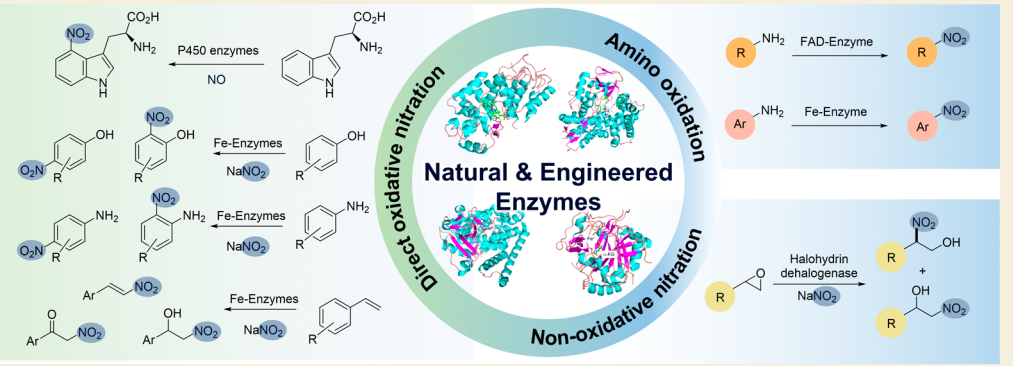
JACS Au
AN OPEN ACCESS JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

pubs.acs.org/jacsau
Perspective

Biocatalytic Strategies for Nitration Reactions

Xiling Wang, Matteo Aleotti, Mélanie Hall,* and Zhiqi Cong*

Cite This: JACS Au 2025, 5, 28−41
Read Online
ACCESS | Metrics & More | Article Recommendations



ABSTRACT: Nitro compounds are key synthetic intermediates used as enabling tools in synthesis and found in a large range of essential compounds, including pharmaceuticals, pesticides, and various organic dyes. Despite recent methodological developments, the industrial preparation of nitro compounds still suffers from harsh reaction conditions, along with poor selectivity and a problematic environmental footprint. Although biological enzymatic methods exist, mild approaches for bionitration are still underexplored. Enzymes, with their exquisite selectivity and compatibility with mild reaction conditions, have the potential to revolutionize the way nitro compounds are prepared. In this perspective, we systematically analyze currently available biological/enzymatic methods, including the oxidation of an amine precursor or methods consisting of direct oxidative nitration and non-oxidative nitration. By examining both the scope and mechanism of these reactions, we aim to present an update on the state-of-the-art while highlighting current challenges in this emerging field. The goal of this perspective is to inspire innovation in enzymatic nitration for sustainable organic synthesis, providing chemists with a valuable guide.

KEYWORDS: *Nitration, Biocatalysis, Nitro compounds, N-Oxygenase, Peroxidase, Monooxygenase*

1. INTRODUCTION

Nitro compounds are not only important synthetic intermediates that can easily be transformed into amines and ketones[1,2] but are also widely used in pharmaceuticals, pesticides, explosives, and various organic dyes.[3,4] For example, nitroimidazoles are used as antibiotic and antiprotozoal agents, such as metronidazole, which is on the WHO model's list of essential medicines and shows, among others, antitubercular properties.[5] Dinitrophenol is a highly potent chemical component historically found in all categories of pesticides (ovicides, insecticides, herbicides, fungicides)[6] and is now used for the manufacturing of dyes. Trinitrotoluene (TNT) is a major constituent of many composite explosives, while nitroglycerin, made famous by Alfred Nobel, is the major component of dynamite.[7] Besides the profusion of synthetic nitro compounds, an estimated 200 nitro-containing natural products only have been isolated from plants, fungi, bacteria, and mammals.[8] Chloramphenicol, for instance, is an antibiotic isolated from *Streptomyces venezuelae* and widely used in molecular biology, while several bacterial strains can produce pyrrolomycin antibiotics, such as pyrrolomycin B (Figure 1).

One of the most established methods used industrially for the preparation of nitro compounds, especially nitro aromatics, relies on the "mixed acid" method, in which sulfuric acid is used to protonate nitric acid, thereby releasing the active electrophilic nitronium species NO_2^+. Large amounts of waste acid and wastewater are generated during the reaction process.[9] The need for harsh conditions leads to poor regioselectivity and overnitration, limiting the presence of other sensitive functional groups. Nitration reactions have undergone a rather lengthy and challenging process of improvement in industry, for example, by using nitrate and

Received: October 21, 2024
Revised: November 22, 2024
Accepted: November 26, 2024
Published: December 16, 2024

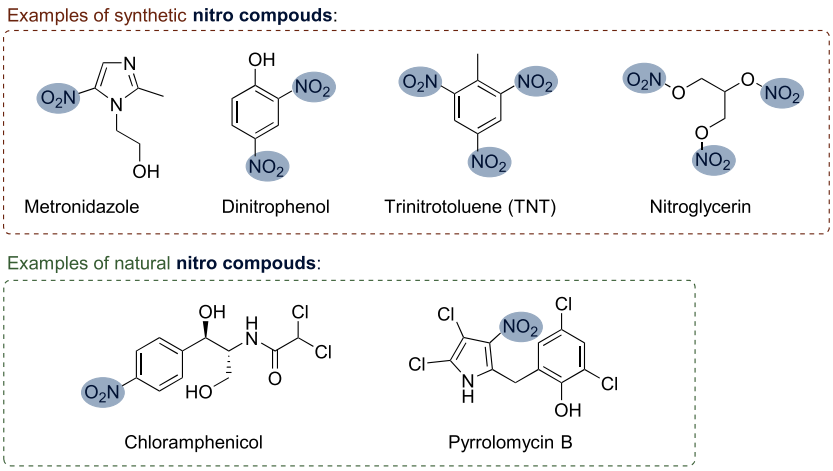
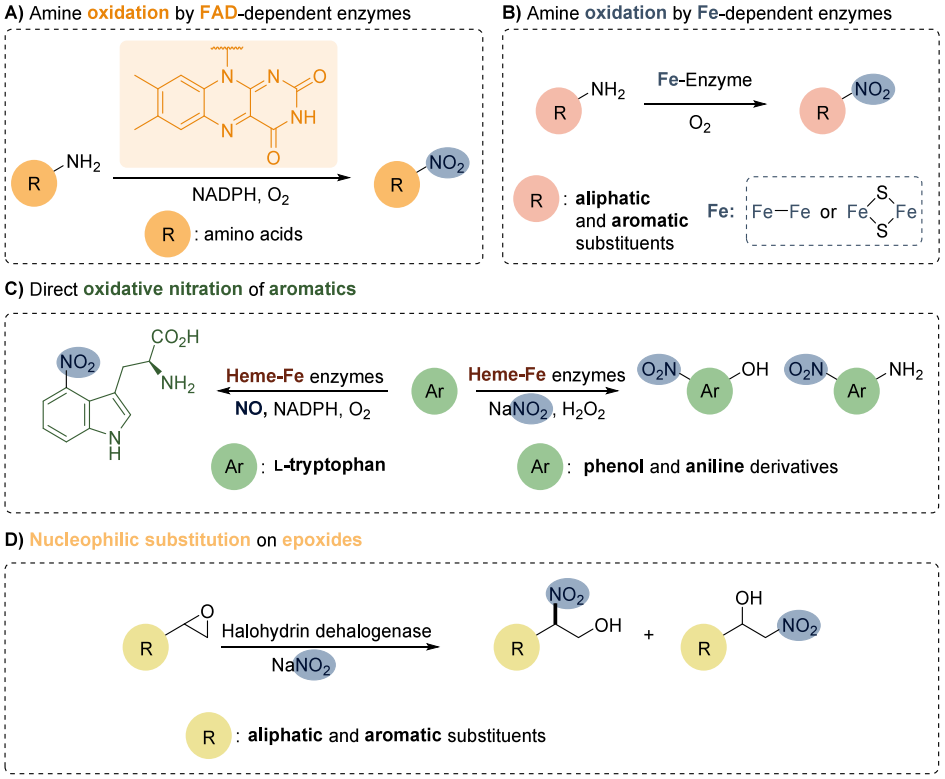


Figure 1. Structures of synthetic and natural nitro compounds.

Scheme 1. Overview of Biocatalytic Nitration Strategies Relying on (A) FAD-Dependent Enzymes for Oxidation of Aliphatic Amines; (B) Heme and Nonheme Diiron-Dependent Enzymes for Oxidation of Aliphatic and Arylamines; (C) Heme-Iron-Dependent Enzymes with NO/O_2 or NO_2^-/H_2O_2 as Oxidative Nitrating Agents of Aromatics; and (D) Cofactor-Independent Halohydrin Dehalogenases via Nucleophilic Substitution on Epoxides with NO_2^-



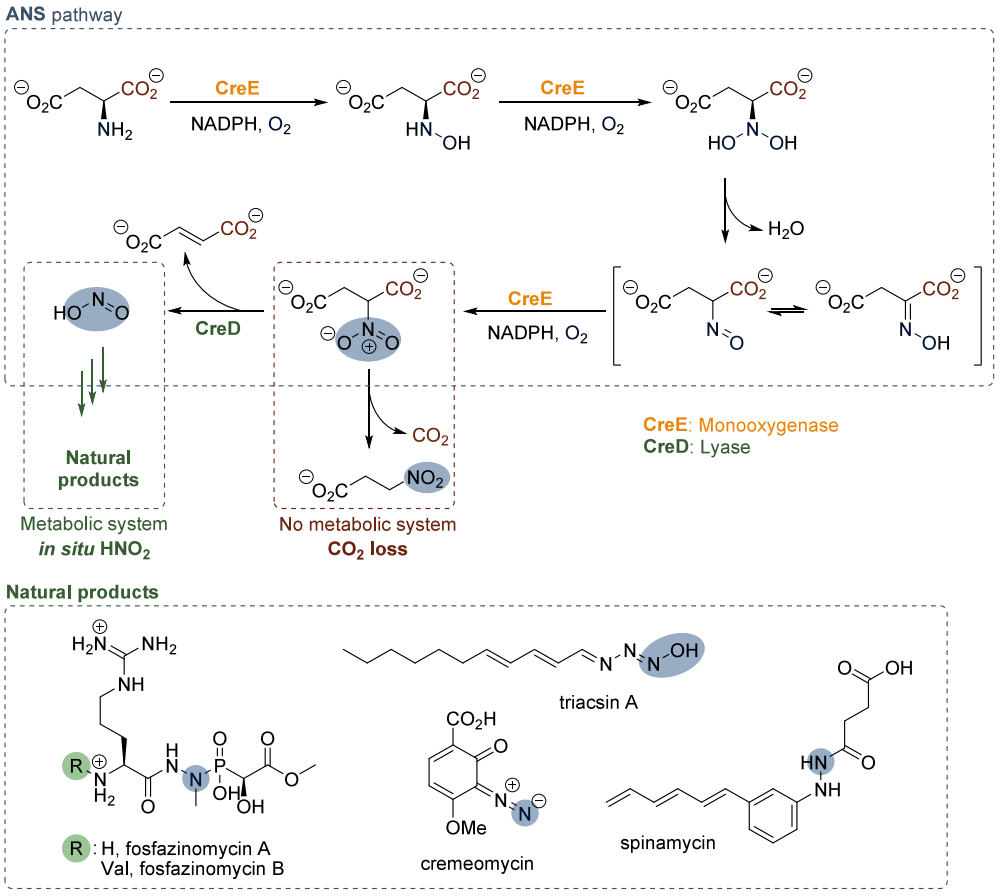
other diazo salts instead of acid or using lanthanide(III) or group IV metal triflates as catalysts.[10] Although these methods show a reduced environmental footprint to some extent, they are still plagued by inadequate cost, controllability, and conversion efficiency.[11]

With the rapid development of free radical chemistry, free radical reactions involving nitro groups have also been studied. Salts such as $AgNO_2$, $NaNO_2$, and $Fe(NO_3)_3{\cdot}9H_2O$ or tBuONO have been used as sources of nitro radicals to achieve the nitration of aromatic compounds under mild conditions.[12−15] In recent years, new strategies have been developed to enable compatibility with sensitive functional groups. For example, the *ipso*-nitration of boronic acids and carboxylic acids has emerged as an attractive method for the direct nitration of aromatic compounds.[16] In addition, aromatic C−H bond activation/nitration catalyzed by transition metals such as palladium, rhodium, ruthenium, or copper has undergone rapid development due to its favorable atom-economy and is mostly applicable to *ortho*-nitration.[17] A mild metal-free photocatalytic nitration method applicable to protected anilines was recently reported,[18] while electrochemical nitration methods are attracting renewed interest.[19,20]

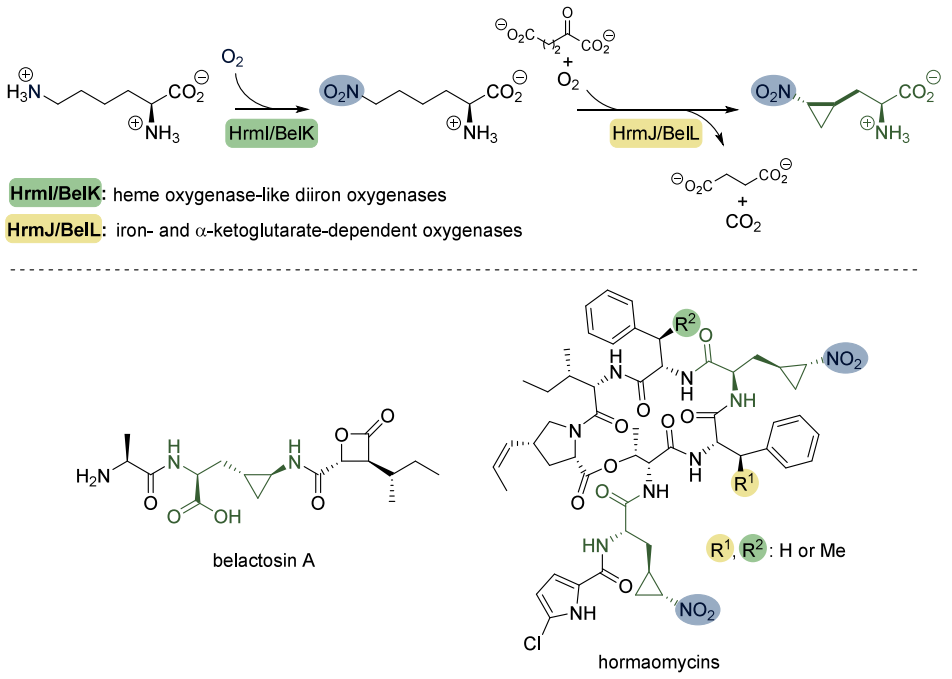
The field of biocatalytic nitration is, in contrast, underexplored, despite the attractive features of enzymes connected to

https://doi.org/10.1021/jacsau.4c00994
JACS Au 2025, 5, 28−41

Scheme 2. Aspartate-Nitrosuccinate (ANS) Pathway Exemplified with the Pair CreE/CreD from *Streptomyces cremeus* and Range of Natural Products Involving the *In Situ* Release of HNO_2 by the ANS Pathway[23]



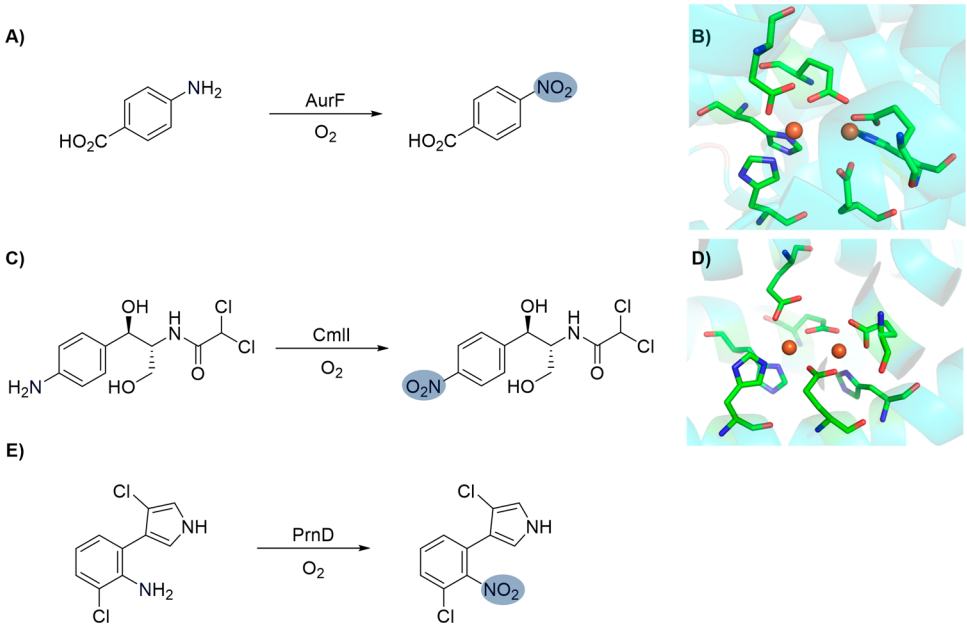
Scheme 3. Oxidation of L-Lysine to 6-Nitronorleucine and Subsequent Formation of 3-(*trans*-2-Nitrocyclopropyl)-alanine, a Precursor to Belactosin A and Hormaomycins[32−35]



their selectivity and the mild reaction conditions. Enzymatic nitration has predominantly been applied to aromatics, with most reported approaches relying on oxidative strategies. The diversity of these methods is reflected by the involvement of a

https://doi.org/10.1021/jacsau.4c00994
JACS Au 2025, 5, 28−41

Scheme 4. (A) AurF-Catalyzed Conversion of Ar-NH_2 to Ar-NO_2;[44] (B) Diiron Form of AurF (PDB 3CHT);[44] (C) Conversion of the Amino Precursor of Chloramphenicol to Chloramphenicol Catalyzed by CmlI;[43,46] (D) Diiron Form of CmlI (PDB 5HYH);[48] and (E) Oxidative Nitration of Aminopyrrolnitrin to Pyrrolnitrin Catalyzed by PrnD[38]



variety of cofactor (usually iron)-dependent enzymes, the use of O_2 or H_2O_2 as an oxidant, and the use of NO_2^- or NO as a nitro source. This perspective provides an overview of currently available nitration methods, classified according to their chemistry: (i) six-electron oxidation of an amine precursor, (ii) direct oxidative nitration, and (iii) non-oxidative methods by nucleophilic substitution (Scheme 1). By focusing on the scope and mechanism of these reactions, we aim to provide an update on the state-of-the-art and highlight the current challenges in this emerging field.

2. ENZYMATIC NITRATION VIA OXIDATION OF AN AMINE PRECURSOR

2.1. Oxidation of Aspartate by Flavin-Dependent Monooxygenase

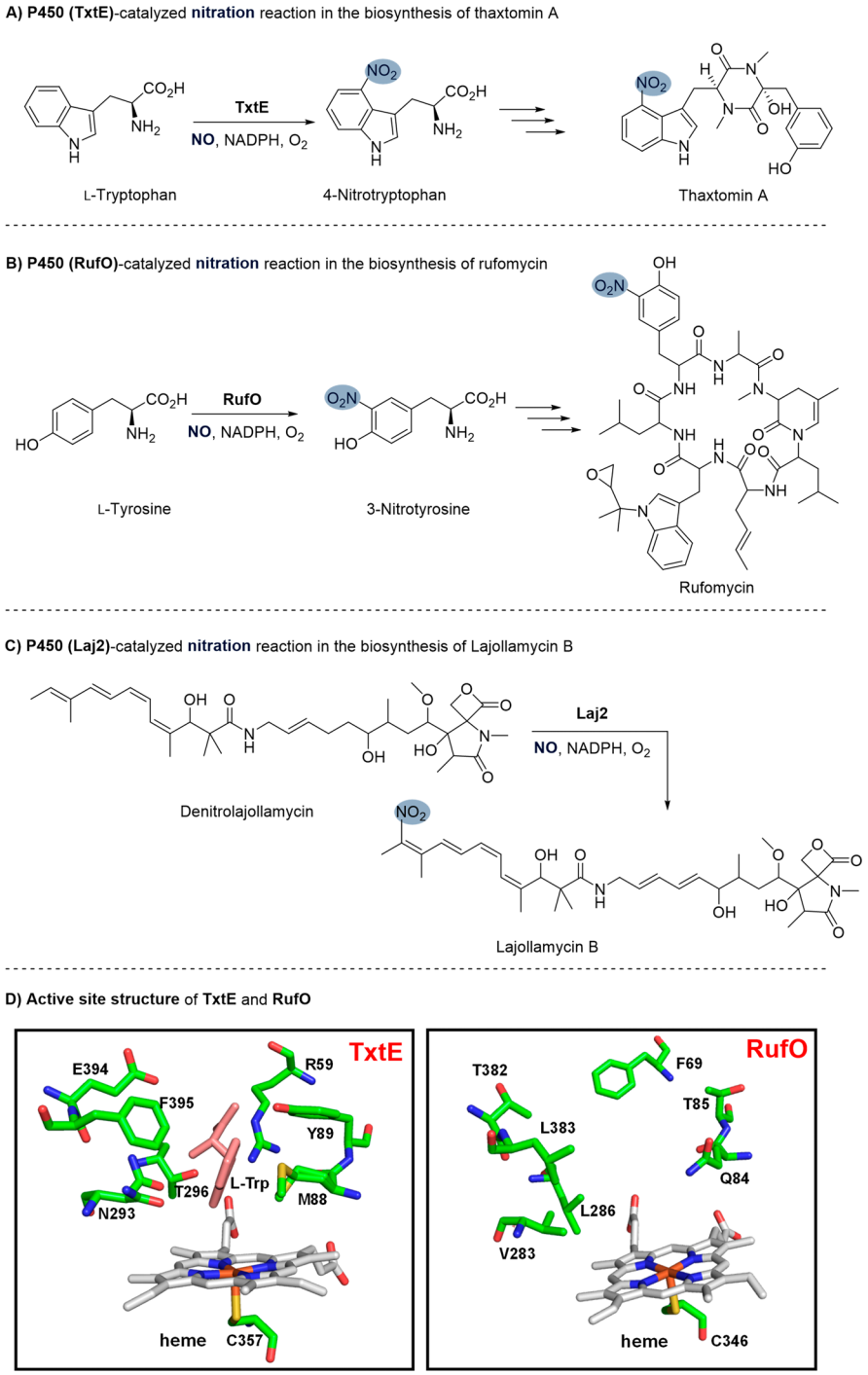
The oxidation of L-aspartate to nitrosuccinate via three successive two-electron oxidative steps was first discovered as part of the biosynthetic pathway of cremeomycin, a diazo compound with cytotoxicity and antibiotic properties produced by the bacteria *Streptomyces cremeus*.[21] The reaction is catalyzed by the FAD-dependent monooxygenase CreE at the expense of NADPH. Nitrosuccinate then undergoes dehydronitration by the action of the nitrosuccinate lyase CreD to liberate nitrous acid,[22] which is required for the formation of the diazo functionality of cremeomycin. This pathway, called the aspartate-nitrosuccinate (ANS) pathway,[23] liberates nitrous acid initiated by the oxidation of aspartate to generally promote the formation of N−N bonds in a variety of biosyntheses (Scheme 2). The majority of the ANS pathways are involved in the formation of diazo compounds, and homologues of CreE include AzpE, which participates in the formation of the diazo compound alazopeptin,[24] and SpiE and AvaE, which are involved in the synthesis of a diazo precursor of spinamicyn[25] and avenalumic acid,[26] respectively. In the case of FzmM, involved in the biosynthesis of fosfazinomycin A,[27] nitrous acid has been proposed to participate in the formation of hydrazinosuccinate via a hypothetical aliphatic diazo precursor; however, the exact pathway remains unclear. As an exception, the enzymatic formation of HNO_2 is a crucial step for the formation of the *N*-hydroxytriazene moiety in triacsin A in *Streptomyces aureofaciens*.[28]

The mechanism involved in the oxidation of aspartate to 2-nitrosuccinate has been investigated with the monooxygenase from *Streptomyces* sp. V2 and revealed the well-known oxygen activation mechanism by FAD-dependent class B monooxygenases at the expense of NADPH to form the C4a-hydroperoxyflavin,[29] which is the electrophilic species attacked by the amino group of aspartate.[30] This initiates the first oxidation step to the hydroxylamine intermediate, followed by a second oxidation to the dihydroxylamine, which dehydrates to the nitroso/oxime tautomers. Finally, the last oxidation step yields nitrosuccinate. The enzyme can prevent leakage of oxidation intermediates owing to the presence of an "entry chamber",[30] thereby ensuring full oxidation to nitrosuccinate. Since this compound is unstable, it undergoes decarboxylation to 3-nitropropanoate.[31] However, in nature, this process is usually coupled to a nitrosuccinate lyase so that dehydronitration prevails and the β-elimination of nitrous acid results in the formation of fumarate.[22]

The enzymatic oxidation of the ε-amino group of L-lysine to form 6-nitronorleucine was shown to precede the formation of 3-(*trans*-2-nitrocyclopropyl)-alanine, a compound found in the biosynthetic pathway of hormaomycin and belactosin A. The first step of amine oxidation is catalyzed by HrmI and BelK, respectively, which are heme-oxygenase-like dinuclear iron-dependent enzymes identified as members of the family of heme-oxygenase-like diiron oxygenases (HDO).[32,33] The consecutive cyclopropanation of 6-nitronorleucine is catalyzed by HrmJ and BelL, respectively, two nonheme iron- and α-ketoglutarate-dependent oxygenases (Scheme 3).[34,35]

https://doi.org/10.1021/jacsau.4c00994
JACS Au 2025, 5, 28−41

Scheme 5. (A) L-Tryptophan Nitration Catalyzed by TxtE;[58] (B) L-Tyrosine Nitration Catalyzed by RufO;[59,60] (C) Olefin Nitration Catalyzed by Laj2;[61] and (D) Active Sites in the Crystal Structures of L-Tryptophan-Bound TxtE (PDB 4TPO)[64] and Substrate-Free RufO (PDB 8SPC)[72,73]



2.2. Arylamine Oxidation by Diiron-Dependent *N*-Oxygenases

N-oxygenases that catalyze nitration by oxidation of an aromatic amine precursor are rare in nature and include *p*-aminobenzoate *N*-oxygenase (AurF) from *Streptomyces thioluteus*,[36] α-*N*-dichloroacetyl-*p*-aminophenylserinol *N*-oxygenase (CmlI) from *Streptomyces venezuelae*,[37] aminopyrrolnitrin oxygenase (PrnD) from *Pseudomonas fluorescens* Pf5,[38] and RohS from *Kitasatospora azatica*.[39] AurF and CmlI are both nonheme diiron monooxygenases that share only 34% amino acid identity and catalyze the six-electron oxidation of arylamines,[40] while PrnD represents a unique case of a Rieske oxygenase that transforms arylamines into nitro compounds by a proposed sequence of two two-electron oxidation steps and one dehydrogenation step.

AurF catalyzes the sequential oxidation of aromatic amines to nitroarenes via the hydroxylamine intermediate. In *Streptomyces thioluteus*, AurF catalyzes the oxidation of *p*-aminobenzoate to *p*-nitrobenzoate in the biosynthesis of aureothin. Zhao and co-workers first reported the crystal

https://doi.org/10.1021/jacsau.4c00994
JACS Au 2025, 5, 28−41

structure of AurF with the product *p*-nitrobenzoate.[41] In this structure, the active site consists of two ferric ions bridged by an oxo group and Glu136. Fe1 is ligated to Glu101, His139, and His223, while Fe2 is coordinated to Glu196, Glu227, and His230. The reaction mechanism for the oxidation of the amino group by AurF has been heavily debated. While the oxidative conversion of the amino group into a nitro group was suggested in two different proposals to proceed through the hydroxylamine and nitroso intermediate in three consecutive two-electron processes involving each the consumption of one equivalent of molecular oxygen,[42,43] Bollinger et al. demonstrated that the oxidation of *p*-hydroxylaminobenzoate to *p*-nitrobenzoate is a four-electron oxidation that involves one dioxygen molecule only (Scheme 4A,B).[44] This mechanism was later investigated by density functional calculations, which supported the Bollinger mechanism and the involvement of a diferric peroxo species as an oxygenating intermediate.[45]

CmlI is a *N*-oxygenase involved in the biosynthesis of chloramphenicol.[37,42] It catalyzes the ultimate step of the pathway, which is a six-electron oxidation of the arylamine precursor D-threo-1-(4-aminophenyl)-2-dichloroacetylamino-1,3-propanediol (Scheme 4C,D). Upon exposure of the diferrous cluster to O_2, CmlI forms a long-lived peroxo intermediate, which reacts with the arylamine precursor to form chloramphenicol.[43,46]

The catalytic mechanism of CmlI remains controversial. For example, some studies suggest that the reactive species of CmlI is a peroxo species rather than a high-valent iron-oxo species proposed for most other nonheme diiron oxygenases.[47] The X-ray crystal structure of CmlI was solved after truncation of the 33 *N*-terminal residues.[48] The overall structure of CmlIΔ33 is similar to that reported for Fe-AurF, but the detailed structure of the active site diiron cluster has distinct differences, which may account for the respective substrate preference of the two enzymes.

Aminopyrrolnitrin oxygenase (PrnD) is involved in the last step of the biosynthesis of the broad-spectrum antifungal agent pyrrolnitrin and catalyzes the oxidation of aminopyrrolnitrin to pyrrolnitrin (Scheme 4E).[38] PrnD, which contains a [2Fe−2S] cluster and nonheme Fe(II), also showed activity on 4-aminobenzylamine. The catalytic mechanism includes two monooxygenation steps and one proposed dehydrogenation step as well as the formation of the hydroxylamine and nitroso compounds as intermediates.[49,50]

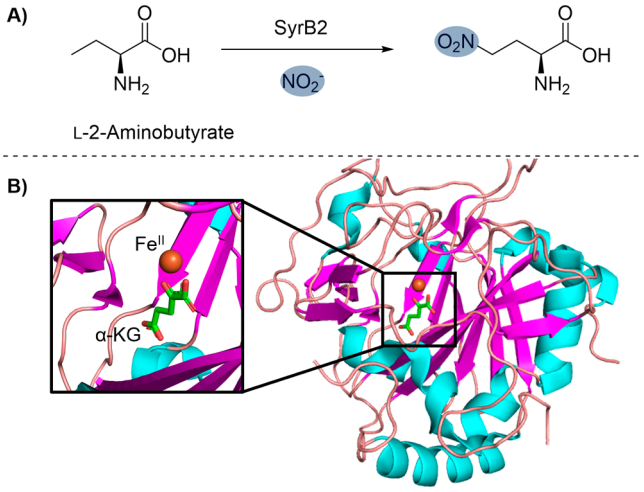
Finally, the iron-dependent *N*-oxygenase RohS was demonstrated to catalyze the oxidation of 2-aminoimidazole to the nitroimidazole azomycin *in vitro* in the presence of phenazine methosulfate,[39] NADH, and $FeSO_4$. It has been classified as a member of the emerging family of heme-oxygenase-like diiron oxygenases (HDO).[32,33]

3. DIRECT ENZYMATIC OXIDATIVE NITRATION

3.1. Nitration of Unsaturated Hydrocarbons by Cytochrome P450s with NO/O_2

Cytochrome P450 monooxygenases are well recognized as multifunctional biological oxidation catalysts distributed ubiquitously in nature,[51−56] but so far, only a few natural P450 enzymes have been reported to catalyze the direct nitration of aromatics and olefins using nitric oxide (NO) and O_2.[57] For instance, P450 TxtE involved in the biosynthesis of thaxtomin A in *Streptomyces scabies*, catalyzes the direct nitration of the indole group of L-tryptophan to 4-nitro-L-tryptophan in the presence of NO, O_2, and NADPH (Scheme 5A).[58] Similarly, P450 RufO, found in the biosynthesis of rufomycins/ilamycins in *Streptomyces atratus*, catalyzes the regioselective 3-nitration of tyrosine with NO, O_2, and NADH (Scheme 5B).[59,60] Very recently, an olefin nitrating P450 enzyme (Laj2) has been mined from the biosynthetic gene cluster of lajollamycin B's isomers from strain *Streptomyces qinglanensis* CGMCC 4.6825, which also uses NO, O_2, and NADPH to drive the nitration reaction (Scheme 5C).[61]

Scheme 6. (A) Nitration of L-2-Aminobutyrate Catalyzed by Halogenase SyrB2 and (B) Crystal Structure of the Iron- and α-Ketoglutarate (α-KG)-Dependent Halogenase SyrB2 (PDB 2FCT)[77]

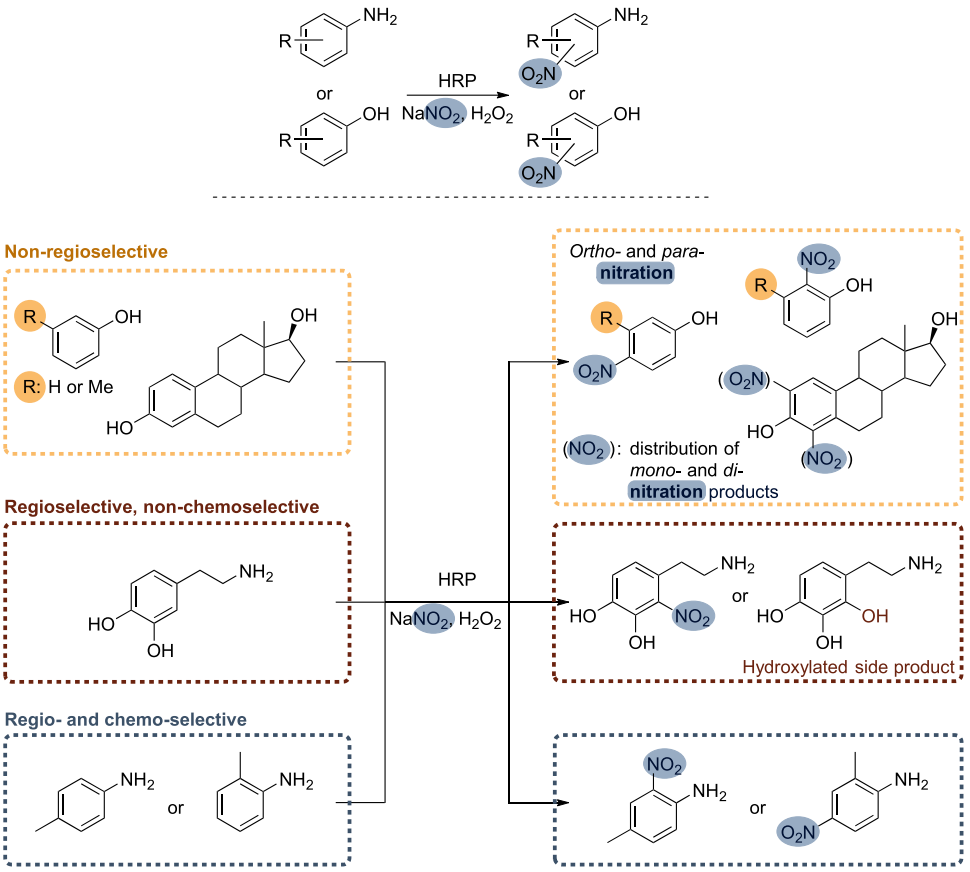


Given the application potential of the nitrating P450 enzyme for designing novel routes to aromatic nitro compounds, the Arnold group pioneered the investigations on the structure−function relationship and protein engineering of P450 TxtE.[62,63] They first determined the high-resolution crystal structures of the substrate-free and L-tryptophan-bound forms of TxtE, which show almost the same canonical fold as that of the already reported inhibitor imidazole-bound form (Scheme 5D).[64] The analysis of crystal structures and molecular docking revealed that the substrate L-tryptophan was recognized and bound by TxtE through polar contacts with as many as five amino acid residues, including Arg59, Tyr89, Asn293, Thr296, and Glu394. Subsequent spectroscopic and functional characterization indicated the narrow substrate scope of TxtE, which includes compounds structurally similar to L-tryptophan. With the structural information in hand, Arnold and co-workers performed molecular dynamic simulation-guided protein engineering of TxtE, shifting the regioselectivity from 4-nitro-L-tryptophan to 5-nitro-L-tryptophan.[63]

Additionally, the Ding group was able to improve the catalytic efficiency of TxtE and broaden its substrate scope.[65−68] An artificial self-sufficient nitrating P450 enzyme was constructed by fusing TxtE with the reductase domain of P450BM3 (BM3R), largely improving the nitration performance of fluorinated tryptophan analogues over the wild-type TxtE.[65] By tuning the linker length between TxtE and BM3R, the coupling efficiency and total turnover number were further improved.[66] In addition, the substrate scope was also expanded to 4-Me-L-Trp to yield 4-Me-5-NO_2-L-Trp and 4-Me-7-NO_2-L-Trp. A whole-cell nitration system was then constructed in *Escherichia coli* based on the best chimeric TxtE-BM3R variant (P450 TB14), resulting in 4-nitro-L-tryptophan titers of 192

https://doi.org/10.1021/jacsau.4c00994
JACS Au 2025, 5, 28−41

Scheme 7. Horseradish Peroxidase-Catalyzed Nitration Reaction[89−92]



mg/L within 20 h.[67] Interestingly, an *E. coli*-based cell-free protein synthesis system was later reported to produce around 360 μM 4-NO_2-L-Trp within 16 h, through the combination of TxtE with the nitric oxide synthase TxtD for the *in situ* production of NO from L-arginine.[69] Recently, Challis and co-workers applied the TxtE-reductase fusion approach to an R59C mutant of TxtE, successfully expanding the substrate scope to a range of tryptamine analogues, a substrate not accepted by native TxtE.[70]

To improve the electron transfer efficiency, Qian et al.[71] constructed the electrochemistry-driven ferredoxin-TxtE system with methyl viologen as the electron mediator, which directly avoids the use of the expensive nicotinamide cofactor NADPH. Interestingly, it was found that the electric field not only improves catalytic performance and product yield but also regulates the regioselectivity of the nitration reactions. The product distributions of 4-nitro-L-Trp and 5-nitro-L-Trp varied depending on the external electrode constant potential. In addition, the polarity of the electron mediators directly influenced the product distribution.

In comparison with TxtE, the protein engineering of RufO and the olefin nitrating P450 enzyme has not yet been reported. However, Davis and Wang independently reported the substrate-free crystal structure of RufO in high resolutions of 1.87 and 1.89 Å, respectively (Scheme 5D).[72,73] According to spectroscopic characterization and biochemical and theoretical simulation analysis, both studies suggested that a peptide tethered with tyrosine was the actual substrate of RufO, not free tyrosine. In the case of TxtE, it is generally accepted that the binding of tryptophan is the first key step in initiating the catalytic cycle. Although a ferric-peroxynitrite active species has been proposed to be responsible for the nitration of tryptophan, the exact mechanism of TxtE still remains unclear.[74−76] The discovery of further nitrating P450 enzymes nevertheless provides a promising platform for aromatic nitration.

3.2. Aliphatic Nitration by Nonheme Iron- and α-Ketoglutarate-Dependent Halogenases

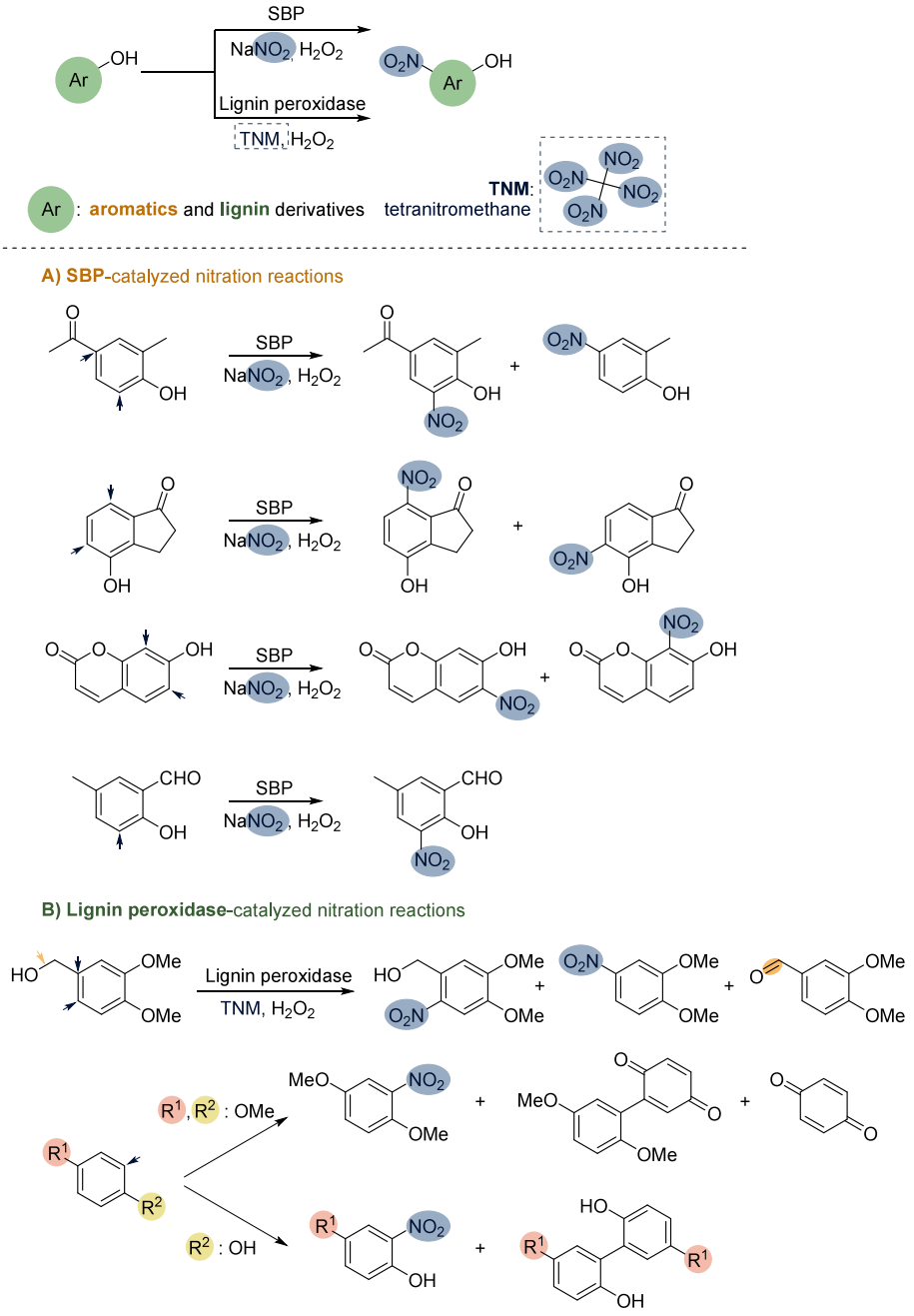
While the oxyfunctionalization of C(sp^3)−H bonds is already a demanding task for synthetic chemists, the direct nitration of unactivated aliphatic carbon atoms, whether by chemical or biological catalysis, poses an even greater challenge due to its extreme difficulty. Inspired by the nonheme iron- and α-ketoglutarate-dependent halogenase-catalyzed mechanism of chlorination/bromination, Matthews and co-workers reported the direct nitration of the terminal sp^3 carbon atom of L-2-aminobutyrate by halogenase SyrB2 in the presence of nitrite (Scheme 6).[77] Although this approach is limited by the requirement for a carrier protein, it sheds light on new strategies for the enzymatic nitration of aliphatic hydrocarbons. Recently, the freestanding nonheme iron aliphatic halogenase WelO5* was reported to incorporate nitrite into the macrolide soraphen A.[78]

3.3. Aromatic Nitration by Heme Peroxidases with NO_2^-/H_2O_2

Peroxidases are heme-iron-dependent oxidoreductases[79] that are widely present in animals, plants, fungi, and bacteria and include, for instance, myeloperoxidase in animals[80] or the well-known horseradish peroxidase (HRP) in plants.[81] Peroxidase activity is also found in microperoxidases, which are heme peptides obtained from the digestion of cytochrome c.[82−85]

https://doi.org/10.1021/jacsau.4c00994
JACS Au 2025, 5, 28−41

Scheme 8. Soybean Peroxidase (SBP)- and Lignin Peroxidase-Catalyzed Oxidative Nitration of Aromatics[93,94]



Peroxidases generally oxidize a variety of exogenous substances in the presence of hydrogen peroxide. Eosinophil peroxidase and myeloperoxidase were first proposed to be involved in the nitration of protein tyrosyl residues during post-translational modifications.[86] The potential of peroxidases for the nitration of aromatic compounds using nitrite and hydrogen peroxide as the nitrating pair was then uncovered.

The heme-containing octapeptide (amino acid residues 14−21) microperoxidase 8 (MP8) is the most widely studied micropeptidase[87] and catalyzes the nitration of phenolic compounds to produce *o*-nitrophenol and *p*-nitrophenol.[88]

Horseradish peroxidase (HRP) has been used widely for enzymatic nitration (Scheme 7). Dai and co-workers reported the HRP-catalyzed nitration of phenol and *m*-cresol in the presence of hydrogen peroxide and sodium nitrite.[89] In both cases, a mixture of regioisomers was obtained by attack on the *ortho* and *para* positions relative to the hydroxy group. In addition to simple phenol derivatives, more complex substrates have also been investigated. For example, Pezzella and co-workers[90] achieved the enzymatic nitration of 17β-estradiol by HRP and obtained 2-nitroestradiol, 4-nitroestradiol, and 2,4-dinitroestradiol when excess nitrite was used. Palumbo and co-workers[91] studied the enzyme-catalyzed oxidation of dopamine in the presence of nitrite and observed the formation of 6-hydroxydopamine and 6-nitrodopamine using HRP. Increasing the concentration of NO_2^- led to increased nitration and decreased formation of 6-hydroxydopamine. Dai and co-workers also reported the formation of 2-nitroaniline and 4-nitroaniline through the nitration of aniline by HRP, which

https://doi.org/10.1021/jacsau.4c00994
JACS Au 2025, 5, 28−41

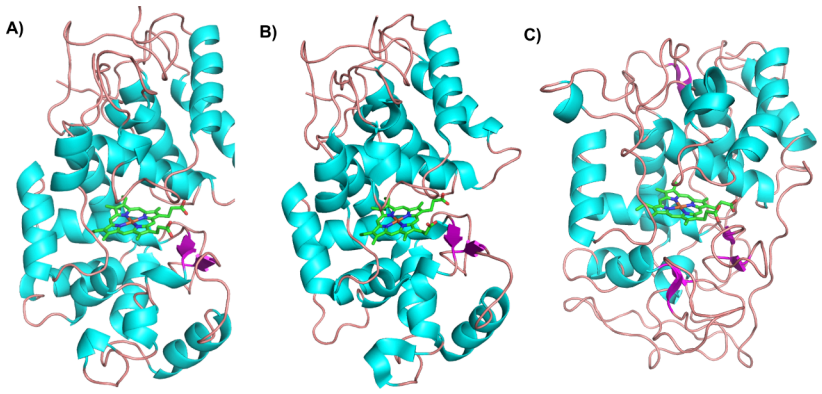


Figure 2. Crystal structures of (A) HRP (PDB 1ATJ),[95] (B) SBP (PDB 1FHF),[96] and (C) lignin peroxidase (PDB 1LGA).[97] Panel (A) is reproduced from ref 95. Copyright 1997 Nature. Panel (B) is reproduced with permission from ref 96. Copyright 2008 John Wiley & Sons, Inc. Panel (C) is reproduced from ref 97. Copyright 1993 Elsevier.

indicates that both hydroxy and amino groups allow sufficient activation of the ring for nitration.[92]

In addition to MP8 and HRP, several other peroxidases are also capable of nitration (Scheme 8). Budde et al. reported the direct nitration of 4-hydroxy-3-methylacetophenone catalyzed by soybean peroxidase (SBP) in the presence of hydrogen peroxide and nitrite to 4-hydroxy-3-methyl-5-nitroacetophenone.[93] An interesting case of *ipso*-substitution was also monitored, leading, in addition, to the formation of 2-methyl-4-nitrophenol. Other phenolic compounds were accepted, including 4-hydroxy-1-indanone, 7-hydroxycoumarin, and 2-hydroxy-5-methylbenzaldehyde. Finally, lignin peroxidase was reported to catalyze the nitration of veratryl alcohol, 1,4-dimethoxybenzene, and tyrosine. Here, in place of nitrite, tetranitromethane was used in combination with hydrogen peroxide.[94]

Notably, the regioselectivity of these peroxidases is usually poor, regardless of the substrates. This may be due to the fact that their active centers are mostly exposed or located close to the surface of the protein (Figure 2), leading to unspecific orientation of the substrates. In addition, the free radical cross-coupling mechanism used by peroxidases further increases the difficulty of controlling the regioselectivity. A main reaction pathway of peroxidases has been proposed, according to which peroxidases react with H_2O_2 to form an iron(IV)-oxo porphyrin π-cation radical (compound I).[98] Compound I could then be reduced to the iron(IV)-oxo porphyrin compound II by phenol, leading to the formation of phenoxy radicals, or by NO_2^-, leading to $NO_2^\bullet$ radicals. Compound II of peroxidases could also be reduced by nitrite or phenol to the resting iron(III) porphyrin, together with the formation of $NO_2^\bullet$ and phenoxy radicals. The coupling reaction of $NO_2^\bullet$ radicals with phenoxy radicals then gives the nitration products. Due to the resonance equilibrium of phenoxy radicals, the coupling reactions typically yield two or more mixed products. Therefore, one of the biggest challenges in using peroxidases in organic synthesis is controlling the regioselectivity of their reactions. Another key challenge is expanding the typically narrow substrate spectrum.

3.4. Nitration of Unsaturated Hydrocarbons by a DFSM-Facilitated P450 Peroxidase with NO_2^-/H_2O_2

In 2018, the Cong group developed a dual-functional small molecule (DFSM) strategy to enable the peroxygenase activity of P450BM3 monooxygenase (Scheme 9A). The DFSM molecule is composed of an acyl amino acid as an anchoring group, an imidazolyl as a catalytic group, and a carbon chain as a length-adjustable linker group. By binding with P450BM3, the imidazolyl group of DFSM plays a general acid−base catalyst role assisting in the activation of H_2O_2.[52,99,100] Subsequently, the same group found that the DFSM-facilitated P450 peroxygenase can be well switched to a peroxidase mode by a redox-sensitive residue engineering approach, resulting in efficient one-electron oxidations toward phenolic and arylamine substrates.[101,102] Given multiple pieces of evidence that heme peroxidases can catalyze aromatic nitration with NO_2^-/H_2O_2 (see section 3.3), the above results open up the possibility for nitration reactions by P450 enzymes.

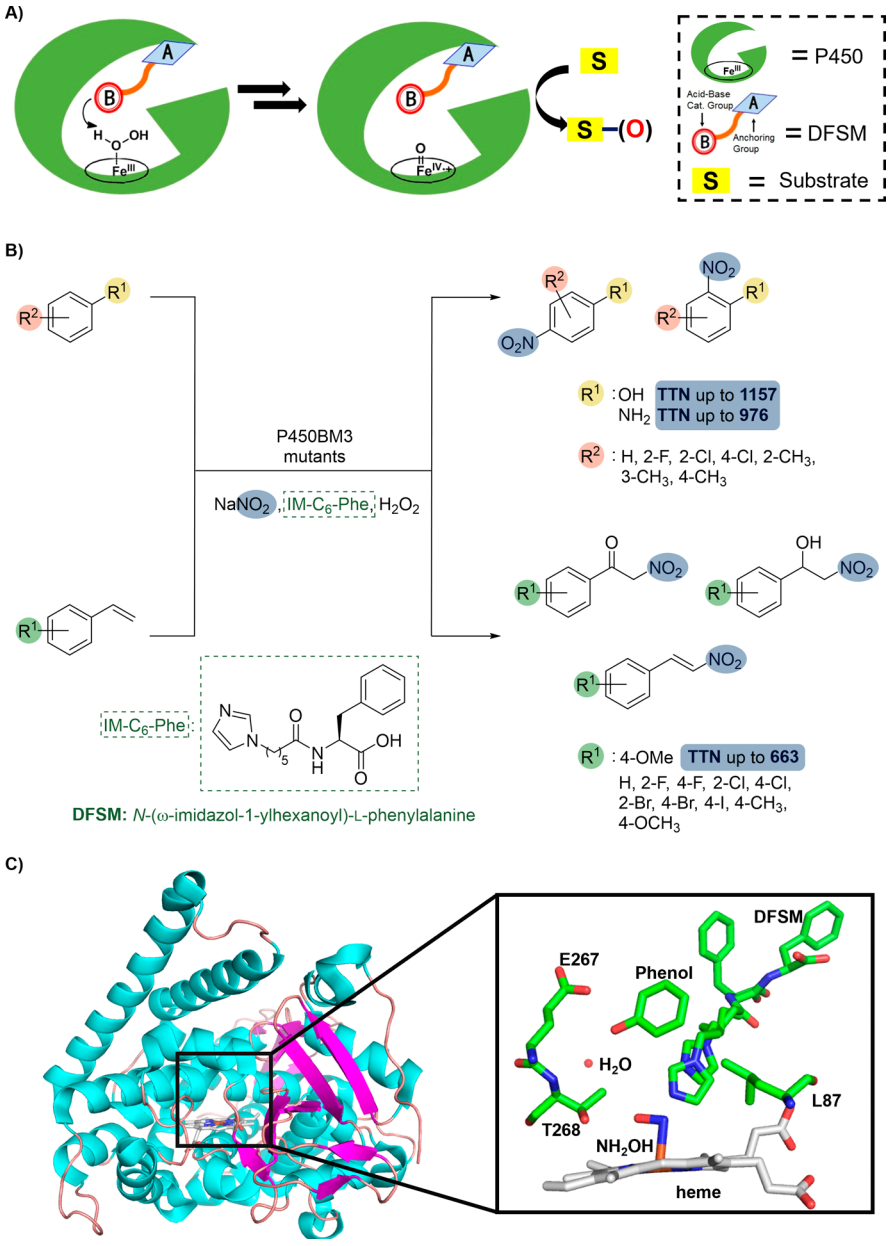
Cong and co-workers reported the first example of unsaturated hydrocarbon nitration by the DFSM-facilitated P450BM3 peroxidase with styrenes and nitrite in the presence of *N*-(*ω*-imidazol-1-ylhexanoyl)-L-phenylalanine (Im-C6-Phe, so-called DFSM) and H_2O_2 (Scheme 9B).[103] Like other heme peroxidases, the engineered P450 peroxidase system also widely accepted phenolic compounds and aromatic amines as substrates (up to 19 examples) to give *ortho*- and *para*-nitration products with moderate to high total turnover numbers. Despite a deeper substrate binding pocket compared to other peroxidases and the participation of DFSM in substrate recognition and fixation (Scheme 9C), the regioselectivity of the P450 could not be controlled, and the distribution of *ortho*- and *para*-nitration products was similar to that of HRP. Combined with the example of olefin nitration by the P450 monooxygenase Laj2 reported shortly after,[61] these studies indicate the large potential of P450 enzymes for developing practical nitrating biocatalysts.

4. NON-OXIDATIVE NITRATION BY HALOHYDRIN DEHALOGENASES VIA NUCLEOPHILIC SUBSTITUTION

An unusual non-oxidative direct enzymatic nitration was reported with halohydrin dehalogenases (HHDHs), which catalyzed the ring-opening of epoxides by nucleophilic substitution using nitrite.[104,105] Unexpectedly, nitrite could act as an ambident nucleophile that can attack through its nitrogen or oxygen. Attack via the O atom formed *β*-hydroxy alkylnitrites, which hydrolyzed to vicinal diols, while attack via the N atom produced chiral *β*-nitroalcohols (Scheme 10A). In 2022, Wang et al.[106] reported the discovery of an unusual halohydrin dehalogenase, HHDHamb, which exhibited high chemoselectivity through N-attack, *α*-regioselectivity, and (*S*)-

https://doi.org/10.1021/jacsau.4c00994
JACS Au 2025, 5, 28−41

Scheme 9. (A) Illustration of the DFSM-Facilitated P450 Peroxygenase System;[a] (B) Nitration of Anilines, Phenols, and Styrene Compounds Catalyzed by Engineered Cytochrome P450 with Assistance from DFSM; and (C) Crystal Structure of P450BM3 F87L Mutant in Complex with Phenol and DFSM (PDB 7WDG)[103]



[a]Panel (A) is reproduced with permission from ref 99. Copyright 2018 John Wiley & Sons, Inc.

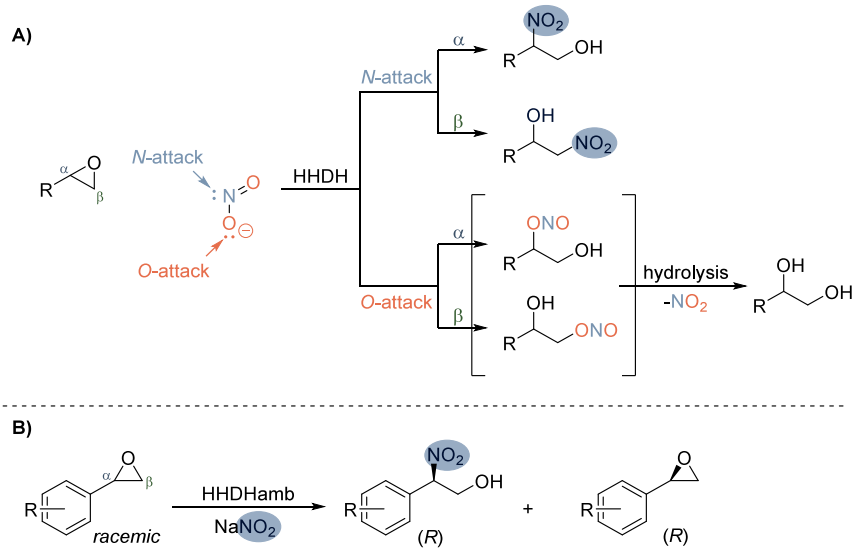
enantioselectivity in the bionitration of styrene oxides, affording various aryl-substituted chiral β-nitroalcohols in up to 41% isolated yield and >99% enantiomeric excess (ee) (Scheme 10B). The differences in chemoselectivity in nitrite-mediated epoxide ring-opening reactions were investigated by comparing the monomer structures of HheC (with a high O-attack selectivity), HheG (with a moderate N-attack selectivity), and HHDHamb (with a high N-attack selectivity). Due to a narrow active site, HHDHamb displayed higher enantioselectivity in the kinetic resolution of styrene oxides than HheG with a larger active site. Additionally, differences in the electrostatic potentials in the halide binding site regions of the three HHDHs allowed the nucleophile nitrite to adopt different binding conformations and contributed to the different chemoselectivity patterns.

5. SUMMARY AND OUTLOOK

Despite the scarcity of nitro compounds in nature, enzymatic approaches to access a variety of nitrated molecules exist, predominantly for nitro aromatics. These strategies offer attractive alternatives to current chemical methods, which are still plagued by poor selectivity and environmental footprint. The variety of enzymatic machinery combined with various nitro sources allows for the nitration of diverse functional groups, either by oxidation of amine precursors or by direct nitration reactions. Biocatalytic nitration reactions are, however, still limited to a few substrates, and the

https://doi.org/10.1021/jacsau.4c00994
JACS Au 2025, 5, 28−41

Scheme 10. Ring-Opening Reactions of Epoxides with Nitrite Catalyzed by Halohydrin Dehalogenases HHDHs (A) and HHDHamb (B)[a]



[a]Reproduced with permission from ref 106. Copyright 2022 John Wiley & Sons, Inc.

regioselectivity problem and low yields remain major hurdles to their establishment in synthetic applications. In response to these limitations, efforts in developing biological nitration methods are mainly directed toward improving the catalytic efficiency and regioselectivity of current nitrating enzymes and extending their substrate scope. Additionally, approaches that use gene mining to discover new nitrating enzymes or create isoenzymes with a nitration function will become key to advance the field. Further progress may lead to the development of sustainable and complementary routes to nitro compounds as precursors of pharmaceuticals, organic dyes, and other relevant molecules.

AUTHOR INFORMATION

Corresponding Authors

Zhiqi Cong − *Key Laboratory of Photoelectric Conversion and Utilization of Solar Energy, Qingdao New Energy Shandong Laboratory, CAS Key Laboratory of Biofuels, Shandong Provincial Key Laboratory of Synthetic Biology, Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences, Qingdao 266101, China*; orcid.org/0000-0002-0449-3821; Email: congzq@qibebt.ac.cn

Mélanie Hall − *Institute of Chemistry and BioHealth, University of Graz, Graz 8010, Austria*; Email: melanie.hall@uni-graz.at

Authors

Xiling Wang − *Key Laboratory of Photoelectric Conversion and Utilization of Solar Energy, Qingdao New Energy Shandong Laboratory, CAS Key Laboratory of Biofuels, Shandong Provincial Key Laboratory of Synthetic Biology, Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences, Qingdao 266101, China*

Matteo Aleotti − *Institute of Chemistry, University of Graz, Graz 8010, Austria*; orcid.org/0000-0002-6016-0175

Complete contact information is available at: https://pubs.acs.org/10.1021/jacsau.4c00994

Author Contributions

Z.C. and M.H.: conceptualization and supervision. Z.C., M.H., and X.W.: funding acquisition. X.W., M.A., M.H., and Z.C.: writing−review and editing. CRediT: **Xiling Wang** funding acquisition, writing - original draft, writing - review & editing; **Matteo Aleotti** writing - original draft, writing - review & editing; **Mélanie Hall** conceptualization, funding acquisition, supervision, writing - original draft, writing - review & editing; **Zhiqi Cong** conceptualization, funding acquisition, project administration, supervision, writing - review & editing.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

Acknowledgment is made to the National Key Research and Development Program of China (2023YFA0915500), Natural Science Foundation of Shandong Province (ZR2022QB053), National Natural Science Foundation of China (32371311 and 22307125), QIBEBT/SEI/QNESL (S202302), Taishan Scholars Program, and ACS GCI Pharmaceutical Roundtable Research Grant for partial support of this research and to the University of Graz for funding.

REFERENCES

(1) Formenti, D.; Ferretti, F.; Scharnagl, F. K.; Beller, M. Reduction of nitro compounds using 3d-nonnoble metal catalysts. *Chem. Rev.* **2019**, *119*, 2611−2680.

(2) Liu, J.; Zhu, X. R.; Ren, J.; Chen, W.; Zeng, B. Copper-catalyzed one-pot oxidation-aldol/henry reaction of benzylic-amines to *α,β*-unsaturated methyl ketone/nitro compounds. *Synlett* **2013**, *24*, 2740−2742.

(3) Calvo, R.; Zhang, K.; Passera, A.; Katayev, D. Facile access to nitroarenes and nitroheteroarenes using N-nitrosaccharin. *Nat. Commun.* **2019**, *10*, 3410.

(4) Zlotin, S. G.; Dalinger, I. L.; Makhova, N. N.; Tartakovsky, V. A. Nitro compounds as the core structures of promising energetic materials and versatile reagents for organic synthesis. *Russ. Chem. Rev.* **2020**, *89*, 1−54.

(5) Mukherjee, T.; Boshoff, H. Nitroimidazoles for the treatment of TB: past, present and future. *Future Med. Chem.* **2011**, *3*, 1427−1454.
(6) Ware, G. W. In *The pesticide book*, 4th ed.; Thompson Publications, Fresno, CA, USA, 1994.
(7) Alfred Nobel's life and work. NobelPrize.org. https://www.nobelprize.org/alfred-nobel/alfred-nobels-life-and-work/.
(8) Parry, R.; Nishino, S.; Spain, J. Naturally occurring nitro compounds. *Nat. Prod. Rep.* **2011**, *28*, 152−167.
(9) Shi, M.; Cui, S. C. Electrophilic aromatic nitration using a mixed catalyst of lithium, molybdenum, ytterbium on silica gel. *Adv. Synth. Catal.* **2003**, *345*, 1329−1333.
(10) Mellor, J. M.; Mittoo, S.; Parkes, R.; Millar, R. W. Improved nitrations using metal nitrate-sulfuric acid systems. *Tetrahedron* **2000**, *56*, 8019−8024.
(11) Mulla, S. I.; Bharagava, R. N.; Belhaj, D.; Saratale, G. D.; Bagewadi, Z. K.; Saxena, G.; Kumar, A.; Mohan, H.; Yu, C.-P.; Ninnekar, H. Z. An overview of nitro group-containing compounds and herbicides degradation in microorganisms. In *Microbial Metabolism of Xenobiotic Compounds*; Arora, P. K., Ed.; Springer, Singapore, 2019.
(12) Sabbasani, V. R.; Lee, D. Nitration of silyl allenes to form functionalized nitroalkenes. *Org. Lett.* **2013**, *15*, 3954−3957.
(13) Rokade, B. V.; Prabhu, K. R. Synthesis of substituted nitroolefins: A copper catalyzed nitrodecarboxylation of unsaturated carboxylic acids. *Org. Biomol. Chem.* **2013**, *11*, 6713−6716.
(14) Zhu, X.; Qiao, L.; Ye, P.; Ying, B.; Xu, J.; Shen, C.; Zhang, P. Copper-catalyzed rapid C-H nitration of 8-aminoquinolines by using sodium nitrite as the nitro source under mild conditions. *RSC Adv.* **2016**, *6*, 89979−89983.
(15) Maity, S.; Manna, S.; Rana, S.; Naveen, T.; Mallick, A.; Maiti, D. Efficient and stereoselective nitration of monoand disubstituted olefins with $AgNO_2$ and TEMPO. *J. Am. Chem. Soc.* **2013**, *135*, 3355−3358.
(16) Yan, G.; Yang, M. Recent advances in the synthesis of aromatic nitro compounds. *Org. Biomol. Chem.* **2013**, *11*, 2554−2566.
(17) Song, L. R.; Fan, Z. L.; Zhang, A. Recent advances in transition metal-catalyzed C(sp2)-H nitration. *Org. Biomol. Chem.* **2019**, *17*, 1351−1361.
(18) Dusel, S. J. S.; König, B. Visible-light-mediated nitration of protected anilines. *J. Org. Chem.* **2018**, *83*, 2802−2807.
(19) Kurig, N.; Palkovits, R. Electrochemical nitration for organic C-N bond formation: a current view on possible N-sources, mechanisms, and technological feasibility. *Green Chem.* **2023**, *25*, 7508−7517.
(20) Blum, S. P.; Nickel, C.; Schäffer, L.; Karakaya, T.; Waldvogel, S. R. Electrochemical Nitration with Nitrite. *ChemSusChem* **2021**, *14*, 4936−4940.
(21) Sugai, Y.; Katsuyama, Y.; Ohnishi, Y. A nitrous acid biosynthetic pathway for diazo group formation in bacteria. *Nat. Chem. Biol.* **2016**, *12*, 73−75.
(22) Katsuyama, Y.; Sato, Y.; Sugai, Y.; Higashiyama, Y.; Senda, M.; Senda, T.; Ohnishi, Y. Crystal structure of the nitrosuccinate lyase CreD in complex with fumarate provides insights into the catalytic mechanism for nitrous acid elimination. *FEBS J.* **2018**, *285*, 1540−1555.
(23) Hagihara, R.; Katsuyama, Y.; Sugai, Y.; Onaka, H.; Ohnishi, Y. Novel desferrioxamine derivatives synthesized using the secondary metabolism-specific nitrous acid biosynthetic pathway in *Streptomyces davawensis*. *J. Antibiot.* **2018**, *71*, 911−919.
(24) Kawai, S.; Sugaya, Y.; Hagihara, R.; Tomita, H.; Katsuyama, Y.; Ohnishi, Y. Complete biosynthetic pathway of alazopeptin, a tripeptide consisting of two molecules of 6-diazo-5-oxo-l-norleucine and one molecule of alanine. *Angew. Chem., Int. Ed.* **2021**, *60*, 10319−10325.
(25) Kawai, S.; Yamada, A.; Du, D.; Sugai, Y.; Katsuyama, Y.; Ohnishi, Y. Identification and analysis of the biosynthetic gene cluster for the hydrazide-containing aryl polyene spinamycin. *ACS Chem. Biol.* **2023**, *18*, 1821−1828.
(26) Kawai, S.; Hagihara, R.; Shin-ya, K.; Katsuyama, Y.; Ohnishi, Y. Bacterial avenalumic acid biosynthesis includes substitution of an aromatic amino group for hydride by nitrous acid dependent diazotization. *Angew. Chem., Int. Ed.* **2022**, *61*, No. e202211728.
(27) Wang, K.-K. A.; Ng, T. L.; Wang, P.; Huang, Z.; Balskus, E. P.; van der Donk, W. A. Glutamic acid is a carrier for hydrazine during the biosyntheses of fosfazinomycin and kanamycin. *Nat. Commun.* **2018**, *9*, 3687.
(28) Del Rio Flores, A.; Twigg, F. F.; Du, Y.; Cai, W.; Aguirre, D. Q.; Sato, M.; Dror, M. J.; Narayanamoorthy, M.; Geng, J.; Zill, N. A.; Zhai, R.; Zhang, W. Biosynthesis of triacsin featuring an *N*-hydroxytriazene pharmacophore. *Nat. Chem. Biol.* **2021**, *17*, 1305−1313.
(29) Valentino, H.; Sobrado, P. Characterization of a nitro-forming enzyme involved in fosfazinomycin biosynthesis. *Biochemistry* **2021**, *60*, 2851−2864.
(30) Rotilio, L.; Boverio, A.; Nguyen, Q. T.; Mannucci, B.; Fraaije, M. W.; Mattevi, A. A biosynthetic aspartate N-hydroxylase performs successive oxidations by holding intermediates at a site away from the catalytic center. *J. Biol. Chem.* **2023**, *299*, 104904−104918.
(31) Huang, Z.; Wang, K.-K. A.; van der Donk, W. A. New insights into the biosynthesis of fosfazinomycin. *Chem. Sci.* **2016**, *7*, 5219.
(32) Fan, C.; Zhou, F.; Huang, W.; Xue, Y.; Xu, C.; Zhang, R.; Xian, M.; Feng, X. Characterization of an efficient N-oxygenase from Saccharothrix sp. and its application in the synthesis of azomycin. *Biotechnol. Biofuels* **2023**, *16*, 194.
(33) McBride, M. J.; Sil, D.; Ng, T. L.; Crooke, A. M.; Kenney, G. E.; Tysoe, C. R.; Zhang, B.; Balskus, E. P.; Boal, A. K.; Krebs, C.; Bollinger, J. M., Jr A eroxodiiron(III/III) intermediate mediating both N-hydroxylation steps in biosynthesis of the N-nitrosourea pharmacophore of streptozotocin by the multi-domain metalloenzyme SznF. *J. Am. Chem. Soc.* **2020**, *142*, 11818−11828.
(34) Li, X.; Shimaya, R.; Dairi, T.; Chang, W.-c.; Ogasawara, Y. Identification of cyclopropane formation in the biosyntheses of hormaomycins and belactosins: sequential nitration and cyclopropanation by metalloenzymes. *Angew. Chem., Int. Ed.* **2022**, *61*, No. e202113189.
(35) Engelbrecht, A.; Wolf, F.; Esch, A.; Kulik, A.; Kozhushkov, S. I.; de Meijere, A.; Hughes, C. C.; Kaysser, L. Discovery of a cryptic nitro intermediate in the biosynthesis of the 3-(trans-2'-aminocyclopropyl)-alanine moiety of belactosin A. *Org. Lett.* **2022**, *24*, 736−740.
(36) He, J.; Hertweck, C. Biosynthetic origin of the rare nitroaryl moiety of the polyketide antibiotic aureothin: involvement of an unprecedented N-oxygenase. *J. Am. Chem. Soc.* **2004**, *126*, 3694−3695.
(37) Pacholec, M.; Sello, J. K.; Walsh, C. T.; Thomas, M. G. Formation of an aminoacyl-S-enzyme intermediate is a key step in the biosynthesis of chloramphenicol. *Org. Biomol. Chem.* **2007**, *5*, 1692−1694.
(38) Zhu, X.; van Pée, K.-H.; Naismith, J. H. The ternary complex of PrnB (the second enzyme in the pyrrolnitrin biosynthesis pathway), tryptophan, and cyanide yields new mechanistic insights into the indolamine dioxygenase superfamily. *J. Biol. Chem.* **2010**, *285*, 21126.
(39) Hedges, J. B.; Ryan, K. S. In vitro Reconstitution of the Biosynthetic Pathway to the Nitroimidazole Antibiotic Azomycin. *Angew. Chem., Int. Ed.* **2019**, *58*, 11647−11651.
(40) Ju, K. S.; Parales, R. E. Nitroaromatic compounds, from synthesis to biodegradation. *Microbiol. Mol. Biol. R.* **2010**, *74*, 250−272.
(41) Choi, Y. S.; Zhang, H.; Brunzelle, J. S.; Nair, S. K.; Zhao, H. In vitro reconstitution and crystal structure of p-aminobenzoate N-oxygenase (AurF) involved in aureothin biosynthesis. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 6858−6863.
(42) Lu, H.; Chanco, E.; Zhao, H. CmlI is an N-oxygenase in the biosynthesis of chloramphenicol. *Tetrahedron* **2012**, *68*, 7651−7654.
(43) Komor, A. J.; Rivard, B. S.; Fan, R.; Guo, Y.; Que, L.; Lipscomb, J. D. Mechanism for six-electron aryl-N-oxygenation by the nonheme diiron enzyme CmlI. *J. Am. Chem. Soc.* **2016**, *138*, 7411−7421.

https://doi.org/10.1021/jacsau.4c00994
JACS Au 2025, 5, 28−41

(44) Li, N.; Korboukh, V. K.; Krebs, C.; Bollinger, J. M. Four-electron oxidation of *p*-hydroxylaminobenzoate to *p*-nitrobenzoate by a peroxodiferric complex in AurF from Streptomyces thioluteus. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 15722−15727.
(45) Wei, W. J.; Siegbahn, P. M.; Liao, R. Z. Mechanism of the dinuclear iron enzyme p-aminobenzoate N-oxygenase from density functional calculations. *ChemCatChem.* **2019**, *11*, 601−613.
(46) Komor, A. J.; Rivard, B. S.; Fan, R.; Guo, Y.; Que, L.; Lipscomb, J. D. CmlI N-oxygenase catalyzes the final three steps in chloramphenicol biosynthesis without dissociation of intermediates. *Biochemistry* **2017**, *56*, 4940−4950.
(47) Makris, T. M.; Vu, V. V.; Meier, K. K.; Komor, A. J.; Rivard, B. S.; Münck, E.; Que, L.; Lipscomb, J. D. An unusual peroxo intermediate of the arylamine oxygenase of the chloramphenicol biosynthetic pathway. *J. Am. Chem. Soc.* **2015**, *137*, 1608−1617.
(48) Knoot, C. J.; Kovaleva, E. G.; Lipscomb, J. D. Crystal structure of CmlI, the arylamine oxygenase from the chloramphenicol biosynthetic pathway. *J. Biol. Inorg. Chem.* **2016**, *21*, 589−603.
(49) Lee, J.; Simurdiak, M.; Zhao, H. Reconstitution and characterization of aminopyrrolnitrin oxygenase, a Rieske N-oxygenase that catalyzes unusual arylamine oxidation. *J. Biol. Chem.* **2005**, *280*, 36719.
(50) Lee, J.; Zhao, H. Mechanistic studies on the conversion of arylamines into arylnitro compounds by aminopyrrolnitrin oxygenase: Identification of intermediates and kinetic studies. *Angew. Chem., Int. Ed.* **2006**, *45*, 622−625.
(51) Schröder, G. C.; Smit, M. S.; Opperman, D. J. Harnessing heme chemistry: Recent advances in the biocatalytic applications of cytochrome P450 monooxgenases. *Curr. Opin. Green Sust. Chem.* **2023**, *39*, 100734−100746.
(52) Fan, S.; Cong, Z. Emerging strategies for modifying cytochrome P450 monooxygenases into peroxizymes. *Acc. Chem. Res.* **2024**, *57*, 613−624.
(53) Wolf, M. F.; Hinchen, D. J.; DuBois, J. L.; McGeehan, J. E.; Eltis, L. D. Cytochromes P450 in the biocatalytic valorization of lignin. *Curr. Opin. Biotechnol.* **2022**, *73*, 43−50.
(54) Grogan, G. Hemoprotein Catalyzed Oxygenations: P450s, UPOs, and Progress toward Scalable Reactions. *JACS Au* **2021**, *1*, 1312−1329.
(55) Urlacher, V. B.; Girhard, M. Cytochrome P450 Monooxygenases in Biotechnology and Synthetic Biology. *Trends Biotechnol.* **2019**, *37*, 882−897.
(56) Fansher, D. J.; Besna, J. N.; Fendri, A.; Pelletier, J. N. Choose Your Own Adventure: A Comprehensive Database of Reactions Catalyzed by Cytochrome P450 BM3 Variants. *ACS Catal.* **2024**, *14*, 5560−5592.
(57) Caranto, J. D. The emergence of nitric oxide in the biosynthesis of bacterial natural. *Curr. Opin. Chem. Biol.* **2019**, *49*, 130−138.
(58) Barry, S. M.; Kers, J. A.; Johnson, E. G.; Song, L.; Aston, P. R.; Patel, B.; Krasnoff, S. B.; Crane, B. R.; Gibson, D. M.; Loria, R.; Challis, G. L. Cytochrome P450-catalyzed L-tryptophan nitration in thaxtomin phytotoxin biosynthesis. *Nat. Chem. Biol.* **2012**, *8*, 814−816.
(59) Tomita, H.; Katsuyama, Y.; Minami, H.; Ohnishi, Y. Identification and characterization of a bacterial cytochrome P450 monooxygenase catalyzing the 3-nitration of tyrosine in rufomycin biosynthesis. *J. Biol. Chem.* **2017**, *292*, 15859−15869.
(60) Ma, J.; Huang, H.; Xie, Y.; Liu, Z.; Zhao, J.; Zhang, C.; Jia, Y.; Zhang, Y.; Zhang, H.; Zhang, T.; Ju, J. Biosynthesis of ilamycins featuring unusual building blocks and engineered production of enhanced anti-tuberculosis agents. *Nat. Commun.* **2017**, *8*, 391.
(61) Li, H.; Li, W.; Song, K.; Liu, Y.; Zhao, G.; Du, Y.-L. Nitric oxide synthase-guided genome mining identifies a cytochrome P450 enzyme for olefin nitration in bacterial specialized metabolism. *Synth. Sys. Biotechnol.* **2024**, *9*, 127−133.
(62) Dodani, S. C.; Cahn, J. K. B.; Heinisch, T.; Brinkmann-Chen, S.; Mcintosh, J. A.; Arnold, F. H. Structural, functional, and spectroscopic characterization of the substrate scope of the novel nitrating cytochrome P450 TxtE. *ChemBioChem.* **2014**, *15*, 2259−2267.
(63) Dodani, S. C.; Kiss, G.; Cahn, J. K. B.; Su, Y.; Pande, V. S.; Arnold, F. H. Discovery of a regioselectivity switch in nitrating P450s guided by molecular dynamics simulations and Markov models. *Nat. Chem.* **2016**, *8*, 419−425.
(64) Yu, F.; Li, M.; Xu, C.; Wang, Z.; Zhou, H.; Yang, M.; Chen, Y.; Tang, L.; He, J. Structural insights into the mechanism for recognizing substrate of the cytochrome P450 enzyme TxtE. *PLoS One* **2013**, *8*, No. e81526.
(65) Zuo, R.; Zhang, Y.; Huguet-Tapia, J. C.; Mehta, M.; Dedic, E.; Bruner, S. D.; Loria, R.; Ding, Y. An artificial self-sufficient cytochrome P450 directly nitrates fluorinated tryptophan analogs with a different regio-selectivity. *Biotechnol. J.* **2016**, *11*, 624−632.
(66) Zuo, R.; Zhang, Y.; Jiang, C.; Hackett, J. C.; Loria, R.; Bruner, S. D.; Ding, Y. Engineered P450 Biocatalysts Show Improved Activity and Regio-Promiscuity in Aromatic Nitration. *Sci. Rep.* **2017**, *7*, 842.
(67) Zuo, R.; Ding, Y. Direct Aromatic Nitration System for Synthesis of Nitrotryptophans in Escherichia coli. *ACS Synth. Biol.* **2019**, *8*, 857−865.
(68) Jiang, G.; Zuo, R.; Zhang, Y.; Powell, M. M.; Zhang, P.; Hylton, S. M.; Loria, R.; Ding, Y. One-Pot Biocombinatorial Synthesis of Herbicidal Thaxtomins. *ACS Catal.* **2018**, *8*, 10761−10768.
(69) Tian, X.; Liu, W. Q.; Xu, H.; Ji, X.; Liu, Y.; Li, J. Cell-free expression of NO synthase and P450 enzyme for the biosynthesis of an unnatural amino acid L-4-nitrotryptophan. *Synth. Sys. Biotechnol.* **2022**, *7*, 775−783.
(70) Saroay, R.; Roiban, G. D.; Alkhalaf, L.; Challis, G. Expanding the substrate scope of nitrating cytochrome P450 TxtE by active site engineering of a reductase fusion. *ChemBioChem.* **2021**, *22*, 2262−2265.
(71) Qian, Y.; Jiang, Z.; Long, Y.; Fei, H.; Song, H.; Guo, C. Electroenzymatic aromatic nitration via an electric field and electromediator. *ACS Sustain. Chem. Eng.* **2022**, *10*, 6667−6674.
(72) Dratch, B. D.; McWhorter, K. L.; Blue, T. C.; Jones, S. K.; Horwitz, S. M.; Davis, K. M. Insights into Substrate Recognition by the Unusual Nitrating Enzyme RufO. *ACS Chem. Biol.* **2023**, *18*, 1713−1718.
(73) Jordan, S.; Li, B. N.; Traore, E.; Wu, Y. F.; Usai, R.; Liu, A. M.; Xie, Z. R.; Wang, Y. F. Structural and spectroscopic characterization of RufO indicates a new biological role in rufomycin biosynthesis. *J. Biol. Chem.* **2023**, *299*, 105049−105061.
(74) Louka, S.; Barry, S. M.; Heyes, D. J.; Mubarak, M. Q. E.; Ali, H. S.; Alkhalaf, L. M.; Munro, A. W.; Scrutton, N. S.; Challis, G. L.; de Visser, S. P. Catalytic mechanism of aromatic nitration by cytochrome P450 TxtE: involvement of a ferric-peroxynitrite intermediate. *J. Am. Chem. Soc.* **2020**, *142*, 15764−15779.
(75) Martin, C. P.; Chen, M.; Martinez, M. F.; Ding, Y.; Caranto, J. D. The ferric-superoxo intermediate of the TxtE nitration pathway resists reduction, facilitating its reaction with nitric oxide. *Biochemistry* **2021**, *60*, 2436−2446.
(76) Mondal, P.; Udukalage, D.; Mohamed, A. A.; Wong, H. P. H.; de Visser, S. P.; Wijeratne, G. B. A Cytochrome P450 TxtE Model System with Mechanistic and Theoretical Evidence for a Heme Peroxynitrite Active Species. *Angew. Chem., Int. Ed.* **2024**, *63*, No. e202409430.
(77) Matthews, M. L.; Chang, W.; Layne, A. P.; Miles, L. A.; Krebs, C.; Bollinger, J. M. Direct nitration and azidation of aliphatic carbons by an iron-dependent halogenase. *Nat. Chem. Biol.* **2014**, *10*, 209−215.
(78) Büchler, J.; Malca, S. H.; Patsch, D.; Voss, M.; Turner, N. J.; Bornscheuer, U. T.; Allemann, O.; Le Chapelain, C.; Lumbroso, A.; Loiseleur, O.; Buller, R. Algorithm-aided engineering of aliphatic halogenase WelO5* for the asymmetric late-stage functionalization of soraphens. *Nat. Commun.* **2022**, *13*, 371.
(79) Poulos, T. L. Heme Enzyme Structure and Function. *Chem. Rev.* **2014**, *114*, 3919−3962.
(80) Jacquet, A.; Garciaquintana, L.; Deleersnyder, V.; Fenna, R.; Bollen, A.; Moguilevsky, N. Site-directed mutagenesis of human

https://doi.org/10.1021/jacsau.4c00994
JACS Au 2025, 5, 28−41

myeloperoxidase: Further Identification of Residues Involved in Catalytic Activity and Heme Interaction. *Biochem. Biophys. Res. Commun.* **1994**, *202*, 73.
(81) van der Vliet, A.; Eiserich, J. P.; Halliwell, B.; Cross, C. E. Formation of reactive nitrogen species during peroxidase-catalyzed oxidation of nitrite: a potential additional mechanism of nitric oxide-dependent toxicity. *J. Biol. Chem.* **1997**, *272*, 7617−7625.
(82) Balkus, K. J., Jr.; Pisklak, T. J.; Huang, R. Microperoxidase-11 immobilized in a metal organic framework. In *Biomolecular Catalysis*; ACS Symposium Series, Vol. *986*; American Chemical Society, 2008; pp 76−98. .
(83) Low, D. W.; Yang, G.; Winkler, J. R.; Gray, H. B. Modification of heme peptides by reverse proteolysis: Spectroscopy of microperoxidase-10 with C-terminal histidine, tyrosine, and methionine residues. *J. Am. Chem. Soc.* **1997**, *119*, 4094−4095.
(84) Prieto, T.; Nantes, I. L.; Nascimento, O. R. Microperoxidase-9 cycle in the presence of cetyltrimethylammonium bromide micelles: tert-butyl hydroperoxide as both an oxidizing and a reducing agent. *Prog. Colloid Polym. Sci.* **2004**, *128*, 193−198.
(85) Low, D. W.; Abedin, S.; Yang, G.; Winkler, J. R.; Gray, H. B. Manganese microperoxidase-8. *Inorg. Chem.* **1998**, *37*, 1841−1843.
(86) Ischiropoulos, H. Protein tyrosine nitration. *Redox Biol.* **2024**, *8*, 100030−100035.
(87) Lecomte, S.; Ricoux, R.; Mahy, J. P.; Korri-Youssoufi, H. Microperoxidase-8 adsorbed on a roughened silver electrode as a monomeric high-spin penta-coordinated species: characterization by SERR spectroscopy and electrochemistry. *J. Biol. Inorg. Chem.* **2004**, *9*, 850−858.
(88) Ricoux, R.; Boucher, J.-L.; Mansuy, D.; Mahy, J.-P. Microperoxidase 8 catalyzed nitration of phenol by nitrogen dioxide radicals. *Eur. J. Biochem.* **2001**, *268*, 3783−3788.
(89) Dai, R. J.; Huang, H.; Chen, J.; Deng, Y. L.; Xiao, S. Y. Nitration reaction catalyzed by horseradish peroxidase in the presence of H_2O_2 and $NaNO_2$. *Chin. J. Chem.* **2007**, *25*, 1690−1694.
(90) Pezzella, A.; Manini, P.; Di Donato, P.; Boni, R.; Napolitano, A.; Palumbo, A.; d'Ischia, M. 17b-Estradiol nitration by peroxidase/H_2O_2/NO_2^-: a chemical assessment. *Bioorg. Med. Chem.* **2004**, *12*, 2927−2936.
(91) Palumbo, A.; Napolitano, A.; Barone, P.; d'Ischia, M. Nitrite- and peroxide-dependent oxidation pathways of dopamine: 6-nitrodopamine and 6-hydroxydopamine formation as potential contributory mechanisms of oxidative stress- and nitric oxide induced neurotoxicity in neuronal degeneration. *Chem. Res. Toxicol.* **1999**, *12*, 1213−1222.
(92) Liu, C.; Dai, R. J.; Lin, J.; Sun, J. H.; Chen, S.; Deng, Y. L. Synthesis of nitroanilines catalyzed by horseradish peroxidase in the presence of $NaNO_2$ and H_2O_2. *Chem. Res. Chin. Univ.* **2013**, *29*, 1164−1166.
(93) Budde, C. L.; Beyer, A.; Munir, I. Z.; Dordick, J. S.; Khmelnitsky, Y. L. Enzymatic nitration of phenols. *J. Mol. Catal. B: Enzym.* **2001**, *15*, 55−64.
(94) Sheng, D. W.; Joshi, D. K.; Gold, M. H. Nitration of veratryl alcohol by lignin peroxidase and tetranitromethane. *Arch. Biochem. Biophys.* **1998**, *352*, 121−129.
(95) Gajhede, M.; Schuller, D. J.; Henriksen, A.; Smith, A. T.; Poulos, T. L. Crystal structure of horseradish peroxidase C at 2.15 A resolution. *Nat. Struct. Biol.* **1997**, *4*, 1032−1038.
(96) Henriksen, A.; Mirza, O.; Indiani, C.; Teilum, K.; Smulevich, G.; Welinder, K. G.; Gajhede, M. Structure of soybean seed coat peroxidase: a plant peroxidase with unusual stability and haem-apoprotein interactions. *Protein Sci.* **2001**, *10*, 108−115.
(97) Poulos, T. L.; Edwards, S. L.; Wariishi, H.; Gold, M. H. Crystallographic refinement of lignin peroxidase at 2 A. *J. Biol. Chem.* **1993**, *268*, 4429−4440.
(98) Dunford, H. B.; Stillman, J. S. On the function and mechanism of action of peroxidases. *Coordin. Chem. Rev.* **1976**, *19*, 187−251.
(99) Ma, N.; Chen, Z.; Chen, J.; Chen, J.; Wang, C.; Zhou, H.; Yao, L.; Shoji, O.; Watanabe, Y.; Cong, Z. Dual-functional small molecules for generating an efficient cytochrome P450BM3 peroxygenase. *Angew. Chem., Int. Ed.* **2018**, *57*, 7628−7633.
(100) Qin, X.; Jiang, Y.; Yao, F.; Chen, J.; Kong, F.; Zhao, P.; Jin, L.; Cong, Z. Anchoring a Structurally Editable Proximal Cofactor-like Module to Construct an Artificial Dual-center Peroxygenase. *Angew. Chem., Int. Ed.* **2023**, *62*, No. e202311259.
(101) Ma, N.; Fang, W.; Liu, C.; Qin, X.; Wang, X.; Jin, L.; Wang, B.; Cong, Z. Switching an artificial P450 peroxygenase into peroxidase via mechanism- guided protein engineering. *ACS Catal.* **2021**, *11*, 8449−8455.
(102) Jiang, F.; Wang, Z.; Cong, Z. Tuning peroxidase activity of artificial P450 peroxygenase by engineering redox-sensitive residues. *Faraday Discuss.* **2024**, *252*, 52−68.
(103) Wang, X.; Lin, X.; Jiang, Y.; Qin, X.; Ma, N.; Yao, F.; Dong, S.; Liu, C.; Feng, Y.; Jin, L.; Xian, M.; Cong, Z. Engineering cytochrome P450BM3 enzymes for direct nitration of unsaturated hydrocarbons. *Angew. Chem., Int. Ed.* **2023**, *62*, No. e202217678.
(104) Hasnaoui, G.; Lutje Spelberg, J. H.; de Vries, E.; Tang, L.; Hauer, B.; Janssen, D. B. Nitrite-mediated hydrolysis of epoxides catalyzed by halohydrin dehalogenase from Agrobacterium radiobacter AD1: a new tool for the kinetic resolution of epoxides. *Tetrahedron: Asymmetr.* **2005**, *16*, 1685−1692.
(105) Janssen, D.B.; Majeric-Elenkov, M.; Hasnaoui, G.; Hauer, B.; Spelberg, J.H. L. Enantioselective formation and ring-opening of epoxides catalyzed by halohydrin dehalogenases. *Biochem. Soc. Trans.* **2006**, *34*, 291−295.
(106) Wang, H.-H.; Wan, N.-W.; Miao, R.-P.; He, C.-L.; Chen, Y.-Z.; Liu, Z.-Q.; Zheng, Y.-G. Identification and structure analysis of an unusual halohydrin dehalogenase for highly chemo-, regio- and enantioselective bionitration of epoxides. *Angew. Chem., Int. Ed.* **2022**, *61*, No. e202205790.

https://doi.org/10.1021/jacsau.4c00994
JACS Au 2025, 5, 28−41