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# Preparative scale Achmatowicz and aza-Achmatowicz rearrangements catalyzed by *Agrocybe aegerita* unspecific peroxygenase<sup>†</sup>

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The unspecific peroxygenase (UPO) from *Agrocybe aegerita* (rAaeUPO-PaDa-I-H) is an effective and practical biocatalyst for the oxidative expansion of furfuryl alcohols/amines on a preparative scale, using the Achmatowicz and aza-Achmatowicz reaction. The high activity and stability of the enzyme, which can be produced on a large scale as an air-stable lyophilised powder, renders it a versatile and scalable biocatalyst for the preparation of synthetically valuable 6-hydroxypyranones and dihydropiperidinones. In several cases, the biotransformation out-performed the analogous chemo-catalysed process, and operates under milder and greener reaction conditions.

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#### Introduction

The oxidative rearrangement of furyl-alcohols to dihydropyranones – generally termed the Achmatowicz reaction<sup>1</sup> – is one of the most important and widely used furan transformations in organic chemistry (*e.g.*  $1 \rightarrow 3$ , Scheme 1).<sup>2,3</sup> Most synthetic Achmatowicz reactions rely on the use of strong stoichiometric chemical oxidants<sup>4,5</sup> such as *m*CPBA, which in this case promotes the reaction *via* an initial epoxidation (to form 2) and subsequent rearrangement as shown (Scheme 1). Various other Achmatowicz-type methods are also known using other oxidants, *e.g.* employing electrochemistry,<sup>6</sup> singlet oxygen<sup>7</sup> and photocatalysis.<sup>8</sup> Apart from the latter, catalytic methods performed under ambient conditions are rare.

Biocatalysis offers significant advantages compared with chemical methods, to enable milder and more sustainable Achmatowicz reactions (Scheme 2). The first enzymatic Achmatowicz reaction reported was developed by Beifuss *et al.*, and is based on the use of laccase from *Trametes versicolor* and hydroxy-TEMPO as a redox mediator (*e.g.*  $4 \rightarrow 5a$ , Scheme 2A).<sup>9</sup> After this, Deska *et al.* reported that *C. fumago* chloroperoxidase (*Cfu*CPO), catalyzes the Achmatowicz reaction on a range of substrates and with moderate enantioselectivity.<sup>10</sup> A subsequent study by the same group utilized *Cfu*CPO, assisted by

glucose oxidase (GOx), for the generation of peracid from an acetic acid buffer, for Achmatowicz reactions of the type  $4 \rightarrow 5b$  (Scheme 2B).<sup>11</sup> The authors also demonstrated that *C. antarctica* lipase B (CAL-B) can also be used to generate per-



Scheme 1 The Achmatowicz reaction promoted by *meta*-chloroperbenzoic acid.



Scheme 2 Biocatalytic Achmatowicz protocols.

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acetic acid from the acetate present in the buffer and the added  $H_2O_2$  (*e.g.*  $4 \rightarrow 5c$ ; Scheme 2C).<sup>11</sup> Each of these reactions should proceed through an initial oxidation of the furan and subsequent rearrangement, *via* a mechanism similar to that depicted in Scheme 1.

The aza-Achmatowicz reaction for the transformation of aza-analogues such as **6** into products **8** is another important synthetic transformation, with a general reaction depicted in Scheme 3A.<sup>12</sup> Biocatalytic aza-Achmatowicz reactions are rare however, although Hollman and co-workers have reported successful examples using the vanadium-dependent peroxidase from *C. inaequalis* (*Cin*VPO) in the presence of  $H_2O_2$  and KBr in aqueous media with ethanol.<sup>13</sup> In this case, the reactive oxidant is likely to be hypohalous acid generated biocatalytically from KBr (Scheme 3B). Notably, reactions such as the formation of **5d** were performed at up to 100s milligram scale, although relatively limited scope was demonstrated to date. The CAL-B-mediated *in situ* peracetic acid generation system developed by the Deska group can also be used to perform aza-Achmatowicz reactions in limited cases.<sup>11</sup>

Unspecific Peroxygenases (UPOs) have recently been shown to possess catalytic characteristics bridging those of cytochromes P450 (P450s) and haloperoxidases such as CfuCPO, as well as exhibiting superior stability and catalytic activity.<sup>14-17</sup> In contrast to P450s however, these heme dependent enzymes are able to catalyze oxygenation reactions at the expense of only hydrogen peroxide as the external oxidant. These attributes, coupled with advances in heterologous expression,<sup>18-20</sup> improved protocols for protein engineering<sup>21-23</sup> and also methods for gradual hydrogen peroxide delivery that reduce oxidative stress on the enzyme,<sup>24-26</sup> have established them as attractive biocatalysis for scalable oxygenation reactions. We reasoned therefore that UPOs could also be good biocatalysts for both Achmatowicz and aza-Achmatowicz reactions initiated by epoxidation, especially as UPOs have been shown previously to be capable of promoting the epoxidation of a range of alkenes aliphatic and alicyclic alkenes<sup>27</sup> and styrene derivatives.<sup>28</sup> Therefore, in this study, both reaction classes were



Scheme 3 Biocatalytic aza-Achmatowicz reaction.



Scheme 4 Preparative scale Achmatowicz and aza-Achmatowicz rearrangements catalyzed by rAaeUPO-PaDa-I-H in this study.

explored, using a variant of the PaDa-I mutant of Agrocybe aegerita UPO originally described by Alcalde and coworkers,<sup>18,19</sup> which was expressed in *Pichia pastoris* using a modified vector and previously used by our groups as r*Aae*UPO-PaDa-I-H.<sup>29–32</sup> Our studies confirm that rAaeUPO-PaDa-I-H is an effective biocatalyst for both transformations (Scheme 4). Both reaction series gave high synthetic yields and were readily scaled-up. The UPO reactions show an improved substrate scope over CfuCPO-catalyzed reactions, with 23 preparative scale reactions (up to 1 g scale) successfully demonstrated and further expand our knowledge of the ever-growing promiscuous activity of UPOs.

#### Results and discussion

rAaeUPO-PaDa-I-H was expressed and lyophilized using our reported method.<sup>29</sup> The 2-furylcarbinol substrates **9a–v** used in this study were prepared using standard methods, with full synthetic details described in the ESI (ESI, section 7†). Synthetic standards for the Achmatowicz products **10a–i** were also prepared from the 2-furylcarbinols *via* oxidation with *N*-bromosuccinimide (NBS) or potassium hypobromite (ESI, section 7†).

We started by examining the reactions of 2-furylcarbinols 9a-i on small scale. The substrates (10 mM) were reacted with H<sub>2</sub>O<sub>2</sub> (3.3 mM in a single portion, or 10 mM added in three portions at 10 min intervals) in the presence of rAaeUPO-PaDa-I-H, on 1 mL scale in a pH 5.5 buffer solution, with 10% t-BuOH as co-solvent at RT<sup>33</sup> (Table 1). The reaction extracts were analysed by GC-FID. The results of biotransformations of the first substrates tested were somewhat disappointing; for example, the simplest furyl-carbinol 9a (entry 1), was not converted into the Achmatowicz product 10a and a complex mixture of side products was obtained. Substitution with an ethyl group on the furan 5-position (9b) resulted in a simpler product mixture, but aldehyde 13 was the only product identified (entries 2 and 3). 2-Furylcarbinol 9c was not oxidized by rAaeUPO-PaDa-I-H (entry 4). In this regard, rAaeUPO-PaDa-I-H is similar to CPO as that enzyme was also reported not to convert a 2,5-disubstituted substrate in the original report.<sup>10</sup>

Table 1 GC conversions for the rAaeUPO-PaDa-I-H-catalyzed Achmatowicz reaction. Products were identified by comparing their retention times to authentic standards

	$R^{1} \xrightarrow{O} R^{2} = R^{3}$	AaeUPO-PaDa-I-H H <sub>2</sub> O <sub>2</sub> , t-BuOH pH 5.5. buffer	$ \begin{array}{c}                                     $
Entry	Substrate	$[H_2O_2]/mM$	Product <sup>a</sup>
	ОН		носо
1	9а	3.3	10a, 0%
2	9b	3.3	13, 29%
3	ОН	10	13, 85%
4	9c	3.3	10c, 0%
5	9d	3.3	<b>10d</b> , 13%
7 8	OH 9e	3.3 10	100, 80% HO 10e, 20% 10e, 59%
9 10	9f	3.3 10	HO 10f, 27% 10f, 70%
11	о ОН 9g	3.3	HO O 10g, 21%
12		10	10g, 59%
13 14	ОН 9h	3.3 10	10h, 16% 10h, 47%
	ОН		HOOO
15	9i	3.3	<b>10i</b> , 30%
16		10	10i. 84% <sup>b</sup>

Reaction conditions: 10 mM substrate, 4.4 U mL<sup>-1</sup> rAaeUPO or 20 U mL<sup>-1</sup> CPO, 1 mL total volume, 10% v/v <sup>t</sup>BuOH, 50 mM sodium citrate buffer (pH 5.5) at RT.<sup>33</sup> Reactions were extracted with 2 × 1 mL EtOAc, dried over MgSO<sub>4</sub> and analysed by GC-FID (HP-5MS column). <sup>*a*</sup> Yields based on analysis by GC-FID. <sup>*b*</sup> H<sub>2</sub>O<sub>2</sub> was added dropwise by a syringe pump over 7 h. Control reactions, to which no enzyme was added, gave no conversion.<sup>34</sup>

However, more promisingly, rAaeUPO-PaDa-I-H successfully converted monosubstituted 2-furylcarbinols 9d-g into the analogous pyranones 10d-g (entries 5-16; the products were positively identified using authentic standards). This initial screen showed that less bulky furyl-carbinols are transformed more readily by the enzyme, with similar results obtained in published studies using CfuCPO as the biocatalyst.<sup>11</sup> It is also noteworthy that, using the same H<sub>2</sub>O<sub>2</sub> addition method, in our hands CfuCPO failed to convert any of 2-furylcarbinols 9d-i at 3.3 mM and 10 mM H<sub>2</sub>O<sub>2</sub> loading (data not shown). This may be due to the instability of CfuCPO in the presence of the comparatively higher peroxide loadings used, and serves as a useful illustration of the practicality and robustness of rAaeUPO-PaDa-I-H. The >50% conversions observed in several cases show that the UPO is capable of converting both enantiomers of the chiral 2-furylcarbinols tested, at least to some degree. This observation, coupled to near-zero specific rotation measurements for the products (vide infra), suggests that achieving an enantioselective rAaeUPO-PaDa-I-H-catalysed Achmatowicz reaction through kinetic resolution of racemic 2-furylcarbinols is unlikely to be feasible using this enzyme.

Next, we set out to optimize the reaction conditions prior to increasing the reaction scale. First, a pH screen was carried out using 1-(2-furyl)-ethanol **9d**. A pH between 5.0 and 7.0 was found to be optimal for its conversion into **10d** (Fig. 1), hence a pH 5.5 citrate buffer was taken forward and used in the preparative reactions.<sup>24</sup> The effect of different co-solvents was also examined, with acetone identified as the best co-solvent based solely on conversion, especially at higher concentrations (Fig. 2).

However, *t*-BuOH performed similarly at 10% and 20% (v/v), and it was later found that product purification in the



Fig. 1 pH profile of the rAaeUPO-PaDa-I-H catalysed transformation of 9d into 10d. Reactions were performed in 150 µL total volume, extracted with 2 × 200 µL EtOAc and analysed by GC-FID on a HP-5MS column (30 m) at RT.<sup>33</sup> Citrate buffers were used between pH 3.50 and 5.50 (50 mM) and above this KPi buffers (50 mM) were employed. Error bars show ±one standard deviation from the mean.

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**Fig. 2** Effect of co-solvent on the rAaeUPO-PaDa-I-H catalysed transformation of **9d** into **10d**. Tests were performed in 150 µL total volume in sodium citrate buffer (pH = 5.50, 50 mM) at 10 mM substrate concentration at RT.<sup>33</sup> Reactions were extracted with 2 × 200 µL EtOAc after 15 min and the extract was directly analysed by GC-FID (30 m HP-5MS column). Error bars indicate ±one standard deviation. MeCN – acetonitrile; DMF – dimethylformamide; DMSO – dimethylsulfoxide.

preparative reactions was simpler from reactions containing *t*-BuOH compared with those containing acetone. Therefore, *t*-BuOH was preferred for the preparative studies that follow (*vide infra*, Scheme 5).

The optimised reaction conditions were then tested at preparative scale. A range of monosubstituted 2-furylcarbinols **9d-v** was explored (Scheme 5), with a syringe pump used to control slow  $H_2O_2$  addition at a rate of 5 mM h<sup>-1</sup>, at 10 mM substrate concentration. In all cases the reactions were run for 2 h at room temperature. Pleasingly, the preparative results for 2-furylcarbinols **9d-i** were in line with the small scale experiments, with pyranones **10d-i** all being formed and isolated in good to excellent yields. Longer chain homologues **10j** and **10k** were also prepared in the same way. In the case of **10k** the cosolvent was switched to acetonitrile, as the pyranone product was found to be too poorly soluble when using *t*-BuOH. Notably, such bulky substrates were not reported to be transformed by *CfuCPO* in previous studies.<sup>11</sup>

Products containing bulkier cyclic hydrocarbon substituents (10l and 10m) and aryl (10o and 10p) were also obtained in acceptable yields, while ester-containing pyranone 10n was isolated in 70% yield. Conversely, alkyne and alkene containing starting materials 9q-9s were not converted into the expected products (10g-s), with unreacted 2-furylcarbinol starting material recovered in each case and no epoxidation of the double bond detected during the reactions. We postulated that this may be a consequence of enzyme inhibition by these substrates, and indeed, this notion was supported by the observation that 2-furylcarbinols 9r and 9s significantly reduced the conversion of 9e into 10e in inhibition studies (ESI section 4, Fig. S1<sup>†</sup>). However, a mechanism for this inhibition was not clear from these experiments. Bulky tertiary alcohol containing starting materials were well-tolerated, with pyranones 10t-v isolated in good yields using the standard protocol. Finally, pyranone 10c was obtained from 2,5-disubstituted starting material 9c, although the isolated yield was modest in this case (18%). This example is notable as 9c was not converted in the initial small-scale screen. This highlights the synthetic advantages that can be realised by using the slow  $H_2O_2$ addition method.

Notably, in our hands, these biocatalytic Achmatowicz reactions using r*Aae*UPO-PaDa-I-H tended to give similar or superior yields to those obtained for the analogous reactions promoted by *N*-bromosuccinimide (NBS, ESI section 5†). The biocatalytic method also generally gave rise to much cleaner reaction mixtures; indeed, column chromatographic purification was not required in several cases. As expected, all of the pyranone products formed from chiral alcohols were obtained as mixtures of diastereoisomers (dr between 1:1 and 3:1; see ESI†). The dr of the products produced by the enzymatic method was the same as in the material synthesised using NBS, strongly indicating that these are simply thermodynamic



**Scheme 5** Achmatowicz reactions promoted by r*Aae*UPO-PaDa-I-H and  $H_2O_2$ . Reaction conditions as shown in the box above (see ESI section 7<sup>†</sup> for more detail). 10 mM substrate concentration was used, with  $H_2O_2$  supplied *via* syringe pump at 5 mM h<sup>-1</sup> (2 h reaction time in total) at RT.<sup>33 a</sup> No chromatographic purification was needed in these cases. <sup>b</sup> Performed with 10 v/v % MeCN in place of *t*-BuOH.

outcomes associated with the relative energies of the two equilibrating diastereoisomers. It is therefore very unlikely that other UPOs would affect the dr of the products formed. However, it remains possible that the application of sequence diverse UPOs or engineered enzymes may be capable of delivering enantiomerically enriched products, *via* the kinetic resolution of chiral starting materials.

#### UPO-catalyzed aza-Achmatowicz reactions

To our knowledge, there have been few reports of biocatalytic aza-Achmatowicz reactions reported in the literature,<sup>11,13</sup> and those examples relied on the formation of a diffusible oxidants by either lipase-catalysed perhydrolysis of acetate<sup>11</sup> or the vanadium-dependent peroxidase from Curvularia inequalis (CinVPO).13 Based on our results with the standard Achmatowicz reaction, we reasoned that the active site of rAaeUPO-PaDa-I-H may permit the binding and direct oxidation of aza-Achmatowicz substrates within the enzyme active site. To probe this, we challenged the enzyme with a series of furan-2-ylmethanamine substrates N-protected (11a-f, Scheme 6A), on preparative scale, using the method established for the Achmatowicz reaction of 2-furylcarbinols (ESI section 5<sup>†</sup>).

Substrates bearing a *tert*-butyloxycarbonyl (Boc) protecting group in the amine (**11a–f**) were explored first (Scheme 3A).



Scheme 6 Aza-Achmatowicz reactions promoted by rAaeUPO-PaDa-I-H and H<sub>2</sub>O<sub>2</sub>. Reaction conditions as shown in the box above. 10 mM substrate concentration was used, with H<sub>2</sub>O<sub>2</sub> supplied using a syringe pump at a rate of 5 mM h<sup>-1</sup> at RT.<sup>33 a</sup> **12a** could not be isolated, due to its propensity to rearrange into *tert*-butyl pyridine-3-yl carbonate product **14**. <sup>*b*</sup> KBr (100 mM) was added to the buffer. <sup>*c*</sup> 1 mM h<sup>-1</sup> H<sub>2</sub>O<sub>2</sub> addition rate was used.

Pleasingly, the smaller substrates in this series were accepted by rAaeUPO-PaDa-I-H. However, in the case of the smallest substrate in this series (11a, R = H), while we believe that the aza-Achmatowicz took place, the product 12a could not isolated as it was observed to be unstable and to convert into pyridine 14, which was isolated in 60% yield. Pleasingly, substituted analogues 12b and 12c were more stable, and using the standard method, the expected aza-Achmatowicz products were obtained in acceptable yields. To the best of our knowledge, these represent the first biocatalytic aza-Achmatowicz reactions to be performed without using diffusible oxidants. Substrate 11c apparently represents the limit of the N-Boc series with respect to size however, as bulkier substrates 11d-f were not converted into the respective products 12d-f. This result was not wholly surprising, especially considering the additional steric imposition of the Boc protecting group.

Nonetheless, in these more challenging cases, we found that a biocatalytic aza-Achmatowicz can still be achieved, demonstrated by the successful synthesis of **12e** and **12f**, by simply adding KBr to the reaction medium. Presumably, these modified examples operate *via* enzyme-catalysed oxidation of the added bromide to form hypobromite, which then diffuses from the enzyme active site to promote the oxidative rearrangement. This discovery is useful in expanding the scope the method to include bulkier substrates. However, this is be balanced against the strong likelihood that the oxidation takes place outside of the UPO active site when KBr is used, and hence the opportunity for the enzyme to impart stereocontrol on the reaction is lost.

N-Mesyl protected substrates 11g-k (Scheme 6B) were also examined, in the hope that the switch to a smaller protecting group would allow more flexibility to incorporate bulkier substituents. Although yields from substrates 11g and 11h gave results comparable to analogous N-Boc protected substrates 11b and 11c, in the case of substrate 11i, this was transformed with 40% yield, whereas the NBoc-protected analog 11e was not. No conversion was observed when trying to convert the larger ester containing substrate 11k however. In most cases (12b and 12i being the exceptions) the products were isolated as single diastereoisomers. It is not always clear whether the 2,6-cis or 2,6-trans diastereoisomer will predominate in aza-Achmatowicz reactions, although it is relatively common for there to be a thermodynamic preference for one diastereoisomer.<sup>2</sup> Based on specific rotation measurements, all products 12b-j appear to be racemic, indicating little or no kinetic resolution of the chiral amine precursors 11b-j, as was the case for the 2-furylcarbinol substrates.

Finally, having demonstrated the feasibility of both the r*Aae*UPO-PaDa-I-H-catalyzed Achmatowicz and aza-Achmatowicz reactions, we wanted to further demonstrate the scalability of the protocol by performing the oxidation of **9e** on a 1 g scale (Scheme 7). To do this, the concentration of the substrate was raised radically to 400 mM. The enzyme tolerated this change well, but it was necessary to switch the co-solvent to acetone better solubilise **9e**. The use of acetone as co-solvent introduces the possibility of unwanted dimethyl-

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Scheme 7  $\,$  rAaeUPO-PaDa-I-H catalyzed oxidation of 9e on a 1 g scale at RT.  $^{33}$ 

dioxirane (DMDO – potentially explosive) being formed in the reaction, although the slow addition of  $H_2O_2$  and the quick enzymatic reaction both minimise this risk. Under these conditions, 0.92 g of the product **10e** was isolated, which corresponds to 82% isolated yield. This transformation showcases well the robustness of r*Aae*UPO-PaDa-I-H under challenging biocatalytic conditions.

#### Conclusions

We have demonstrated that rAaeUPO-PaDa-I-H is an easy-touse catalyst for Achmatowicz and aza-Achmatowicz reactions. A simple, slow-addition H2O2 delivery method has been established for preparative scale reactions, and the lyophilized enzyme is practical, easy to use and is stable for years when stored at -20 °C. The transformations are performed under mild reaction conditions, and are generally highly chemoselective - the biocatalytic method often out-performed the standard chemo-promoted method (NBS), and in several cases afforded products that were sufficiently clean that column chromatography was not needed. The scope of the transformations is relatively broad, with substrate specificity primarily influenced by steric factors. Notably, the scalability of the rAaeUPO-PaDa-I-H catalyzed Achmatowicz reaction was further demonstrated by performing the oxidation of 1-(2-furyl) propan-1-ol 9e at 1 g scale, with the desired product 10e isolated in 82% yield. This study expands the reaction scope of UPOs and provides further substantial evidence of practicality and ever-growing practical synthetic utility as green oxidants.

#### Author contributions

All authors contributed to the design and execution of experiments. BP, WPU and GG wrote the manuscript, with contributions also from ADR.

#### Data availability

The data supporting this article have been included as part of the ESI.†

### Conflicts of interest

There are no conflicts to declare.

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- 33 Throughout this manuscript, 'RT' refer to the ambient temperature in out laboratory, which is typically  $\approx$ 21 °C.
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