Enantioselective [2+2]-cycloadditions with triplet photoenzymes

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Organic molecules with their electrons promoted to the excited state have frontier orbitals fundamentally different from those of the ground state. This feature underpins many important photochemical transformations that are thermochemically forbidden11–12. In this regard, energy transfer (EnT) catalysis provides a powerful tool to populate the triplet state and enable various prominent photochemical transformations, including [2+2]-cycloaddition, electrocyclization, isomerization and others13–20. In a typical EnT catalysis scenario, a relatively long-lived triplet photosensitizer, which is generated from intersystem crossing from its excited singlet state (S1), promotes the substrate from the ground state (S0) to the triplet state (T1) via (Dexter) energy transfer (Fig. 1a). Unlike the well-established ground-state asymmetric catalysis, wherein the chiral catalyst-associated transition states typically have a lowered activation barrier, which ensures predominance of the enantioselective pathway, the molecule at the T1 state promoted by the photosensitizer gains sufficient energy to spontaneously undergo subsequent reactions without needing further catalysis. Therefore, to achieve triplet state enantio-induction, the reacting substrate molecule must already be associated in a chiral complex before photosensitization (Fig. 1a). To this end, one viable strategy is using affinitive chiral photosensitizers. Seminal studies by Bach and coworkers demonstrated that capitalizing on the two-point hydrogen bonding with a class of designer lactam-containing chiral diarylketones could mediate the high enantioselectivity of different EnT photoreactions (for example, [2+2]-cycloadditions, aza Paternò–Büchi reaction, deracemization) of lactam or amide substrates (Fig. 1b, top)15–18. Chromophore activation, in which the substrate alters its photophysical properties on complexation with a chiral catalyst, provides an alternative approach19–22. Pioneering work by the Yoon group showed that the triplet energy (Et) of hydroxychalcones is significantly lowered when coordinated to a chiral scandium complex, thereby attenuating racemic background reactions and enabling enantioselective intermolecular [2+2]-photocycloaddition of indole derivatives with good substrate generality and excellent enantioselectivities (up to 99% enantiomeric excess). A crystal structure of the photoenzyme–substrate complex elucidated the non-covalent interactions that mediate the reaction stereochemistry. This study expands the energy transfer reactivity23–26 of artificial triplet photoenzymes in a supramolecular protein cavity and unlocks an integrated approach to valuable enantioselective photochemical synthesis that is not accessible with either the synthetic or the biological world alone.

Naturally evolved enzymes, despite their astonishingly large variety and functional diversity, operate predominantly through thermochemical activation. Integrating prominent photocatalysis modes into proteins, such as triplet energy transfer, could create artificial photoenzymes that expand the scope of natural biocatalysis1–3.

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chemocatalysts (Fig. 1c). The chiral cavity optimized through directed evolution is accurately disposed with multiple functional elements around the photocatalytic centre, rendering efficient EnT catalysis for a collection of indole substrates with high yields and enantionic excess (e.e.).

To begin the study, the multidrug resistance regulator LmrR was selected as the protein scaffold for photoenzyme development. This protein features a large hydrophobic binding pocket at its dimer interface. The intramolecular [2+2]-photocycloaddition of indole derivative 1a was selected to prove our concept. The derived cyclobutane-fused tetracyclic spiroindoline 2a represents an interesting member of the large family of valuable polycyclic indole derivatives, but its enantioselective preparation remains unknown (Fig. 2a)41. 4-Benzoylphenylalanine (BpA; Fig. 2a) was used as a photosensitizer to construct an artificial photocatalytic centre using genetic code expansion for substrate chiral environment via multiple interactions with the enzyme, which provides enantioselectivity.

Fig. 1 | Design of triplet photoenzyme (TPe) for enantioselective [2+2]-cycloaddition. a, Comparison of enantio-induction of excited-state reactions with that of ground-state reactions. The latter is established by forming a chiral complex between the catalyst and substrate (S) to lower the activation barrier, thus suppressing the racemic background reaction. The triplet excited substrate, generated via triplet EnT, spontaneously transforms to product (P) regardless of complexation. To achieve enantioselective EnT, the spontaneous racemic pathway must be minimized. cat., catalysis. b, Two known strategies to achieve enantioselectivity for EnT [2+2]-cycloaddition. c, The design of TPe. The unnatural photosensitizer BpA or 3′-fluoro-BpA (FBpA) is installed in the reactive centre of TPe by genetic code expansion for substrate activation via EnT, and the resulting triplet state substrate is associated with a chiral environment via multiple interactions with the enzyme, which provides enantioselectivity.

To rapidly evolve the wide-type LmrR into a potent TPe for photocycloaddition of 1a, a semirational iterative-site-specific mutagenesis strategy was used for enzyme optimization based on a small, focused and high-quality library42. Three rounds of direct evolution were performed via single point saturation mutagenesis, for which cell lysates were used directly for photocycloaddition without tedious enzyme purification. First, docking 1a into the interfacial pocket of LmrR on the basis of the available crystal structure (PDB 3F8F) showed the expected promiscuous π–π stacking of the substrate indole moiety in between W96/W96′ (Fig. 2b). Therefore, in the first round of mutagenesis for BpA insertion, W96, D100, and M8, which closely surround the substrate, were preserved to secure the binding cavity. V15, N19, M89 and F93 were preferentially selected on the basis of their spatial distance to the C(2)–C(3) double bond of indole 1a (less than 10 Å) for effective EnT (Extended Data Fig. 1). Accordingly, four mutants were generated and evaluated for the EnT photocycloaddition of 1a, among which TPe_F93BpA (TPe1.0) exhibited substantially higher reactivity (51% yield) than the rest, but no enantioselectivity was observed (Supplementary Table 1). The liquid chromatography with tandem mass spectrometry study confirmed the precise insertion of BpA (Supplementary Fig. 2 and 3). Of note is that the uncalculated background reaction with LmrR gave photoproduct 2a in approximately 5% yield (Fig. 2d). Next, TPe1.0 was chosen as the new parent and residues that lie in proximity to BpA93, namely M8, A11, V15, N19, M89, W96, and D100, were mutated for the second round of directed evolution, and these are also the residues most used for LmrR-based artificial enzyme optimization43. Although the majority of the mutants gave very low e.e. values (less than 10% e.e.; Supplementary Table 2), the catalyst TPe_F93BpA_W96L (TPe2.0), which discards the initially appreciated W96, substantially improved the enantio-induction (36% yield, 46% e.e.). This suggests that the π–π stacking imposed by W96 might be detrimental for enantiomeric differentiation, as the inserted BpA also has aromatic rings. Notably, almost no performance difference was observed between purified TPe2.0 and the respective cell lysate (Fig. 2d). The buffer composition was screened
comparison to BP and LmrR. The reaction time was 12 h. e, Influence of reaction condition with TPe3.0. The reaction time was 1 h. The general reaction conditions, unless otherwise specified, were as follows: 1a (200 μM), TPe lysate or other catalyst (2.5 mol%), MOPS buffer (20 mM MOPS, 150 mM NaCl, pH 7.0), DMF (10% v/v for LmrR, TPe1.0, TPe2.0) or DMSO (10% v/v for TPe3.0), stirred at room temperature with irradiation (λ = 365 nm, 162 mW cm⁻²) under aerobic conditions. Error bars denote the standard deviation from triplicate runs.

Our subsequent studies found that TPe3.0 exhibited pronounced substrate specificity. Substituents on the indole ring significantly deteriorated the enantio-induction (20%, 0%, and 0%, respectively; Fig. 3a and Extended Data Fig. 2a) are distinct in the dimeric interfacial cavity, which ensured efficient EnT from the triplet state benzophenone to indole. This also explains the inferior enantioselectivity in the presence of the original W96, which disfavours the above set of stacking patterns. The stereochemistry presumably arises from the blocking of the enantiotopic face by the symmetrical indole substrate in the dimeric structure (Fig. 3a). The weak π-alky interactions of V15, A92 and L96 with indole, as well as the weak coordination of the olefin moiety by M89, A92 and benzophenone, assist in fixing the conformation of the substrate in favour of the observed enantioface differentiation (Fig. 3b). These presumptions are in line with the experimental findings in the second and third rounds of directed evolution, in which mutation of these residues resulted in a dramatic decrease in enantioselectivity (Supplementary Tables 2 and 3).

The crystal structure of the TPe3.0–1b complex sheds light on further enzyme optimization. The proximity of A11 and L18 to the carbonyl of 1b suggests that establishing new hydrogen bonds between them might be beneficial. To evaluate this hypothesis, indole 1m, a challenging substrate for TPe3.0, was chosen for further enzyme evolution. Selected rationally targeted mutagenesis was conducted to replace A11 and L18 with amino acids with hydrogen bond donor properties (serine, threonine, histidine and asparagine; Fig. 3f). To our delight, TPe3.0:A11N (TPe4.0) was identified as optimal and improved the selectivity of 2m from 18% e.e. to 81% e.e., albeit with slightly decreased yield. As suggested by the computational model (Fig. 3c), such marked performance amelioration could presumably be ascribed to the hydrogen bond of newly imparted N11 with the carbonyl of the substrates. Further optimization was made by using 3′-fluoro-4-benzoylphenylalanine (FBpA)

Fig. 2 | Directed evolution of TPe. a, The model intramolecular [2+2]-cycloaddition reaction and the structural illustration of BpA-inserted TPe. b, Docking of substrate 1a interacting with LmrR. The coloured balls show the π-π interaction of the indole moiety of 1a in between W96/W96’. c, The semirational iterative-site-specific mutagenesis strategy for the direct evolution of TPe. d, Reaction outcomes afforded by different TPe variants in (Supplementary Table 4). The third round of mutagenesis was performed based on TPe2.0 by further optimizing the most prominent residues revealed before, M8, V15, N19 and D100 (Supplementary Table 3). This led to the identification of TPe_F93BpA_W96L_M8L (TPe3.0), which afforded 2a in 58% yield and with 71% e.e. With TPe3.0, shortening the reaction time from 12 h to 1 h indeed improved the product enantioselectivity (Supplementary Table 5). The insignificant conformational changes of relevant residues (V15, L18, M89, A92, BpA, L96) with a root-mean-square distance value of 0.267 Å suggest a lock-and-key binding mode (Extended Data Fig. 2c). As shown in Fig. 3a, layer-to-layer
in lieu of BpA, considering the likelihood of enhancement of indole–Bp π–π stacking43 and the formation of additional H···F hydrogen bond(s), which can increase the cofactor conformational rigidity (Fig. 3e). The latter was supported by theoretical calculations suggesting that FBpA forms a weak hydrogen bond with the C–H of the tert-butyl group of 1b (Supplementary Fig. 10). Indeed, TPe4.0_FBpA turned out to be much superior for converting 1m to 2m (93% yield, 91% e.e.). These optimizations also proved to be rewarding for indole 1a (Fig. 3g) and other substrates (Supplementary Fig. 9). TPe4.0_FBpA follows Michaelis–Menten kinetics, with a Michaelis constant ($K_m$) = 37.46 ± 3.84 μM and an apparent unimolecular rate constant $K_{cat}$ (4.29 ± 0.03 min$^{-1}$) for the reaction with 1b (Supplementary Fig. 11). These results suggest that the two indole molecules in the enzyme pocket are unlikely to be simultaneously processed. In other words, only one substrate is excited at one time and the other probably chaperones the reaction to ensure enantioselectivity. This notion was also supported by further...
Despite the light-intensity-dependent photo-inactivation effect, the photoenzyme is highly efficient in completing the photocycloaddition with the e.e. value still higher than 90% (Supplementary Table 7). 0.25 mol%, and accordingly more than 350 turnovers were achieved. Nevertheless, the loading of TPe4.0_FBpA for indole 1b could be lowered to 0.25 mol%, and accordingly more than 350 turnovers were achieved with the e.e. value still higher than 90% (Supplementary Table 7). Despite the light-intensity-dependent photo-inactivation effect, the photoenzyme is highly efficient in completing the photocycloaddition with excellent enantio-induction before significant photodamage occurs (Extended Data Fig. 3 and Supplementary Table 8).

The substrate generality of the triplet photoenzymes was evaluated (Fig. 4). A range of indole derivatives with methyl or aryl groups of diverse steric and electronic properties at the C2 position were well tolerated by TPe4.0_FBpA, affording products 2a–2f in 82–97% yields and with 90–99% e.e. Comparable results were recorded for indoles containing substituents on the benzene ring (21–2n). The reaction of indole derivative 1o bearing gem-dimethyl, an important moiety that prevails in bioactive natural products, proceeded uneventfully to 2o with four contiguous quaternary carbons (80%, 93% e.e.). In several cases, irradiation of weaker light intensity or longer wavelength was applied to attenuate the competing background reaction via direct photoexcitation (2eh, 2n–o). Considering the ever-present substrate specificity to different extents, further enzyme optimizations were needed to ascertain their practicality. Nevertheless, the loading of TPe4.0_FBpA for indole 1b could be lowered to 0.25 mol%, and accordingly more than 350 turnovers were achieved with the e.e. value still higher than 90% (Supplementary Table 7).
individually performed for those substrates with less than 90% e.e. (2h, 2g, 2j, 2k, 2n), and these efforts were rewarding (Supplementary Section 12). For instance, TPe3.0_FBPα_L96V was found to be better suited than TPe4.0_FBPα for substrates 2j and 2k (Supplementary Table 10). Also notable is the 5-fluorindole product 2n, for which the enantipurity was elevated from 85% e.e. provided by TPe4.0_FBPα to 91% e.e. by using TPe4.0_FBPα_V99C, which was identified by additional saturation mutagenesis targeting residues in the second coordination sphere (Supplementary Fig. 14). Notably, the best enzyme variant TPe4.0_FBPα also holds promise for other N-Boc indole substrates of different molecular geometries, as shown by the reaction of indole-2-carboxylic acid derivative 1p (92% yield, 57% e.e.) without individual optimization. Finally, 10 mg-scale synthesis was conducted for three substrates (1a, 1b, 1m), and comparably good results were obtained.

To summarize, the triplet photoenzymes were developed by encoding synthetic photosensitizer unnatural amino acids to create an entirely new photocatalytic centre and optimized through directed evolution. Rounds of directed evolution based on a focused library identified evolved photoenzyme variants that enabled enantioselective intramolecular [2+2] photocycloadditions of indole derivatives (15 entries, 80–97% yields, all greater than or equal to 90% e.e.) under aerobic conditions. The crystal structure of the TPe–substrate complex elucidates the origin of enantioselectivity induced by multiple interactions of the substrate with the surrounding residues. This study demonstrates that the merger of versatile synthetic photocatalysts with a macromolecular protein can impart new reactivity models that fundamentally expand the catalytic repertoire of enzymes for mediating new-to-nature photochemical reactions. The intricate and delicate protein cavity provides a superlative chiral environment for controlling the notoriously difficult enantiotopic selectivity of excitation-state bonding. Given the power of computation-aided artificial enzyme design and the continuous expansion of reprogrammed genetic codes for incorporating more robust non-canonical amino acid photocatalysts, triplet photoenzymes have the promise to become a general solution for various valuable enantoielective photochemical transformations.

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4. Silvi, M. & Melchorre, P. Enhancing the potential of enantioselective organocatalysis with a macromolecular protein can impart new reactivity models that fundamentally expand the catalytic repertoire of enzymes for mediating new-to-nature photochemical reactions. The intricate and delicate protein cavity provides a superlative chiral environment for controlling the notoriously difficult enantiotopic selectivity of excitation-state bonding. Given the power of computation-aided artificial enzyme design and the continuous expansion of reprogrammed genetic codes for incorporating more robust non-canonical amino acid photocatalysts, triplet photoenzymes have the promise to become a general solution for various valuable enantoielective photochemical transformations.
Methods

General procedure for the photoenzymatic reactions
A reaction mixture (1.0 ml) containing the TPe lysates or purified TPe (2.5–5 mol%) and substrate 1 (200 μM) in MOPS buffer (20 mM MOPS, 150 mM NaCl, pH 7.0) with 10% (v/v) DMSO in a glass tube was illuminated using a photoreactor (WATTCAS, WP-TEC-1020LC, 365 nm, 162 mW cm⁻² or 385 nm, 6.2 mW cm⁻², 4 °C). For the reaction setup and the emission spectrum of the lamp, see Supplementary Fig. 1.

After the reactions were completed, the mixture was extracted with ethyl acetate (3 × 3 ml). The combined organic phase was concentrated by rotary evaporation. Then, the crude product was dissolved in isopropanol (90 μl) and the internal standard thioxanthone in isopropanol (0.01 mg ml⁻¹, 60 μl) was added. Yields and e.e. values of photocycloaddition products 2 were measured using normal-phase HPLC on a chiral stationary phase as the mean of triplicate runs.

The 100-fold scale-up reactions (10 mg scale) were performed with the reaction mixture (100 ml) containing the TPe lysates and substrate 1 (200 μM) in MOPS buffer (20 mM MOPS, 150 mM NaCl, pH 7.0) with 10% (v/v) DMSO in a 250 ml round-bottom flask and illuminated under LED lamps (365 nm, 39 mW cm⁻²) for 20 h in an ice bath. The mixture was extracted with ethyl acetate (100 × 3 ml). The combined organic phase was concentrated by rotary evaporation. The crude products were purified by flash chromatography on silica gel (PE/EtOAc, 20:1 to 10:1) to afford the photocycloaddition products.

Data availability

All data are available in the main text or the Supplementary Information. The crystal structure data of TPe 3.0 and TPe 3.0 in complex with substrate 1b have been deposited in the Protein Data Bank under accession numbers 7XUP and 7XUQ. Source data are provided with this paper.

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Author contributions
Y.W. and F.Z. conceived the project and designed the experiments. N.S. and J.H. performed the experiments and interpreted the data. J.Q. and X.C. performed the crystallography study and interpreted the data. T.Z. and R.L. carried out the computational studies. J.G., L.T., W. Zhang and Y.D. assisted with the molecular biology experiments. G.W. assisted with the substrate synthesis. W. Zhao performed protein mass analysis. Y.W. and F.Z. wrote the manuscript with input from all of the authors.

Competing interests
The authors declare no competing interests.

Additional information

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Extended Data Fig. 1 Docking of substrate 1a interacting with the LmrR dimer using AutoDock4. The blue sticks show substrate 1a. The yellow balls and sticks show the W96 residue. The yellow dashes show the π-π interaction between W96 and the indole moiety of 1a. The red balls show the residues pointed to the inner pocket and surrounding W96, which are reserved in the first round of evolution. The green balls show the residues pointed to the inner pocket and with close spatial distances (<10 Å) to the C(2)-C(3) double bond of indole 1a, which were screened for BpA insertion.
Extended Data Fig. 2 | The crystal structures of TPe3.0 and TPe3.0 in complex with substrate 1b. **a.** The crystal structure of TPe3.0 cocrystallized with 1b (PDB code: 7XUQ). Two molecules forming a dimer are presented. The backbone is shown as grey cartoon. The BpA is shown as sticks with carbon atoms coloured in light blue. 1b is shown as sticks with carbon atoms coloured in orange. Oxygen and nitrogen atoms are shown in red and blue, respectively. The yellow dashes show the π-π interactions between BpA and the substrates with the distances (Å) labelled. **b.** The crystal structure of TPe3.0 (PDB code: 7XUP) in a monomeric form. **c.** Superimposition of the structure of TPe3.0 and the structure of TPe3.0 in complex with substrate 1b. TPe3.0 is shown as pink cartoon while TPe3.0 in complex with 1b is shown as grey cartoon. Interacting residues V15, L18, M89, A92, BpA and L96 are shown as sticks with carbon atoms coloured in light pink and grey respectively. Oxygen, nitrogen and sulfur atoms are shown in red, blue and yellow, respectively.
Extended Data Fig. 3 | The reaction time course of photocycloaddition of 1b catalysed by TPe4.0_FBpA under different light intensity irradiation. Light intensity is (A) 162 mW/cm² and (B) 3.8 mW/cm². Error bars denote the standard deviation from triplicate measurements, and they are not shown when smaller than the data point marker.