high selectivity, P450s present challenges; for example, the requirement for an exogenous reductant (NADPH) and electron transport proteins or domains to channel reducing equivalents from the cofactor to the heme to create the reactive oxygenating intermediate Compound I.[13] While these problems can be addressed to an extent through the use of whole cell catalysts, the use of microbe-free biocatalysts for selective oxygenations would be advantageous.

[a] Department of Chemistry, University of York, Heslington, York, YO10 5DD (UK).
[b] Department of Biology, University of York, Heslington, York, YO10 5DD (UK).
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In this context, Unspecific Peroxygenases (UPOs) have assumed a role as alternatives to P450s for scalable biocatalytic oxygenations.[14-16] UPOs are heme oxygenases that are secreted by fungi and easily prepared at scale using heterologous expression hosts such as *Pichia pastoris*.[17] They depend only upon the addition of hydrogen peroxide (H_2O_2) to generate Compound I and can consequently be used as off-the-shelf lyophilised powders, obviating the need for facilities for the growth and containment of microoorganisms. Following early demonstrations of their activity on a variety of small molecules, including aliphatics,[18] aromatic hydrocarbons[19] and common drugs,[20] their suitability for scalable oxygenations was recently demonstrated with a multi-hectogram scale oxygenation of cyclohexane.[21] In previous reports from our groups, we have shown that UPOs can carry out selective oxygenations of terpenes,[22] toluenes[23] and allylic alcohols[24] on up to 1 g scales, and are also capable of 'promiscuous' reactions such as halogenations,[25] Achmatowicz reactions[26] and even cyclopropanations.[27]

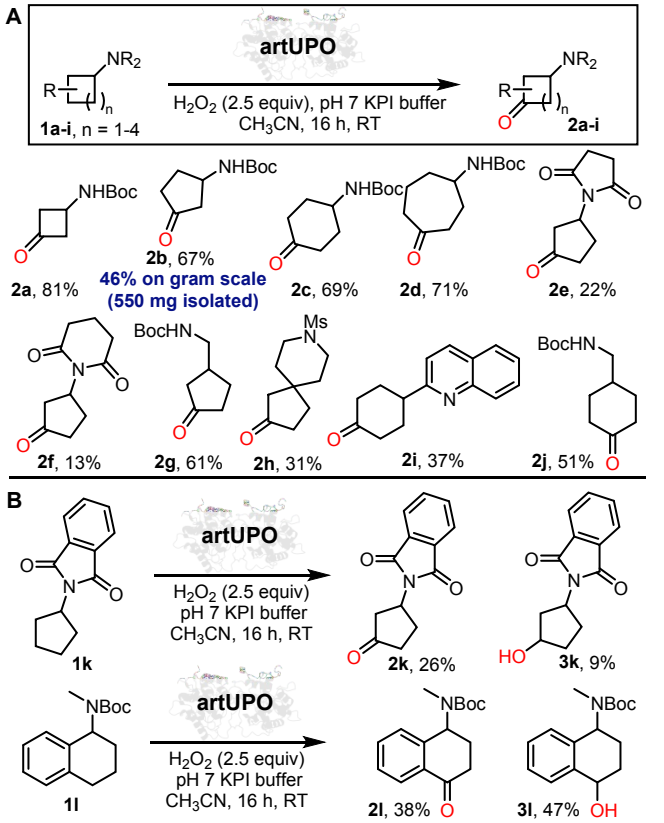
In our previous work we also focused on the oxygenation of aromatic *N*-heterocycles, such as pyridine and indole derivatives, using a lyophilized preparation of a UPO variant from *Agrocybe aegerita* (r*Aae*UPO-PaDa-I-H, Scheme 1B).[28] This new report focuses on extending the capability of UPOs to perform scalable oxygenations of non-aromatic cyclic and acyclic aliphatic amine derivatives, using the Class I UPO, artUPO (Scheme 1C).[29] First, the regioselective biocatalytic conversion of cyclic aliphatic amines **1** into cyclic ketones **2** and alcohols **3** was explored. The selective C–H oxygenation of isopropyl groups on amine derivatives **4** was also examined, enabling the formation of tertiary alcohols **5**. Finally, the generation of enantiomerically enriched amino alcohols has been demonstrated, using a kinetic resolution approach from chiral amine **1m**.

Oxygenation of cyclic aliphatic amines

We began by exploring the oxygenation of cyclic aliphatic amines, with initial experiments performed using cyclobutylamine derivatives (*e.g.* *tert*-butyl cyclobutylcarbamate **1a**). All biotransformations were performed in pH 7 KPi aqueous buffer, with 20% acetonitrile and H_2O_2 as stochiometric oxidant, as similar conditions have been shown to enable a variety of UPO-mediated oxygenations .[22-28] Two UPOs, the Class I artUPO[29] and Class II r*Aae*UPO-PaDa-I-H[30] were screened for activity initially, but only artUPO displayed the desired activities and was subsequently used for all biotransformations. This behaviour is consistent with previous observations of terpene biotransformations[22] in which the ability of artUPO to accept larger substrates was attributed to it having a less sterically restricted active site[29] than r*Aae*UPO-PaDa-I-H. artUPO was prepared by fermentation in *Pichia pastoris* and added to reactions as the crude liquid secretate as previously described.[22,27,29] Different H_2O_2 loadings (1.0–2.5 equivalents) and eight cyclobutylamine substrates with different amine protecting groups/substituents were also screened (see SI section 2.13). Superior conversion was observed using Boc-protected cyclobutylamine **1a** as substrate.

Notably, in contrast to our earlier work on *N*-heterocycle oxygenations,[28] the initial results provided little evidence of oxygenation to form alcohols. Instead, cyclobutanone derivatives (*e.g.* **2a**) formed as the major product; ketone formation presumably arises from initial oxygenation to form an alcohol, followed by fast oxidation *in situ*.[23] Amino ketone derivatives are important building blocks in organic synthesis, and commonly found in bioactive molecules.[31,32] We therefore decided to target the synthesis of amino ketone products, and found that using 2.5 equivalents of H_2O_2 promoted the desired oxygenation most effectively.

Attention now turned to performing preparative scale biotransformations (Scheme 2). First, *tert*-butyl cyclobutylcarbamate **1a** was converted into amino ketone **2a** with 81% isolated yield as a single regioisomer. Homologous 5-, 6- and 7-membered ring systems were also transformed, with amino ketones **2b–2d** isolated in good yields, all as single regioisomers. Reactions were typically performed using 0.2 mmol of the amine **1**, but to demonstrate their scalability, a 1-gram transformation of cyclopentyl derivative **1b** was performed and delivered 550 mg of product **2b**.



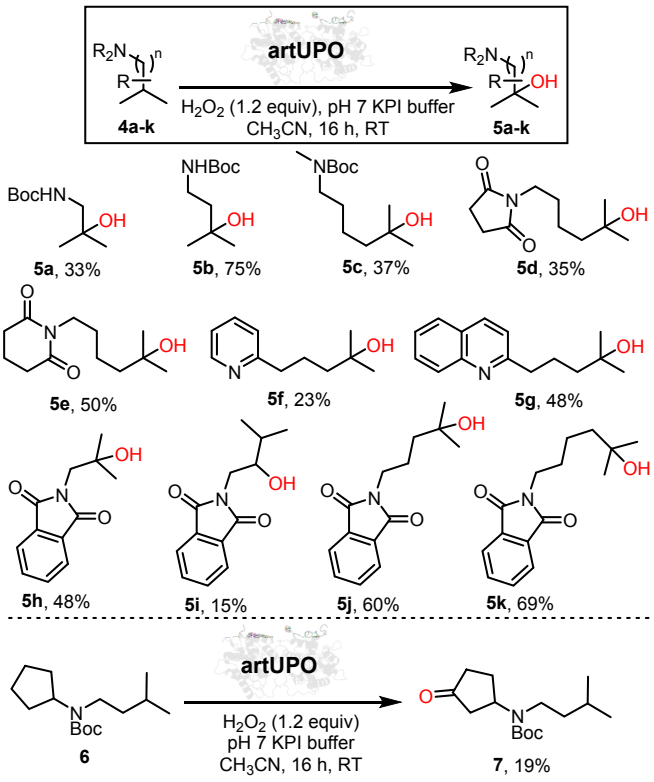
Scheme 2. Biocatalytic synthesis of cyclic amino ketones via the artUPO mediated oxygenation of cyclic amine derivatives. See SI section 2.10 for a general synthetic protocol.

Cyclopentanone formation was also achieved using substrates with cyclic imide substituents, albeit in reduced yields (**2e** and **2f**). Syntheses of cyclopentanone and cyclohexanone derivatives were also demonstrated on substrates containing more remote NBoc groups (to form **2g** and **2j**), a spirocyclic N-mesyl system (**2h**) and a quinoline (**2i**). In all cases, the ketone

was obtained as a single regioisomer, with oxygenation taking places at the methylene furthest from the substituted amine. Substrates **1k** and **1l** (Scheme 2B) were the only systems in which amino alcohol products (**3k** and **3l**) were formed alongside the amino ketones (**2k** and **2l**).

Oxygenation of isopropyl-tethered amine derivatives

We next explored the oxygenation of isopropyl groups on linear aliphatic substrates to form amino alcohols. In our previous work, we showed that UPOs can promote selective and gram-scale C–H oxygenation of the isopropyl group of menthol.[22] In this study, we explored whether preparative scale oxygenation could be performed on a wider array of isopropyl-containing substrates, containing amine derivatives or aza-heterocycles. Similar reaction conditions were used as for ketone formation above, but as only a single oxidation event was needed in this series, H_2O_2 loading was lowered to 1.2 equivalents accordingly (Scheme 3).

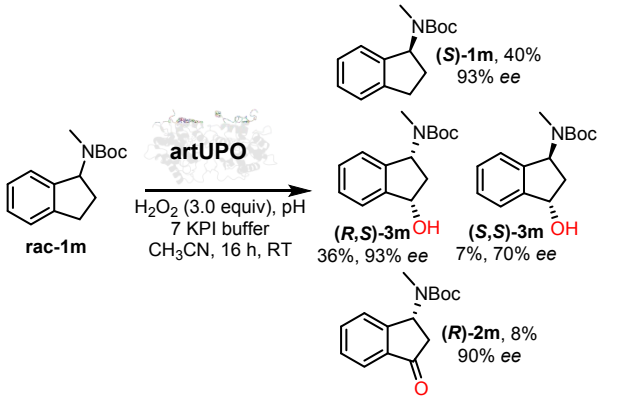


Scheme 3. Biocatalytic synthesis of amino alcohol derivatives *via* artUPO mediated oxygenation of isopropyl-tethered amines. See SI section 2.11 for a general synthetic protocol.

First, C–H oxygenation of the isopropyl group of *N*-Boc substituted linear alkanes was tested, and tertiary alcohol products were formed in 33–75% isolated yield (**5a–5c**). Similarly, selective oxygenation was also achieved using substrates substituted with cyclic imides (**5d** and **5e**), pyridine (**5f**) and quinoline (**5g**) groups. Interestingly, neither benzylic oxidation, nor *N*-oxide formation, were competing reactions for either the pyridine or quinoline substrates. Four homologous phthalimide-containing substrates were then tested, and in this series, the tertiary alcohol products were obtained in three out of four cases (**5h**, **5j** and **5k**), in 48–69% isolated yields. Isopentyl substrate **4i** was anomalous; lower conversion was observed, and unexpectedly, a secondary alcohol product **5i** was formed, *via* C–H oxygenation of the methylene adjacent to the isopropyl. Finally, substrate **6**, containing both a cyclic alkane and an isopropyl group, was tested. The yield was relatively low in this case, but the only product isolated was cyclopentanone **7**, indicating preferential cyclopentane oxygenation over the isopropyl group.

Kinetic resolution

One of the most powerful features of biocatalysis is the ability to generate products with high enantioselectivity. As most products generated in this study are achiral this ability was not utilised hitherto. However, benzannulated substrate **rac-1m** is an interesting exception, being both chiral (racemic) and also possessing a benzylic site available for oxygenation. UPOs are well-capable of effecting the chemo- and enantio-selective oxygenation of benzylic C–H bonds to form chiral alcohols; indeed, ethyl benzene oxygenation is commonly used to benchmark the activity and enantioselectivity of UPOs.[33] We therefore reasoned that oxygenation to form enantiomerically enriched amino alcohols should be possible with this substrate, *via* a kinetic resolution approach. Following optimisation (targeting ≈50% conversion of **rac-1m**, see SI, section 2.12), suitable conditions were found and used in the preparative scale kinetic resolution of **rac-1m** (Scheme 4).



Scheme 4. artUPO mediated enantioselective oxygenation of **rac-1m** via a kinetic resolution approach.

artUPO has a clear preference to accept the (*R*)-enantiomer of **1m**, evidenced by the fact that the unreacted starting material was recovered in 40% isolated yield and enriched to 93% *ee* in favour of the (*S*)-enantiomer (*S*)-**1m**. The absolute stereochemistry of (*S*)-**1m** was assigned by comparison to literature optical rotation data.[34,35] As hoped, artUPO mediated oxygenation to form amino alcohol derivatives was achieved, with (*R*,*S*)-**3m** and (*S*,*S*)-**3m** isolated as an inseparable mixture in 43% combined yield, and with 93% *ee* and 70% *ee* respectively. The relative stereochemical assignments of (*R*,*S*)-**3m** (*cis*) and (*S*,*S*)-**3m** (*trans*) were made based on ^{1}H NMR data; literature data show a clear trend in which the benzyl alcohol C–H signal appears at higher chemical shift for the *trans*-isomer than the *cis*- in a series of closely related compounds, with the same trend seen in our products.[36] A small amount of over-oxidation

was also observed, with (*R*)-**2m** isolated in 8% yield and 90% *ee.* Reduction of (*R*)-**2m** with $NaBH_4$ resulted in clean conversion into (*R*,*S*)-**3m**, confirmed by chiral HPLC (see SI section 4). This result confirmed that both (*R*)-**2m** and (*R*,*S*)-**3m** were derived from the same enantiomer of **1m**, and given their 44% combined yield, their precursor logically must be (*R*)-**1m**, thus enabling the assignment of the absolute stereochemistry of (*R*)-**2m** and (*R*,*S*)-**3m**. The absolute stereochemical assignment of the minor alcohol product (*S*,*S*)-**3m** is less clear. We are confident in assigning its relative stereochemistry to be *trans* as outlined above. The absolute assignment of (*S*,*S*)-**3m** is more tentative, based partly on the overall mass balance of the kinetic resolution, but also based on the expectation that the UPO would more likely promote benzylic oxygenation with the same sense of stereochemistry for both enantiomers of *rac*-**1m**, and form the (*S*)-alcohol product in each case.

In summary, the utility of artUPO to perform preparative scale C–H oxygenation reactions has been extended to encompass the synthesis of amino ketone and amino alcohol derivatives from a range of nitrogen-containing hydrocarbons. Thus, the ever-growing toolbox of synthetically useful UPO-mediated transformations is expanded, further enhancing the reputation of UPOs as biocatalysts with great potential for the selective, safe and scalable oxidation reactions.

Author Contributions

JL designed experiments and JC, WPU and GG designed and supervised experiments. JL and KC performed experiments. WPU and GG wrote the manuscript with contributions from all authors.

Data Availability Statement

The data supporting this article have been included as part of the Supplementary Information.

Conflicts of interest

There are no conflicts to declare.

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