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COMMUNICATION

Enhanced cis- and enantioselective cyclopropanation of styrene catalysed by cytochrome P450BM3 using decoy molecules

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We report the enhanced cis- and enantioselective cyclopropanation of styrene catalysed by cytochrome P450BM3 in the presence of dummy substrates, i.e. decoy molecules. With the aid of the decoy molecule R-Ibu-Phe, diastereoselectivity for the cis diastereomers reached 91%, and the enantiomeric ratio for the (1S,2R) isomer reached 94%. Molecular dynamics simulations underpin the experimental data, revealing the mechanism of how enantioselectivity is controlled by the addition of decoy molecules.

Cyclopropane, the smallest of cycloalkanes, possesses high ring strain, yet is a chemical structure frequently observed in biologically significant natural products.[1] Compounds containing the cyclopropyl moiety are synthetically valuable targets in organic chemistry, as such molecules can be physiologically active and leveraged as pharmaceuticals, e.g. arylcyclopropylamines such as the platelet aggregation inhibitor ticagrelor.[2] Owing to their pharmaceutical value, synthetic chemists have explored numerous avenues to yield cyclopropanes, one of which is the transition metal-catalysed addition of a carbene derived from a diazo compound to an alkene.[3] Although significant progress has been made in the development of catalysts for asymmetric cyclopropanation, elaborate metal catalysts bearing chiral ligands have had to be crafted to achieve sufficiently high stereoselectivities.[4] Biocatalysts are expected to offer an alternative to the currently available challenging and burdensome synthetic methods; however, carbene transfers are abiological reactions and have not been found to occur in nature. Remarkably, seminal work by Arnold and co-workers has established that engineered haemoproteins can be engineered as catalysts for the stereoselective cyclopropanation of olefin compounds with ethyl diazoacetate (EDA) as a carbene source (Fig. 1A).[5] The Fasan group has substantiated that variants of myoglobin can be exploited in cyclopropanation reactions, yielding excellent diastereo- and enantioselectivities.[6] In addition, artificial enzymes incorporating a metallocomplex, which can impart the enzyme with near unlimited reactivity, have also been developed to realise highly stereoselective cyclopropanations.[7]

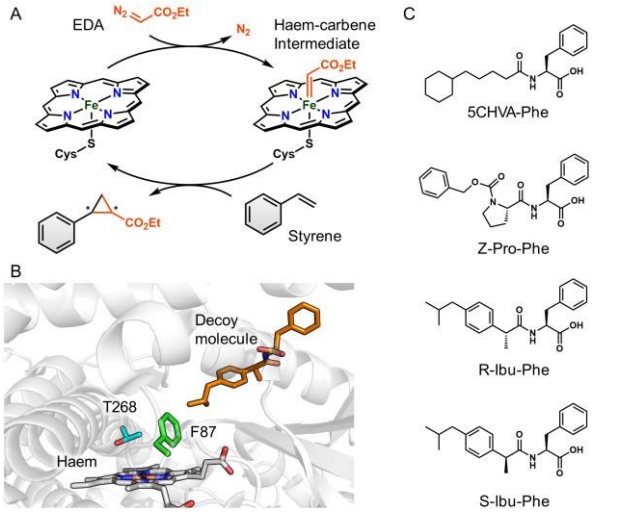

Fig. 1 (A) Styrene cyclopropanation mediated by a haem-carbene intermediate derived from EDA as a carbene source. (B) The active site structure of P450BM3 complexed with a decoy molecule (S-Ibu-Phe) (PDB ID: 6JO1). (C) The chemical structures of the decoy molecules employed in this study.

As a unique and different kind of approach for enzyme engineering, our research group has developed a system, wherein native substrate mimics (decoy molecules) are harnessed to activate unadulterated wild-type cytochrome P450s (CYPs or P450s) for non-native substrate oxidations.[8] Our latest reports revealed that by using *N*-acyl amino acids as decoy molecules, P450BM3, a fatty acid hydroxylase isolated from *Bacillus megaterium*, can be compelled into hydroxylating small organic compounds, such as benzene[9] and propane,[10] which does not occur in the absence of decoy molecules. Furthermore, we have demonstrated that decoy molecules not only trigger hydroxylation but can also control stereoselectivity; for example, depending on the structure of the decoy molecule employed, during the hydroxylation of indane by wild-type P450BM3 the apparent enantioselectivity could be inverted, with the enantiomeric excess ranging from 53% (*R*) with 5CHVA-

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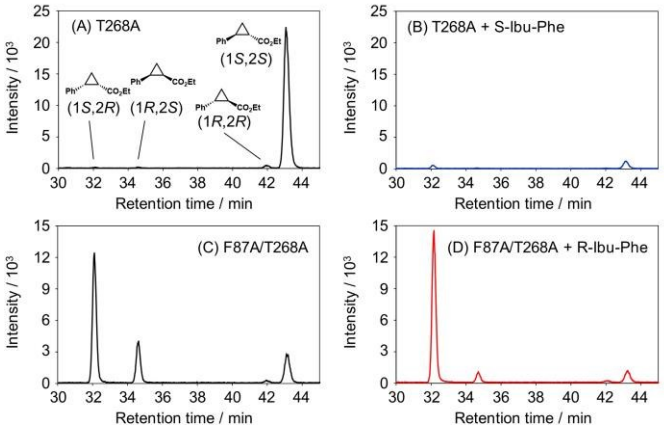


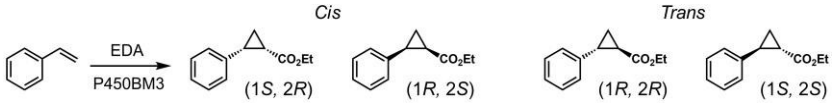
Fig. 2 Chromatograms of the products of styrene cyclopropanation catalysed by P450BM3 mutants in the absence/presence of decoy molecules

Phe to 56% (*S*) with Z-Pro-Phe.[11] Inspired by the recent successes of other groups in the development of biocatalysts for carbene transfer reactions, we envisioned that the application of decoy molecules could be expanded to encompass such abiological cyclopropanations as well as to dictate the stereoselectivity thereof. Herein, we report the stereoselective cyclopropanation of styrene catalysed by mutant P450BM3 in the presence of decoy molecules with EDA as the carbene source.

Initially, we prepared the T268A mutant of the P450BM3 haem domain and examined the stereoselectivity of the target reaction by pairing the T268A mutant with a selection of decoy molecules. The T268A mutant was selected, because threonine in this position has been reported to be deleterious to the catalysis of carbene transfers in P450BM3 (Fig. 1B).[12] The selection of decoy molecules employed in this study were chosen for their previously established potential in controlling the enantioselectivity of benzylic hydroxylation[11] and their structures are depicted in Fig. 1C. The results of the cyclopropanation reactions for the T268A mutant are shown in Table 1. In the absence of a decoy molecule, the T268A mutant catalysed the cyclopropanation of styrene with 99% trans selectivity and an enantiomeric ratio (er) of 98% for the (1*S*,2*S*) isomer, whilst the addition of S-Ibu-Phe increased selectivity for the thermodynamically less favourable cis diastereomer to 27%, with the er for the (1*S*,2*R*) isomer reaching 83% (Fig. 2A and B). This experimental fact indicates that the decoy molecule remains bound to the P450BM3 active site during catalysis, even for abiological reactions, whereby the active site is reshaped and stereoselectivity consequently changed. However, the turnover number (TON) was significantly reduced from 597 to 21 by addition of the decoy molecule, suggesting that the active site is too tight to comfortably accommodate all three molecules, i.e. styrene, the iron haem carbene intermediate, and the decoy molecule, simultaneously. From these observations we can infer that in the case of S-Ibu-Phe being bound to P450BM3, binding of styrene is likely destabilised, thus curbing efficient cyclopropanation. The fact that 5CHVA-Phe and Z-Pro-Phe simply decreased the TON whilst not exerting any appreciable effect upon stereoselectivity implies that these decoy molecules act as weak competitive inhibitors and, unlike S-Ibu-Phe, do not remain in the active site during cyclopropanation.

Given the results of the single mutant, we concluded that it may be necessary to introduce a further mutation to augment the active site space, allowing P450BM3 to stably incorporate decoy molecule, iron haem carbene intermediate, and styrene, simultaneously. We decided to mutate Phe87 into alanine, as this bulky residue is one of the closest to the haem cofactor and the likeliest culprit responsible for obstructing styrene accommodation in the presence of the decoy molecule. Cyclopropanation of styrene by the F87A/T268A double mutant in the presence of R-Ibu-Phe led to enhanced diastereo- and enantioselectivity without a significant loss in activity, with 91% cis selectivity and 94% er for the (1*S*,2*R*) isomer, compared to 81% cis selectivity and 75% er in the absence of a decoy molecule (Fig. 2C, D, and Table 1). In the presence of the decoy molecules tested, both diastereo- and enantioselectivity were changed and somewhat improved, whilst the drops in TON of the double mutant caused by the addition of decoy molecules were less than that of the T268A single mutant. For instance, whereas the addition of R-Ibu-Phe reduced the TON of the T268A mutant to about 5% TON, the F87A/T268A mutant retained about 80%

Table 1 Cyclopropanation of styrene catalysed by P450BM3 mutants in the presence of decoy molecules.[a]



Mutant	Decoy	cis:trans	(1*S*,2*R*):(1*R*,2*S*)	(1*R*,2*R*):(1*S*,2*S*)	TON
T268A	None	1:99	39:61	2:98	597 ± 16
	5CHVA-Phe	2:98	43:57	2:98	188 ± 27
	Z-Pro-Phe	2:98	40:60	2:98	231 ± 16
	R-Ibu-Phe	10:90	76:24	3:97	30 ± 4
	S-Ibu-Phe	27:73	83:17	4:96	21 ± 3
F87A/T268A	None	81:19	75:25	7:93	350 ± 13
	5CHVA-Phe	91:9	86:14	7:93	297 ± 14
	Z-Pro-Phe	87:13	82:18	6:94	350 ± 16
	R-Ibu-Phe	91:9	94:6	15:85	283 ± 10
	S-Ibu-Phe	90:10	90:10	11:89	254 ± 11

[a] Reaction conditions: 10 mM styrene, 10 mM EDA, 10 mM $Na_2S_2O_4$, 200 μM decoy molecule, and 5 μM P450BM3 in 100 mM HEPES buffer (pH 8.0) at 25°C for 14 hours.

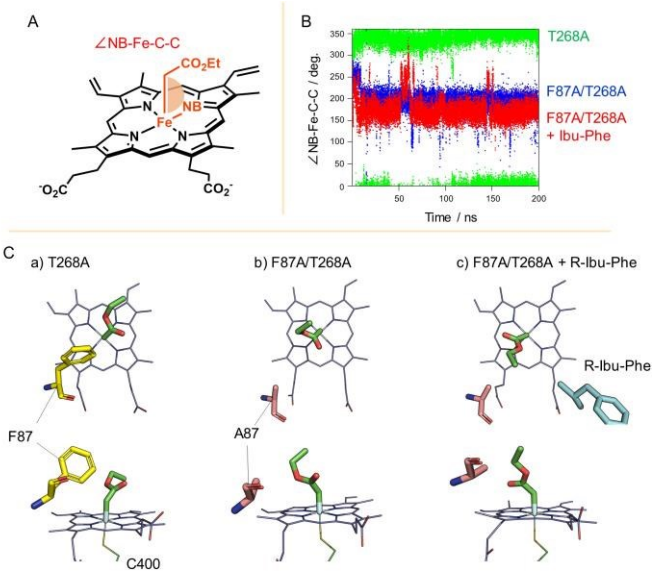


Fig. 4 (A) The dihedral angle in the haem-carbene intermediate monitored during the MD simulations. (B) The plots of the dihedral angle of the mutants during the 200 ns MD simulations. (C) The haem-carbene intermediate structures of the average structures extracted from the 200 ns MD simulations. The hydrogen atoms are omitted for clarity.

TON even in the presence of the decoy molecule. It is worth noting here that regardless of the presence or absence of R-Ibu-Phe, the TON for the (1*S*,2*R*) isomer was almost the same (242 and 213, respectively, calculated from the TONs and the product distribution), revealing that the decoy molecule simply suppresses the production of the other stereoisomers and does not exert a negative effect upon the reaction route to the (1*S*,2*R*) isomer. The 91% selectivity for the cis diastereomer, which remain tough and challenging to access by conventional chemical catalysts,[4] is on par with that of BM3-CIS (a P450BM3 mutant containing 13 mutations: V78A, F87V, P142S, T175I, A184V, S226R, H236Q, E252G, T268A, A290V, L353V, I366V, and E442K) obtained by directed evolution,[5] emphasising the simplicity of our approach.

To understand how the stereoselectivity of cyclopropanation catalysed by the F87A/T268A mutant was improved by the addition of R-Ibu-Phe, we conducted molecular dynamics (MD) simulations of the P450BM3 mutants, wherein the haem carbene intermediate was modelled into the structure manually. We monitored the C—C—Fe—N dihedral angle during 200 ns simulations, and the results revealed with great clarity that the dihedral angles were completely different between the T268A and F87A/T268A mutants, reflecting the inverted cis/trans preference for cyclopropanation between the two mutants (Fig. 3A, B, and C). These observations confirmed that, in the T268A mutant, the Phe87 side chain occludes the active site space, forcing the ester group of the iron-carbene intermediate to point away from Phe87 (Fig. 3C (a)). The MD simulation of the F87A/T268A double mutant with R-Ibu-Phe yielded similar behaviour of the dihedral angle to the simulation without a decoy molecule, indicating that the orientation of the ester group in the iron-carbene intermediate is unperturbed by the presence of a decoy molecule. A successive docking simulation of styrene into the "averaged" structure extracted from the simulation of the F87A/T268A mutant bound to R-Ibu-Phe resulted in styrene adopting a pro-(1*S*,2*R*)-like conformation (Fig. 4A). The benzene ring of styrene is comfortably accommodated in the space created by the F87A mutation, leaning away from the isobutyl group of R-Ibu-Phe to avoid steric repulsion. This docking pose is in harmony with the experimental evidence that formation of the (1*S*,2*R*) isomer was enhanced during cyclopropanation of styrene by the F87A/T268A mutant in the presence of R-Ibu-Phe (Table 1). Moreover, in the case of the T268A mutant, the computationally predicted product configuration of (1*S*,2*S*) is identical to the experimentally observed selectivity. Taken together, we can conclude that the decoy molecule R-Ibu-Phe does not influenced the orientation of the carbenoid species but only that of styrene in the active site, thereby determining how styrene approaches the carbene species and ultimately determining the stereoselectivity of the end products.

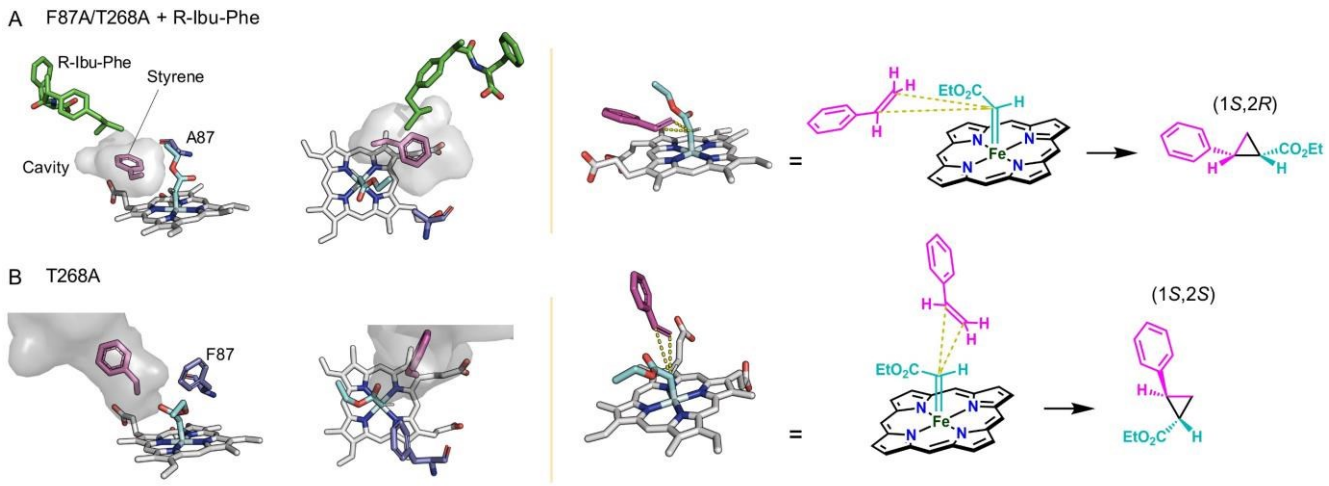


Fig. 3 The results of the docking simulations of styrene into the average structure in the MD simulation of the (A) F87A/T268A double mutant with R-Ibu-Phe and (B) T268A single mutant. (Left) The best docking pose of styrene obtained by the docking simulations. The cavity incorporating styrene is displayed as a surface model. (Right) The predicted product configurations judging from the orientation of styrene and the haem-carbene intermediate. The hydrogen atoms on the carbene carbon are modelled for clarity.

In conclusion, we have disclosed that decoy molecules can be utilised as auxiliaries to modulate the secondary coordination sphere[13] of the P450BM3 active centre to tune stereoselectivity for carbene-mediated cyclopropanations, a reaction type not found to exist in nature. This adds a further milestone to the chemical transformations compatible with our decoy molecule system, greatly expanding their versatility. Moreover, merely two mutations and a decoy molecule were required to deliver highly cis- and enantioselective cyclopropanation, something that has only been traditionally achieved by laborious directed evolution or extensive screening from nature. The work highlighted herein has successfully appended decoy molecules to the repertoire of enzyme engineering techniques available to modulate the stereoselectivity of cyclopropanation. Molecular dynamics simulations of the F87A/T268A mutant with R-Ibu-Phe hinted at the mechanism governing enhanced selectivity, and we believe this may contribute to the future design of P450BM3-based cyclopropanation catalysts in conjunction with decoy molecules.

Although still in its infancy, the present study promises the exploitation of the decoy molecule system to achieve a more selective catalysis, even for a panel of abiological reactions. Various types of carbene and nitrene transfer reactions, besides cyclopropanations, have been showcased by other research groups,[7a,b,14] and we anticipate that the latent potential of P450BM3 for such reactions could be unlocked by combining decoy molecules with conventional protein engineering strategies such as mutagenesis and replacement of haem with artificial metallocomplexes.[15]

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Stereoselectivity of cyclopropanation of styrene catalysed by cytochrome P450BM3 is enhanced in the presence of decoy molecules.

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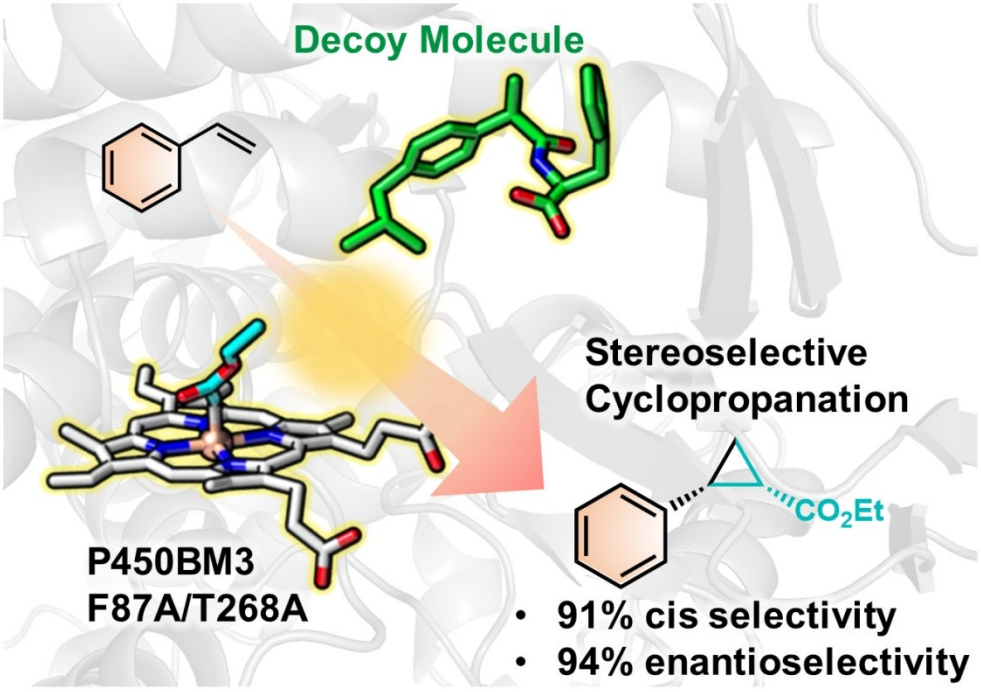

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