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Asymmetric Redox-Neutral Radical Cyclization Catalyzed by Flavin-Dependent 'Ene'-Reductases

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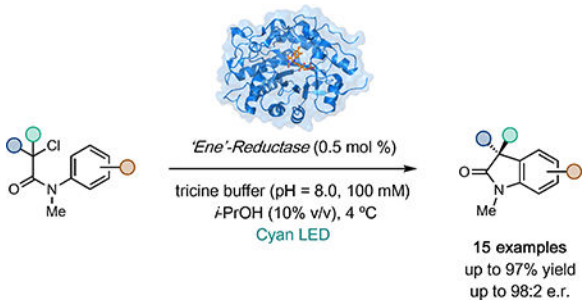
Abstract

Flavin-dependent 'ene'-reductases (EREDs) are exquisite catalysts for effecting stereoselective reductions. While these reactions typically proceed through a hydride transfer mechanism, we recently found that EREDs can also catalyze reductive dehalogenations and cyclizations via single electron transfer mechanisms. Here we demonstrate that these enzymes can catalyze redox-neutral radical cyclizations to produce enantioenriched oxindoles from α-haloamides. This transformation is a C–C bond forming reaction currently unknown in nature and one for which there are no catalytic asymmetric examples. Mechanistic studies indicate the reaction proceeds via the flavin semiquinone/quinone redox couple, where ground state flavin semiquinone provides the electron for substrate reduction and flavin quinone oxidizes the vinylogous α-amido radical formed after cyclization. This mechanistic manifold was previously unknown for this enzyme family, highlighting the versatility of EREDs in asymmetric synthesis.

Graphical Abstract



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Author contributions: T.K.H. conceived and directed the project. T.K.H., M.J.B., A.J.M., and K.F.B. designed the experiments. M.J.B., A.J.M., and K.F.B. performed and analyzed the experiments. D.G.O performed the EPR measurements and B.K. and D.G.O. performed the TCSPC and transient absorption measurements. B.K., D.G.O., and G.D.S. analyzed and interpreted the spectroscopy results. All authors discussed the results and commented on the manuscript. K.F.B., A.J.M., and D.G.O. contributed equally.

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The selective synthesis of stereochemically pure molecules is a goal central to the agrochemical, fragrance, and pharmaceutical industries. Consequently, numerous chiral catalysts have been developed to carry out a diverse range of chemical transformations (1,2). Among these, enzymes are particularly attractive because they can be engineered to provide unparalleled levels of catalyst efficiency and product selectivity (3). Unfortunately, the majority of enzymes are thought to only catalyze a single type of chemical transformation. In contrast, small molecule chiral catalysts are often able to catalyze many mechanistically distinct reactions with good levels of selectivity (4). This generality is due, in part, to their ability to react with a diverse array of substrates. Substrate promiscuity, however, is not a feature exclusive to small molecule catalysts — many enzymes for organic synthesis possess this trait (5). Oxidoreductases, for instance, are renowned for their broad substrate scopes (6,7). We hypothesized that these enzymes could provide prosperous ground on which to discover and develop new reactivity (8,9,10,11). In particular, we are interested in exploiting oxidoreductases to address long-standing selectivity challenges inherent to the reactivity of radicals in chemical synthesis (12,13).

Flavin-dependent ‘ene’-reductases (EREDs) are substrate promiscuous oxidoreductases widely used in chemical synthesis for the stereoselective reduction of activated alkenes (14). Mechanistically, the reduction occurs via hydride transfer from the flavin hydroquinone (FMN_{hq}) to the electrophilic β-position of the alkene (15,16). In other protein scaffolds, such as ferredoxin reductase or P450 reductase, flavin effects the reduction via two single electron transfers (17). We recognized that if this reactivity profile could be achieved with EREDs, they could be used as catalysts in asymmetric radical reactions. In our initial studies, we found that EREDs can catalyze asymmetric radical hydrodehalogenations of α-bromoesters via a single electron transfer (18). Mechanistically, this occurs via electron transfer from FMN_{hq} to the substrate, forming an α-acyl radical, followed by hydrogen atom transfer from the flavin semiquinone (FMN_{sq}). Recently, we demonstrated that these enzymes are also able to catalyze reductive radical cyclizations with high levels of diastero- and enantioselectivity using a related mechanism (19). These examples demonstrate the synthetic versatility of EREDs and highlight their ability to address long-standing challenges in radical-mediated asymmetric synthesis.

Redox-neutral radical reactions are a type of transformation where the substrate is both oxidized and reduced during the reaction mechanism. Catalysts that move between reducing and oxidizing redox states, such as photoredox catalysts (20), are ideally suited for this family of reactivity. We recognized that flavin could serve as reductant and oxidant within the same enzyme active site. As a model, we targeted the cyclization of α-halo-β-amidoesters to furnish 3,3-disubstituted oxindoles. This motif is prevalent in medicinally valuable molecules and there are no known methods for rendering this radical cyclization asymmetric (21,22). Mechanistically, this cyclization occurs via reduction of the α-halo-β-amidoester to afford an α-acyl radical that can react with the pendant aromatic ring and upon oxidation of the resulting vinylogous α-amido radical, followed by deprotonation, afford product (Fig. 1). Central to the development of this reactivity is the identification of a flavin redox pair where the oxidized form is capable of oxidizing the vinylogous α-amido radical without reducing the α-acyl radical. If successful, this reaction would provide a new biocatalytic strategy for stereoselectively preparing Csp^2-Csp^3 bonds (23, 24, 25).

Results and Discussion

We began by exploring the ability of eight structurally diverse wild-type EREDs to catalyze the cyclization of α-bromo-β-amidoester **1** to oxindole **2** under ambient conditions with NADPH to reduce the ERED to the FMN_{hq} oxidation state (Supplementary Table 1). While most EREDs were inactive, morphinone reductase (MorB), with its large active site (26), proved effective in providing racemic oxindole **2** in good yield with the remaining mass balance being reduced amide **3**. Improved enantioselectivity can be achieved with 12-oxophytodienoate reductase (OPR1), although the major product of this reaction is amide **3** (Table 1, entry 1). Under these conditions, we hypothesize the flavin hydroquinone/semiquinone (FMN_{hq}/FMN_{sq}) redox couple to be operative, where FMN_{hq} functions as a reductant and FMN_{sq} serves as the oxidant (Figure 1C). However, FMN_{sq} is also capable of reducing the α-acyl radical, potentially accounting for the formation of amide **3**. We recognized that by moving to an orthogonal redox couple, it may be possible to improve the product selectivity without needing to alter the protein sequence.

The flavin semiquinone/quinone (FMN_{sq}/FMN) redox couple, where FMN_{sq} would serve as the reductant and FMN as the oxidant, has features that make it ideal for redox-neutral radical cyclizations (Figure 1C). FMN_{sq} ($E_{1/2}^{ox}$ = −0.490 V vs SCE) is a slightly stronger reductant than FMN_{hq}, enabling it to reduce activated α-haloacetanilide (27). Moreover, FMN has a comparable oxidation potential to FMN_{sq}, enabling it to oxidize vinylogous α-amido radicals. Importantly, FMN cannot function as a reductant, consequently, formation of amide **3** should be diminished. Massey had previously demonstrated that FMN_{sq} is formed within the active sites of EREDs by irradiating with visible light in the presence of electron donors, such as EDTA (28, 29, 30). Indeed, we found that by removing NADPH and irradiating a sample containing OPR1 in tricine buffer with cyan light, FMN_{sq} is formed (as determined by EPR). When these conditions were tested with α-bromo-β-amidoester **1**, oxindole **2** was formed as the major product, with the remaining mass balance being amide **3** (Table 1, entry 2). Analysis of this reaction by UV-vis spectroscopy revealed formation of FMN_{hq}, providing a mechanism for formation of the dehalogenated amide (Supplementary Figure 3–5). This pathway can be avoided by moving to the less oxidizing α-chloro-β-amidoester **1'**, which provides the desired oxindole in 94% yield with 95:5 er favoring the (*S*)-enantiomer (Table 1, entry 3).

Control experiments confirm both light and enzyme are required to form oxindole (Table 1, entry 4 and 5). A survey of lamps with different emission spectra revealed blue lamps (λ_{max} = 450 nm and 476 nm) to furnish more dehalogenated product than cyan or green ones (λ_{max} = 530 nm) (Supplementary Table 4–5). As the absorption spectrum of OPR1 is red-shifted by comparison to free FMN (Supplementary Figure 6), we hypothesized that photoexcitation of free FMN is facilitating the formation of amide **3**. Indeed, when OPR1 is replaced with FMN under the reaction conditions, only dehalogenated amide **3** is observed (Table S6). Transient absorbance spectroscopy indicates that ground state FMN_{hq}, presumably formed via photoreduction of FMN with tricine, is responsible for substrate dehalogenation (Supplementary Figure 9–11).

Next, we probed the flavin oxidation state responsible for reducing **1'** to the α-acyl radical within the active site of OPR1. While we propose this species to be FMN_{sq}, based on our previous studies, we recognized that ground state or photoexcited flavin hydroquinone (FMN_{hq}) could also be responsible for reduction (23). To determine whether FMN_{hq} is capable of effecting cyclization, reactions were run for 2 hours with 2-fold and 40-fold excess (with respect to enzyme) of NADPH. In the absence of light, neither reaction afforded any consumption of chloroamide **1'**, confirming that ground state FMN_{hq} is not responsible for the observed reaction (Figure 2A). Next, we considered the photoexcited state of FMN_{hq}. Previously, we found that α-chloroamides can be dehalogenated via photoinduced electron transfer if they form electron donor-acceptor (EDA) complexes with FMN_{hq} in the ERED active site. However, quenching of the excited state (FMN_{hq}*) was not observed in the presence of **1'** (Figure 2B), indicating that photoinduced electron transfer from FMN_{hq} to **1'** is not responsible for radical formation. Attempts to observe an EDA complex were unsuccessful because of flavin oxidization on the time-scale of the experiment.

Having ruled out FMN_{hq} as a reductant, we conducted experiments to determine whether FMN_{sq} was responsible for the observed reactivity. First, OPR1 in tricine buffer was irradiated with cyan light and the formation of FMN_{sq} was determined and quantified via EPR spectroscopy (Supplementary Figure 12–14). Next, to determine if ground state FMN_{sq} is responsible for oxindole formation, a Schlenk flask containing OPR1 and tricine buffer was irradiated with cyan light for 1 hr. After FMN_{sq} formation was confirmed and quantified by EPR (Supplementary Figure 13), the reaction was shielded from light and substrate was added. After two hours, oxindole **2** was observed in 3% yield with 95:5 er and negligible formation of the reduced amide **3** (Figure 2C). This experiment indicates that ground FMN_{sq} provides the electron to initiate the cyclization. Moreover, 68 catalyst turnovers are observed (based on FMN_{sq} concentration determined by EPR), suggesting FMN_{sq} is regenerated during the reaction mechanism. Based on these studies, we propose a mechanism in which enzyme-bound FMN is reduced via photoinduced electron transfer to generate FMN_{sq} (24). This species can reduce **1'** to generate an α-acyl radical and FMN. Stereoselective cyclization into the aromatic ring forms a vinylogous α-amidoradical, which is readily oxidized by FMN to form FMN_{sq} and the desired oxindole (Figure 2D)

As light is only required to initiate the reduction of FMN to FMN_{sq}, we questioned why constant irradiation is required to achieve high conversions. Schaller found that OPR1 kinetically stabilizes FMN_{sq}. Consequently, this OPR1⊂FMN_{sq} (⊂indicates binding) can disproportionate to form catalytically inactive FMN_{hq} and FMN (31). Indeed, an EPR spectrum collected at the end of the photoinitiated reaction discussed above (Figure 2C) revealed consumption of FMN_{sq} (Supplementary Figure 13). Moreover, FMN_{sq} concentration steadily decreases over two hours in the dark in the absence of substrate (Supplementary Figure13). Collectively, these results suggest that FMN_{sq} is unstable under the reaction conditions. We considered that the role of irradiation is to oxidize catalytically inactive FMN_{hq} to FMN_{sq}, presumably through oxidation by photoexcited FMN (Supplementary Figure 21). To test this hypothesis, OPR1 is reduced with NADPH to form FMN_{hq}. Upon irradiation with cyan light, formation of an EPR signal consistent with FMN_{sq}

is observed, suggesting light facilitates FMN_{hq} oxidation (Supplementary Figure 14) (32). This feature can be observed in a preparative reaction containing excess NADPH (5-fold excess by comparison to enzyme). When run in the dark, no product is formed, presumably because FMN_{sq} is not present (Figure 2A). Upon irradiation with cyan light, oxindole **2** is formed in 6% yield with <1% formation of the dehalogenated product (Supplementary Figure 15). By comparison, when the same reaction is run in the absence of NADPH, the desired product is formed in 26% yield with 1% formation of **3** (Supplementary Figure 14). These experiments demonstrate that photoinduced electron transfer can be used to regenerate flavin oxidation states with limited stability and in doing so, expand the synthetic utility of flavoenzymes beyond what can be achieved using simple chemical oxidants or reductants.

With ideal reaction conditions in hand, we focused our attention toward the scope of this reaction (Table 2). OPR1 accepts a variety of substituents at the α-position of the amide. While the smaller methyl substituent (Table 2, 18) affords product with lower selectivity, longer linear groups, such as propyl, butyl, or allyl (Table 2, 19,20,22), furnish product in good yield with excellent levels of enantioselectivity. Branched substituents, however, deliver product with lower yield and enantioselectivity (Table 2, 21). A variety of ester substituents are accepted, affording the corresponding oxindoles in high yields and enantioselectivities (Table 2, 23,24). With regard to substitution on the aromatic ring, ortho-substituents proved to be unreactive, failing to provide either oxindole or dehalogenated material, suggesting they may be incapable of binding to OPR1. Electron-donating para-substituents, however, are well tolerated with the corresponding oxindoles accessed in good yield with excellent levels of enantioselectivity (Table 2, 26). Arenes with electron-withdrawing substituents are more challenging substrates, requiring elevated reaction temperatures to furnish product (Table 2, 27–29). As these substrates form a larger amount of the hydrodehalogenated product (40% yield of the hydrodehalogenated product in the case of 28), we hypothesize this low product yield is likely due to the kinetic challenge involved in coupling electron poor arenes with electrophilic α-acyl radicals. Electron-withdrawing meta-substituents were found to be poorly reactive substrates, but electron-donating groups are more reactive. For instance, *m*-methyl amides afford oxindole product as a 1.3:1 ratio of regioisomers (Table 2, 30:31). A preliminary examination of OPR1 variants with mutations made to residues responsible for substrate binding revealed that OPR1-H187N is capable of providing product with modestly improved levels of selectivity (2.1:1), suggesting protein engineering can be used to alter the regioselectivity of the cyclization. Finally, we found that the electron-withdrawing ester group can be removed from the substrate and product formation is still observed, albeit in diminished yields (Table 2, 32). Initial control experiments indicate that ground-state semiquinone is not capable of initiating this reaction (Supplementary Figure 23) and this substrate does not form an EDA complex with FMN_{hq} (Supplementary Figure 24) suggesting this reaction may initiate via a distinct mechanism.

‘Ene’-reductases, enzymes traditionally thought to catalyze only reductive reactions, are able to catalyze redox neutral radical cyclizations with high levels of enantioselectivity under photocatalytic conditions. This new function is achieved without any modification to the

wild-type sequence. The central mechanism for optimization was to change the flavin redox couple. Moving from FMN_{hq}/FMN_{sq} to FMN_{sq}/FMN redox pair disfavors the mechanistic pathway responsible for dehalogenation. Modulating the flavin oxidation state provides a new handle for biocatalyst optimization and highlights the diversity of reactivity available to substrate-promiscuous wild type enzymes. Moreover, identifying enzymes capable of catalyzing non-natural reactions presents an opportunity to address reactivity and selectivity challenges that small molecule catalysts have thus far failed to address (33).

Methods

General procedure for biocatalytic radical cyclization to form oxindoles

Under anaerobic atmosphere, a 4 mL shell vial was charged with 100 mM tricine pH 8 and OPR-1 (0.01 μmol, 0.005 equiv.) to reach a total volume of 1.8 mL. To this mixture was added chloroamide substrate **1** (0.02 mmol, 1.0 equiv.) in 200 μL *i*-PrOH to reach a final total volume of 2 mL. The reaction mixture was capped with a septum and placed on a stir plate in a 4ºC walk in refrigerator (unless otherwise stated). The reaction mixture was irradiated with a 497 nm cyan LED for 48 hours. After 48 hours elapsed, 1 mL acetonitrile containing 0.01 mmol internal standard (either α,α,α−trifluorotoluene or 1,3,5-tribromobenzene) was added, followed by an additional 3 mL acetonitrile to precipitate enzyme. Yield was calculated vs. internal standard on LC-MS. In a scintillation vial, 2 mL quenched reaction mixture was diluted with 10 mL EtOAc, and evaporated to dryness *in vacuo*. The resulting residue was redissolved in 1:1 Hexanes : *i*-PrOH and injected on HPLC to determine enantioselectivity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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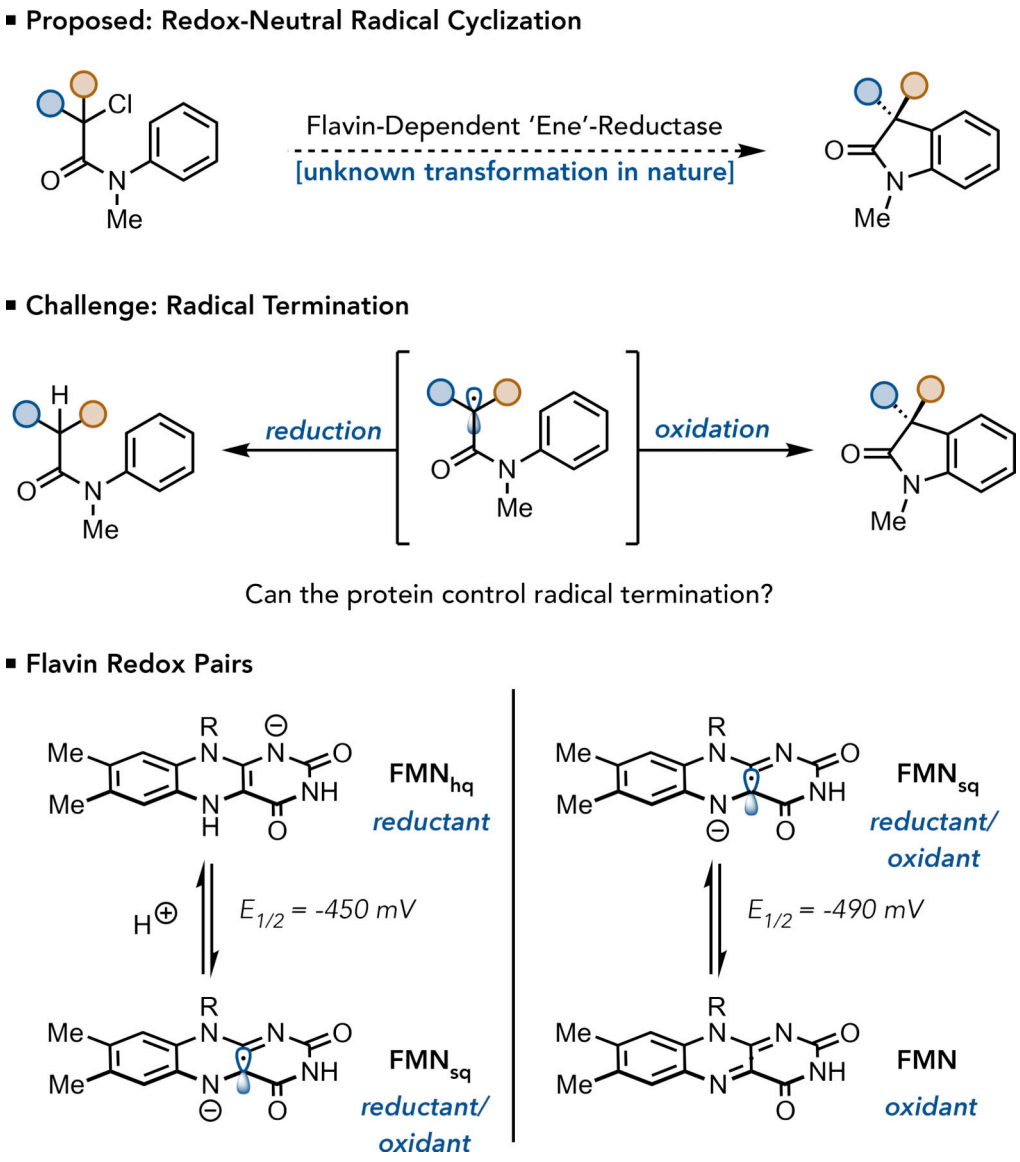


Fig. 1. Strategies and Challenges in Using 'Ene'-Reductases for Redox-Neutral Radical Cyclizations

(a) The desired redox-neutral radical cyclization to prepare oxindoles from α-haloamides, a transformation currently unknown in nature (b) The central challenge to this reactivity is identifying a flavin redox couple that will favor cyclization over reduction. (c) The viable flavin redox pairs for redox-neutral cyclizations. If FMN_{hq} is responsible for reducing the starting material, FMN_{sq} will need to serve as an oxidant for the desired transformation. FMN_{sq} can also function as a reductant, providing access to an undesired reductive mechanism. However, if FMN_{sq} is used to reduce the starting material, FMN is formed in the active site, which can only function as an oxidant.

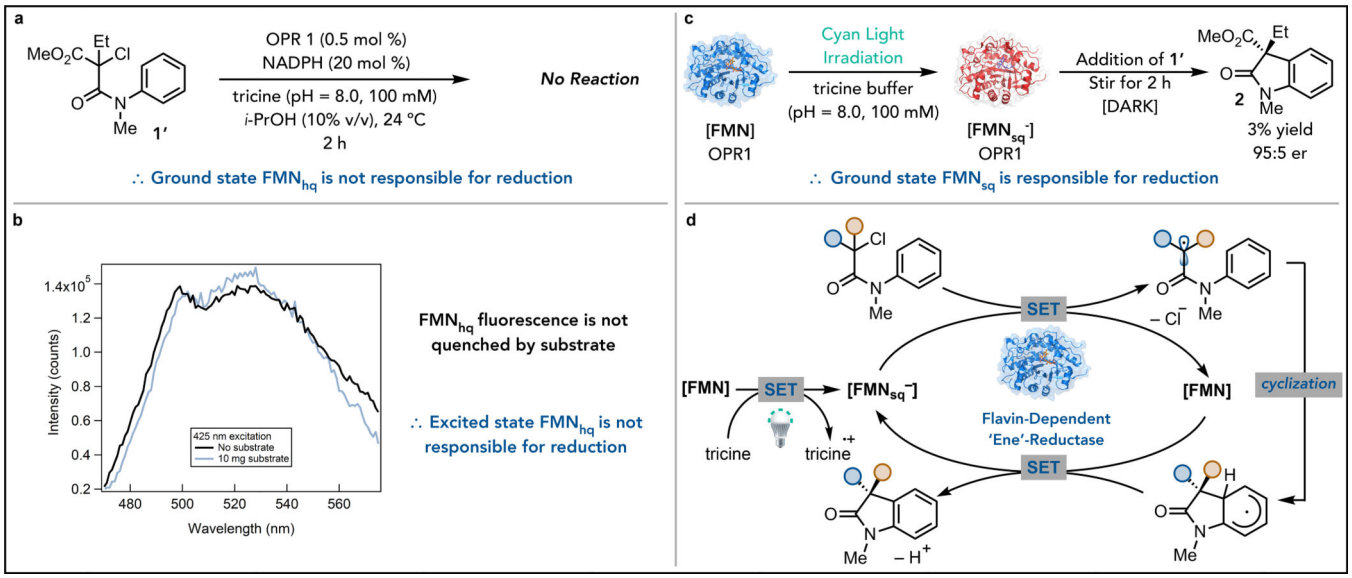
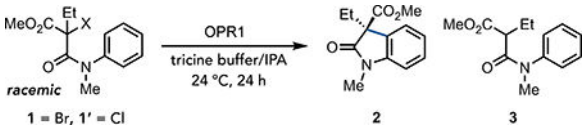


Fig. 2. Studies to determine the mechanism of oxindole formation.

(A) This experiments demonstrates that ground state FMN_{hq} is not able to initiate the radical cyclization. **(B)** The fluorescence spectra indicates that the excited state can be accessed, however, this state is not quenched by the substrate, indicating that FMN_{hq}* is not responsible for initiating the reaction. **(C)** In this experiment, oxidized OPR1 is photoreduced with cyan light and tricine buffer to partially reduce FMN to FMN_{sq}^{-} (as determined by EPR). Then substrate **1'** is introduced to the enzyme in the absence of light. Oxindole is formed under these conditions indicating that gound state FMN_{sq}^{-} is responsible for initiating the reaction. **(D)** This represents a proposed mechanism where light and tricine buffer are responsible for reducing FMN to FMN_{sq}^{-}, which can reduce the substrate to generate an α-acyl radical and FMN. Cyclization of the radical generates a reducing vinylogous amido radical which can be oxidized by FMN to form product and regenerate FMN_{sq}^{-}.

Table 1.

Reaction Optimization



entry	X	LED Irradiation	yield 2 (%)[a]	e.r.[b]	yield 3 (%)[a]
1[c]	Br	none	18	89:11	37
2	Br	Cyan LED	78	80:20	19
3	Cl	Cyan LED	94	95:5	4
4	Cl	none	-	-	-
5[d]	Cl	Cyan LED	-	-	-

Standard Reaction Conditions. Substrate (10 mM), OPR1 (50 μM), tricine buffer (100 mM, pH = 8.0), 10 (v/v)% IPA (iPrOH), Cyan LEDs, 24 h.

[a] Yield determined via reverse phase HPLC relative to an internal standard.

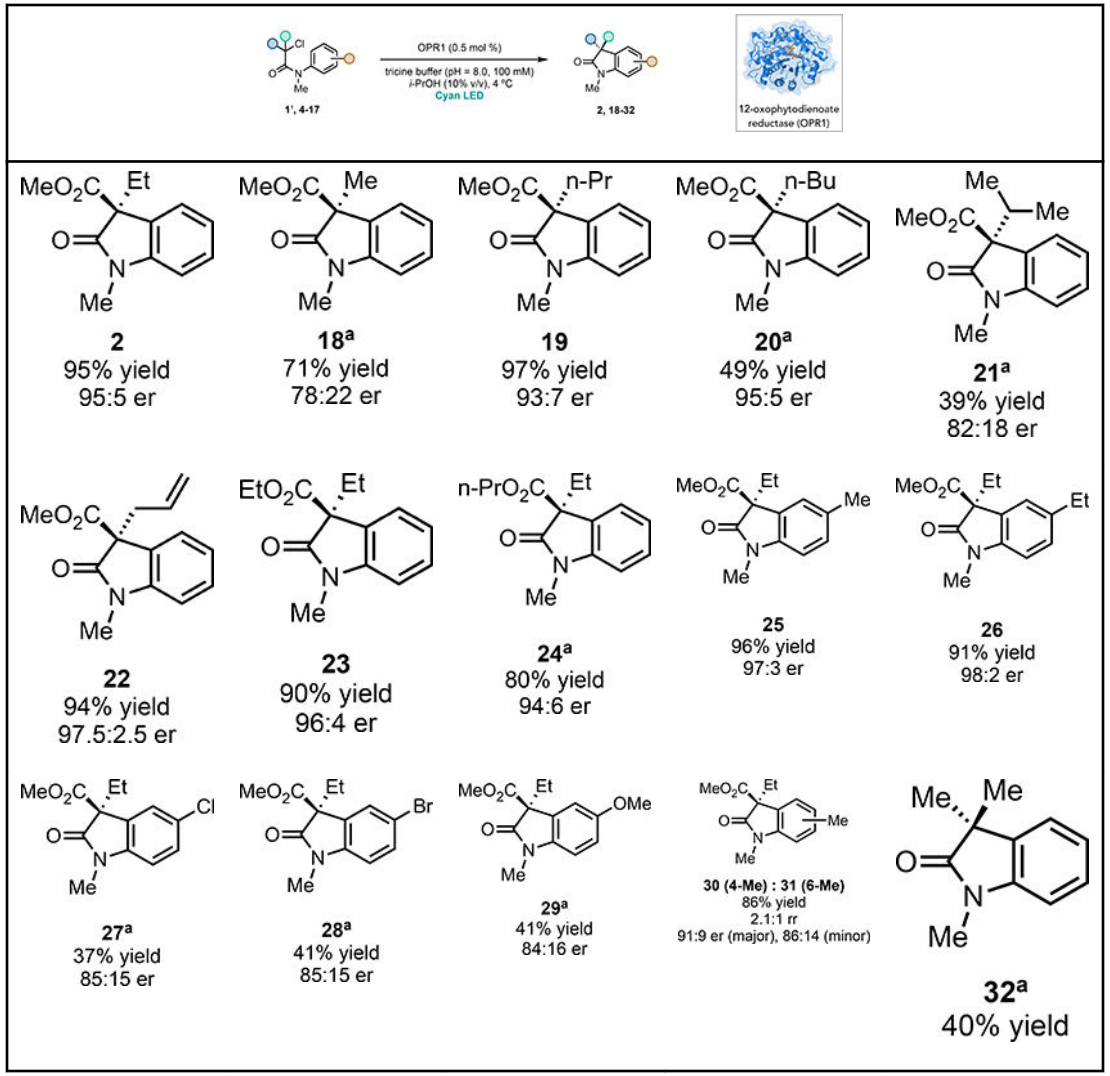
[b] Enantiomeric ratios determined via HPLC on a chiral stationary phase.

[c] $NADP^+$ (1 mol %), Glucose (1 equiv) and GDH-105 (glucose dehydrogenase) added.

[d] No enzyme added.

Table 2.

Biocatalytic radical cyclization to form oxindole scope.



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Standard Reaction Conditions. Substrate (10 mM), OPR1 (50 μM), tricine buffer (100 mM, pH = 8.0), 10 (v/v)% IPA, Cyan LEDs, 24 h. Yield determined via reverse phase HPLC relative to an internal standard. Enantiomeric ratios determined via HPLC on a chiral stationary phase.

[a]Reaction run at 24 °C.