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Cyclopropanation reactions by a class I unspecific peroxygenase†

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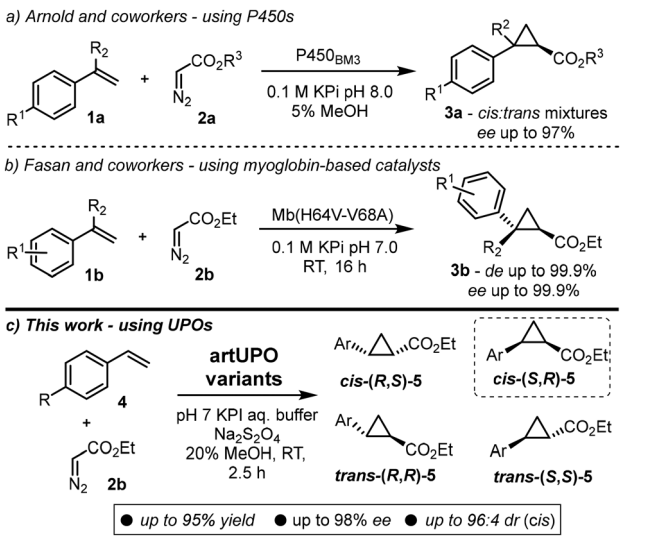
Non-natural biotransformations, such as alkene cyclopropanation through carbene insertion, have been demonstrated for the hemoproteins cytochrome P450 and myoglobin, but have not been investigated for unspecific peroxygenases (UPOs). Here we demonstrate that the diastereo- and enantioselective cyclopropanation of styrenes with ethyldiazoacetate can be performed by the class I enzyme artUPO.

Unspecific peroxygenases (UPOs) are heme-containing oxygenase enzymes secreted by various fungal species.[1] Since their first description in 2004[2] they have been the focus of intense research as biocatalysts for oxyfunctionalisation reactions[3–5] as they provide an attractive alternative to other oxygenase catalysts, such as cytochromes P450 (P450s), for scalable oxygenation reactions. This is because UPOs catalyse their reactions with the assistance of only hydrogen peroxide (H_2O_2), in contrast to the nicotinamide cofactors and auxiliary electron transport systems required by P450s.[6] UPOs have now been applied to the oxygenation of alkanes,[7] aromatics,[8] terpenes[9] and drug molecules[10] and have even on scales as large as 115 mmol and >500 g in the cases of transformations of butane[11] and cyclohexane[12] respectively.

As hemoprotein oxygenases, UPOs catalyse oxygenation reactions through the formation of the crucial catalytic intermediate known as compound I,[13] a reactive heme iron-oxo Fe (IV) cationic intermediate that is able to promote various oxygenation reactions. Many hemoproteins have now also been shown to catalyse 'non-natural' chemical reactions under conditions where a metal-stabilised carbene or nitrene is formed in the active site, in place of compound I, before reacting with a suitable acceptor substrate.[14,15] For example, pioneering studies by Arnold and co-workers first showed that variants of cytochrome $P450_{BM3}$ were capable of converting styrenes of the form **1a** into cyclopropanes **3a**, following reduction of the heme and introduction of a diazocarbonyl carbene donor **2a** (Scheme 1a).[16,17] It has subsequently shown that $P450_{BM3}$ variants are also capable of related reactions, such as C–N bond formation *via* nitrene mediated C–H activation to form amines[18] and amides.[19] Fasan and coworkers have also shown that the heme containing iron storage protein myoglobin (Mb) can also enable biological carbene and nitrene transfer reactions, including the highly diastereo- and enantioselective cyclopropanation of styrenes **1b** with ethyldiazoacetate (EDA, **2b**) to give products **3b** (Scheme 1b).[20–22]

While UPOs have many attractive features as alternative catalysts to P450s and Mb, their capacity for promiscuous biocatalysis has not been explored as extensively, or related enzymes such as chloroperoxidases (CPOs).[23,24] In recent studies, we have shown that the PaDa-I[25,26] variant of the UPO (r-*Aae*UPO-PaDa-I-H)[27] from *Agrocybe aegerita* can catalyse Achmatowicz and aza-Achmatowicz reactions also described



Scheme 1 Biocatalytic cyclopropanation strategies.

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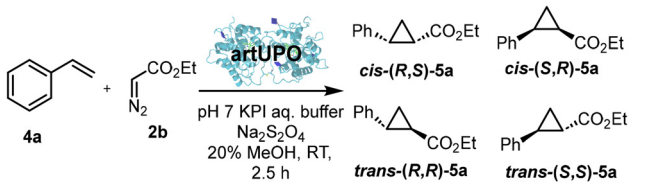
for CPO,[23] on a gram scale, to give ring-expanded products.[28] We have also shown that, as also demonstrated for CPO,[29] r-*Aae*UPO-PaDa-I-H can enable the halogenation of aromatic substrates through the compound I dependent formation of hypobromous acid from hydrogen peroxide and bromide ions.[30] Given these observations of catalytic plasticity within UPOs, we were prompted to investigate whether these enzymes could catalyse carbene insertions of the kind described for both P450s and Mb. Thus, herein we describe the successful application of UPOs in model styrene cyclopropanation reactions (Scheme 1c). To the best of our knowledge, this represents not only the first UPO-promoted styrene cyclopropanation, but also the UPO-promoted carbene insertion reaction of any kind.

The model system for our investigation was, as for P450s and Mb, the cyclopropanation of styrene **4a** with ethyldiazoacetate **2b**, to yield the ethyl-2-phenylcyclopropane **5a**. In each case the reaction contained UPO, ethyldiazoacetate **2b** and styrene **4a** (typically 3 molar equivalents relative to **2b**) in a pH 7.0 potassium phosphate buffer, with 20% methanol as co-solvent. To enable effective reduction of the heme Fe(III) to Fe (II), an argon atmosphere for used for all biotransformations and an excess of sodium dithionite was added as a reducing agent. The yield of product **5a** was measured by ^{1}H NMR analysis of the unpurified reaction mixture, using an internal standard. The relative proportions of the four stereoisomers were measured using chiral GC, with the sample for GC analysis removed before the NMR internal standard was added.

In the first instance, we tested the biotransformation with two published UPOs: artUPO[9,31] and r-*Aae*UPO-PaDa-I-H, which are representatives respectively of the 'short' class I and 'long' class II UPOs, distinguished by their molecular weight and sequence[32] and active site topology.[31,33] We have shown that differences in their active site can have a dramatic impact on their chemo- and regioselectivity in oxygenation reactions. In general, r-*Aae*UPO-PaDa-I-H catalyses more selective reactions on smaller substrates, while artUPO can accept larger substrates, while still offering good selectivity in several cases.[9]

No transformation of **4a** into **5a** was observed using r-*Aae*UPO-PaDa-I, and hence this UPO class was not explored further. However, the class I artUPO successfully catalysed the transformation, with **5a** formed in a 9% yield on a small scale (0.3 mmol **5a**, Table 1, entry 1). A similar yield (8%) was obtained when tested on larger scale (3.0 mmol **4a**, Table 1, entry 3); the ease of scale-up is a key advantage of UPO biocatalysis. The product was formed with reasonable diastereoselectivity (*e.g.* 92 : 8 in favour of the *cis* diastereoisomer for entry 1), with the major diastereoisomer the same as that produced by the published CIS-T438S variants of P450$_{BM3}$,[16] and opposite diastereoselectivity compared to Fasan's Mb system.[21] Modest enantioselectivity was observed for both the *cis*- and *trans*-diastereoisomers, which strongly indicates enzyme-based induction of stereoselectivity. The reaction performed best in pH 7 buffer supplemented with 20% methanol, although conversion into **5a** was also seen with no co-solvent (Table 1, entry 2). In contrast, the use of 20% acetonitrile gave no conversion.

Table 1 Biocatalytic cyclopropanation of styrene **5a** and EDA **2b** using artUPO



Entry	**2b**/ mmol	**4a**/ mmol	Yield[a] **5a**	**5a** isomer proportion[b] %			
				***cis*-(*R*,*S*)**	***cis*-(*S*,*R*)**	***trans*-(*R*,*R*)**	***trans*-(*S*,*S*)**
1	0.1	0.3	9%	24	68	3	5
2[c]	0.1	0.3	7%	19	77	2	2
3[d]	1.0	3.0	8%[e]	15	52	15	17
4	0.3	0.1	2%	3	89	4	4

[a] Unless stated, the yield was measured by ^{1}H NMR, using 2Br-methyl naphthalene as an internal standard and represents a combined yield of all stereoisomers of **5a**. [b] Isomer proportions measured by chiral GC using a BGB175 column. [c] No MeOH co-solvent was used. [d] 16 h reaction time. [e] Isolated yield.

Controlling the pH at 7.0 appeared to be critical, as no conversion was observed when pH 6.0 or 8.0 buffer solutions were used. Interestingly, when the molar equivalence of **4a** and **2b** was inverted from 3 : 1 to 1 : 3 (Table 1, entry 4), the yield was lower (2%), but the selectivity for the formation of ***cis*-(*S*,*R*)-5a** was increased significantly.

While the yields of these class I UPO biotransformations were all low (and compare unfavourably with the P450$_{BM3}$ [16] and Mb[21] cyclopropanation reactions discussed above) it is notable that mutations directed towards improved cyclopropanation had not been made on any of the UPOs tested up to this point. It is known for P450$_{BM3}$ that mutation of nucleophilic residues, notably histidine, in or near the active site can improve their cyclopropanation activity by negating unproductive interactions with ethyl diazoacetate.[34] An analysis of the artUPO structure[31] revealed that, in common with other UPOs,[33,35] it possesses a glutamate residue E164 above the heme that has been implicated in the cleavage of peroxide in the usual UPO oxygenation mechanism using compound I (Fig. 1).[13]

In the interests of improving artUPO activity for cyclopropanation, E164 was therefore mutated to alanine and the E164A mutant assayed for cyclopropanation activity (Table 2). This and other mutants were created using methods described in ESI section 2† and expressed and isolated from *Pichia pastoris* as previously described.[31]

Mutation of the E164 carboxylate side chain was successful in increasing the yield of the reaction by approximately 10-fold, although there was little change in stereoselectivity. We speculated that stereoselectivity may be improved through the mutation of smaller hydrophobic residues I62 and I160 in the tunnel approaching the active site (Fig. 1) to variants with larger phenylalanines I62F and I160F. This indeed proved to

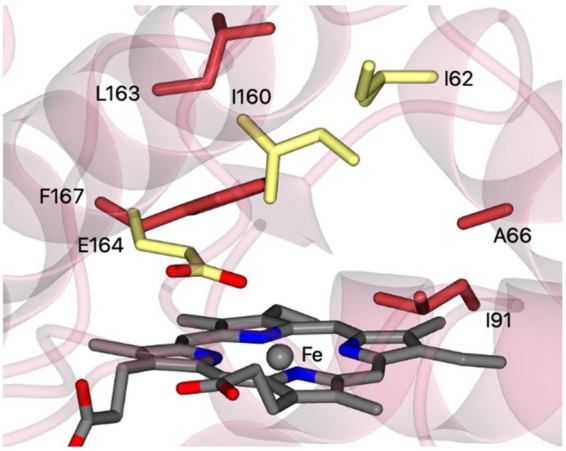
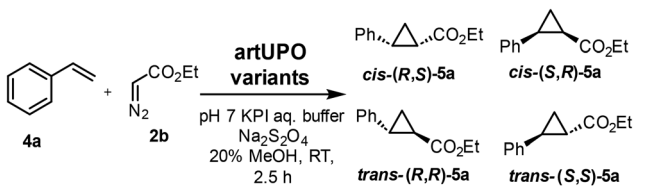


Fig. 1 Active site of artUPO (PDB 7NM)[31] showing the side chains of E164, I62 and I160 mutated in this study highlighted in yellow.

Table 2 Cyclopropanation of styrene **4a** by mutated artUPO variants under different conditions

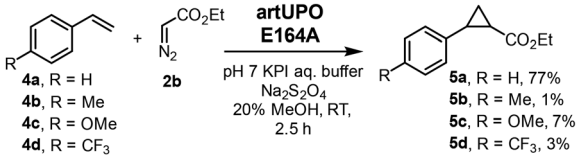


Entry	Variant	Yield[a] **5a**	**5a** isomer proportion[b] %			
			***cis*-(*R*,*S*)**	***cis*-(*S*,*R*)**	***trans*-(*R*,*R*)**	***trans*-(*S*,*S*)**
1	artUPO (wt)	8[c]	15	52	15	17
2	E164A	77	11	45	20	24
3	I62F	9	1	86	6	7
4	I160F	13	1	84	7	8
5	I62F/I160F	6	2	70	13	14
6	E164A/I62F	77[c]	6	53	18	22
7	E164A/I160F	95	17	23	29	30
8	E164A/I62F/I160F	90	6	17	34	43

[a] Unless stated, the yield was measured by ^{1}H NMR, using 2Br-methyl naphthalene as an internal standard and represents a combined yield of all stereoisomers of **5a**. [b] Isomer proportions measured by chiral GC using a BGB175 column. [c] Isolated yield following column chromatography.

be the case, with artUPO I62F (entry 3) and I160F (entry 4) now giving predominantly *cis*-products with very high *ee*s, although with lower activity than E164A. We then decided to combine activity and stereoselectivity mutants, testing I162F/I160F (entry 5) E164A/I62F (entry 6) and E164A/I160F (entry 7) to give double mutants exhibiting conversions of 6%, 77% and 95% respectively, and notably E164/I62F giving the ***cis*-(*S*,*R*)-3** product with an *ee* of 80%. However, combining all three mutations (entry 8) led to a variant with poorer stereoselectivity.

The artUPO E164A mutant was then challenged with a small library of *para*-substituted styrenes (**4a–4d**, Scheme 2).



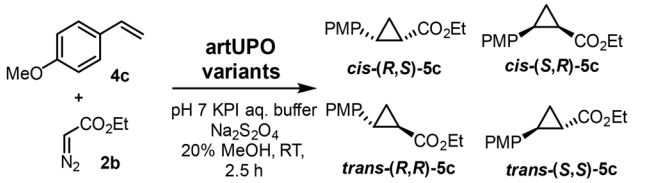
Scheme 2 Cyclopropanation of *para*-substituted styrenes by artUPO E164A mutant, using the same conditions as those used in Table 2 entry 2 with conversion measured by ^{1}H NMR.

The *para*-methyl **4b** and *para*-trifluoromethyl **4d** substrates gave only between 1% and 3% yields as measured by ^{1}H NMR, while the *para*-methoxy derivative **4c** gave the cyclopropane product **5c** in 7% yield with a 1 : 1.3 mixture of *trans* and *cis* isomers having 50% and 60% *ee* respectively (Table 3, entry 2). While there was a marked reduction in yield for the cyclopropanation of substituted styrenes **4b–4d**, mutation will always offer a potential solution to improve the biotransformation of poorly accepted substrates.

Thus, the cyclopropanation of **4c** was explored further with the artUPO mutants (Table 3). Pleasingly, double mutants E164A/I62F and E164A/I160F (entries 3 and 4) offered a stark improvement in yield and were the superior variants for this substrate, giving conversions of 55% and 58% respectively. Stereoselectivity was also improved; E164A/I62F gave the *E*- and *Z*-products with 90% and 76% *ee*; E164A/I160F gave the same products with 91% and 63% *ee*.[36] However, the triple mutant E164A/I62F/I160F (entry 5) was less effective, giving only 11% conversion.

The double mutants were also effective in the transformation of the transformation of 2-vinyl naphthalene **4e** (Table 4), which was transformed with **2b** to cyclopropanes **5e**

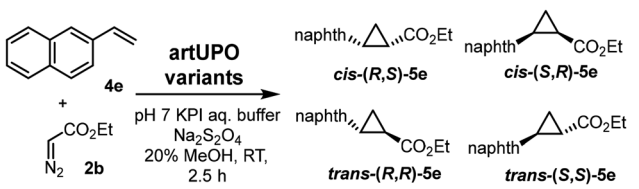
Table 3 Cyclopropanation of *para*-methoxy styrene **4c** by mutated artUPO variants



Entry	Variant	Yield[a] **5c**	**5c** isomer proportion[b] %			
			***cis*-(*R*,*S*)**	***cis*-(*S*,*R*)**	***trans*-(*R*,*R*)**	***trans*-(*S*,*S*)**
1	artUPO (wt)	—	—	—	—	—
2	E164A	7	9	34	43	14
3	E164A/I62F	55	6	42	49	3
4	E164A/I160F	58[c]	7	29	61	3
5	E164A/I62F/I160F	11	1	17	79	3

[a] Unless stated, the yield was measured by ^{1}H NMR, using 2Br-methyl naphthalene as an internal standard and represents a combined yield of all stereoisomers of **5c**. [b] Isomer proportions measured by chiral HPLC using either an IC column or an IA column. [c] Isolated yield. PMP = 4-MeO-C_6H_4.

Table 4 Cyclopropanation of *para*-methoxy styrene **4e** by mutated artUPO variants



			5e isomer proportion[b] %			
Entry	Variant	Yield[a] 5e	*cis*-(*R*,*S*)	*cis*-(*S*,*R*)	*trans*-(*R*,*R*)	*trans*-(*S*,*S*)
1	E164A/I62F	60[c]	4	28	64	4
2	E164A/I160F	58	7	42	47	4

[a] Unless stated, the yield was measured by ^{1}H NMR, using 2Br-methyl naphthalene as an internal standard and represents a combined yield of all stereoisomers of **5e**. [b] Isomer proportions measured by chiral HPLC using an IC column. [c] Isolated yield. naphth = 2-naphthalene.

with approximately 60% conversion in each case and with *E*- and *Z*-products with up to 90% and 75% *ee* respectively.

In conclusion, while UPOs are established biocatalysts for the production of oxygenated intermediates, their scope for 'promiscuous' reactions of the type catalyzed by other notable hemproteins has not been well explored. Although the UPOs here do not exhibit selectivities or performance as high as those of more established P450s and Mb, an exploration of these properties is warranted, as a wider range of improved complementary catalysts may be accessed through high throughput mutation and screening, following the initial identification of the catalytic properties described here. Thus, UPOs can be considered to be an enzyme class with significant potential for stereoselective alkene cyclopropanation, as well as other non-natural biotransformations.

Author contributions

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

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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36 The E164A/I62F and E164A/I160F double mutants were also tested using 3-MeO styrene as the substrate, to test whether meta-substituents can be tolerated. These preliminary results confirm that they can, with cyclopropanation yields of 15% for E164A/I62F, and 48% E164A/I160F obtained.