Elucidation of divergent desaturation pathways in the formation of vinyl isonitrile and isocyanoacrylate

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Two different types of desaturations are employed by iron- and 2-oxoglutarate-dependent (Fe/2OG) enzymes to construct vinyl isonitrile and isocyanoacrylate moieties found in isonitrile-containing natural products. A substrate-bound protein structure reveals a plausible strategy to affect desaturation and hints at substrate promiscuity of these enzymes. Analogs are synthesized and used as mechanistic probes to validate structural observations. Instead of proceeding through hydroxylated intermediate as previously proposed, a plausible carbocation species is utilized to trigger C=C bond installation. These Fe/2OG enzymes can also accommodate analogs with opposite chirality and different functional groups including isonitrile-(D)-tyrosine, N-formyl tyrosine, and phloretic acid, while maintaining the reaction selectivity.

Isonitrile-containing natural products have been identified from terrestrial and marine microorganisms1-8. Among these molecules, several of them are used to construct the core of xanthocillin, hapalindoles, paerucumarin, rhabduscins, and hybeyankacin (Fig. 1A)9-11. Biosynthetic gene cluster analysis reveals that a conserved strategy is employed in assemblies of vinyl isonitrile and isocyanoacrylate, e.g. 1, 2, and 3, wherein the first reaction includes a condensation reaction to furnish the isonitrile group12-19. In the second reaction, iron- and 2-oxoglutarate-dependent (Fe/2OG) oxygenases catalyze two different types of desaturation reactions to install olefin moiety17-19. In the first reaction, cleavage of a C-H bond and a decarboxylation, while production of 2 is a formal dehydrogenation.

Fe/2OG oxygenases catalyze a broad array of reactions. Examples include hydroxylation, epoxidation, endoperoxide formation, halogenation, rearrangement and many more20-25. In all characterized Fe/2OG enzymes, following an Fe(IV)-oxo species triggered hydrogen atom transfer (HAT) or oxygen atom transfer (OAT), diverse pathways are deployed to affect reaction outcomes. While tremendous efforts have been devoted to engineering Fe/2OG hydroxylases and halogenases to prepare valuable molecules26-30, using Fe/2OG enzymes for the production of vinyl isonitrile and isocyanoacrylate moieties remains understudied.

Received: 25 February 2022
Accepted: 19 August 2022
Published online: 12 September 2022

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determinants and to elucidate the plausible pathway accounting for the observed reaction selectivity, substrate analogs (5-8) and mechanistic probes (9-10) are prepared and investigated in vitro. Instead of employing the canonical oxygen-rebound pathway, a benzylic carbocation may potentially be equipped for use en route to vinyl isonitrile and isocyanoacrylate forming enzymes contain decarboxylation-assisted desaturation and formal dehydrogenation to install vinyl isonitrile (1 and 3) and isocyanoacrylate (2) groups, respectively. The position of desaturation is highlighted in red. An orthologue, PIsnB-Ah, with an uncharacterized function is expressed and tested to validate this hypothesis.

**Results and discussion**

**In vitro characterization of PIsnB- and PvcB-catalyzed reactions**

We carried out in vitro assays to establish the reaction profile of the PvcB- and PIsnB-catalyzed reactions. Both enzymes were expressed as NHis<sub>6</sub>-tagged proteins. The reactions include reconstituted enzyme/substrate 4/2OG/ascorbate in a ratio of 1/5/15/10, with a final concentration of 0.1 mM of reconstituted enzyme in Tris-HCl (50 mM, pH 7.7). The enzymatic reactions were analyzed using liquid chromatography coupled mass spectrometry (LC-MS). In the PIsnB-catalyzed reaction, the major product with an m/z value corresponding to the vinyl isonitrile was detected (m/z 190.1→144.1, Fig. 3). Identity of this peak was verified using the synthetic standard of 3. On the other hand, formation of a peak with an m/z value matching isocyanoacrylate (2) was observed in the PvcB-catalyzed reaction (m/z 190.1→188.1, Fig. 3). In both cases, a minor peak with an m/z value consistent with hydroxylation was also detected (m/z 190.1→206.1). Notably, 2 is not very stable and readily decomposes to 11 through hydration of the isonitrile group (Supplementary Fig. 1). This observation is in accordance with the literature where 2 could only be characterized by derivatization.

**X-ray crystal structure of substrate-bound PIsnB**

To understand the molecular insight that leads to the observed selectivity of PvcB and PIsnB, we conducted a structural investigation of PIsnB with its substrate (4). Since the oxidation state of iron can be altered under aerobic conditions, which may lead to heterogeneity affecting crystal growth, PIsnB was reconstituted with various metal ions to obtain stable complexes. Out of a dozen metal ions screened, manganese (Mn) improves the melting temperature (T<sub>m</sub>) of PIsnB by more than ten degrees, suggesting that Mn ion effectively stabilizes the protein, possibly through coordination to the iron-binding site (Supplementary Fig. 2A). The inclusion of compound 4 with P IsnB also has a stabilizing effect by improving the T<sub>m</sub> (35±40 °C) (Supplementary Fig. 2A). The ternary complex (PIsnB·Mn·4) was prepared for structure determination by co-crystallization approach. Specifically, the substrate-bound P IsnB was assembled by incubating recombinant protein at -1 mg/mL with 2.5 mM of Mn ion and 2 mM of 4 for 3 h. The mixture was then concentrated to ~10 mg/mL of protein and subjected to crystallization screening. The diffraction data were collected to a resolution of 1.98 Å, with each asymmetric unit containing one molecule of P IsnB in the space group P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub>. The final model shows that P IsnB exists as a monomer which is consistent with the oligomeric state determined by gel filtration (Supplementary Fig. 3). Analogous to...
The carboxylate moiety of PlsnB has two four-stranded β-sheet in the center, a.k.a., jelly roll or Swiss roll fold (Supplementary Fig. 4A). The metal ion is coordinated by His101, Asp103, His250, and three water molecules (Fig. 4A).

The omit map calculated using protein sequence alone reveals a significant positive density close to the metal center (Supplementary Fig. 4B). We first considered molecules used during Escherichia coli growth and crystallization and modeled 2-(N-morpholinio)ethane-sulfonic acid and L-tyrosine, but significant negative and positive density indicates that they cannot account for the observed density (Supplementary Fig. 4C, E). On the other hand, the chemical structure of 4 is consistent with the shape of the density (Fig. 4B and Supplementary Fig. 4D). Iterative refinement of 4 shows a fairly good fit of the ligand although the density of one of the n-carbons of the benzene ring is slightly weaker compared to the rest of the ligand (average B factor of the phenyl ring is 43.1 Å² compared to an average of ~38.6 Å² for the rest of the ligand) (Supplementary Fig. 4). The benzene ring of 4 locates in a hydrophobic pocket formed by Tyr106, Ile159, Trp102, and Met105 (Fig. 4C). While the ample space of the hydrophobic pocket allows the rotation of the benzene ring, the π–π stacking of the phenyl ring of 4 and His101 may result in a preferred orientation as the observed in the crystal structure. Furthermore, the hydrophobicity of the pocket recognizing the phenyl ring of 4 has an effect for substrate recognition. When we alter the hydrophobicity of the pocket by mutating Met105-Tyr106-Lys107 to Ala-Phe-Ala, the substantial improvement of Tm upon ligand incorporation observed in wild-type protein is no longer retained, suggesting the loss of ligand binding (Supplementary Fig. 2B). Notably, no obvious interactions for the para-hydroxyl group can be identified, thus suggesting other functional groups, e.g., fluoride (7), can be accommodated in the active site pocket.

**Fig. 2** | Plausible reaction mechanisms account for Fe/2OG enzyme-catalyzed hydroxylation and desaturation. A Canonical hydroxylation is initiated by an Fe(IV)-oxo species triggered hydrogen atom transfer (HAT). It is then followed by oxygen-rebound to complete hydroxylation. The HAT site and Fe(IV)-oxo are highlighted in blue and red, respectively. B In the Fe/2OG enzymes, i.e., PlsnB and PvcB, catalyzed desaturation reactions using 4 as the substrate, following HAT step, a hydroxylated intermediate or a benzylic cation may serve as a common species to afford 2 and 3 via deprotonation (pathway a, blue arrow) and decarboxylation (pathway b, red arrow), respectively. Other alternative mechanisms are summarized in the supplementary information (Supplementary Fig. 23).

The active site reveals a plausible strategy to prevent isonitrile chelation to the iron center and hints at the substrate flexibility. While isonitrile is a potent and a common ligand to iron[37–39], coordination of isonitrile to the iron in the active site inactivates PlsnB as demonstrated by Mössbauer spectroscopy previously[40]. The substrate-bound structure reveals how PlsnB prevents isonitrile chelation to the iron while maintaining catalytic efficiency. Since the carboxylate group has hydrogen bonds with Gly104 and an iron-coordinated water molecule (Fig. 4D). It is worth mentioning that a loop region (I159-V162) is disordered in the ternary complex structure. Sequence alignment analysis suggests that this region is not conserved among Fe/2OG enzymes that catalyze vinyl isonitrile or isocyanoacrylate formation (Supplementary Fig. 5). To evaluate the potential role of this capping loop, we mutated this loop to the corresponding loop in PvcB (I159R_N160S_K161A) and characterized the product profile using LC-MS. Under current experimental conditions, while this variant is substantially less active (~20% of 3 was produced compared to the wild-type enzyme), the product distribution is not altered (Supplementary Fig. 6). Therefore, the capping loop likely provides a hydrophobic environment to maintain effective catalysis but does not affect the selectivity.

**POTENTIALLY CATALYTIC ROLE OF THE CAPING LOOP IN PLSN B**

The carboxylate moiety of 4 is well-positioned by hydrophilic interactions. Namely, the carboxylate is anchored by Arg265, which has a cation-π interaction with Trp74 with a distance of 3.5 Å to the indole ring (Fig. 4D). It also forms hydrogen bonds with Gly104 and an iron-coordinated water molecule (Fig. 4D). It is worth mentioning that a loop region (I159-V162) is disordered in the ternary complex structure. Sequence alignment analysis suggests that this region is not conserved among Fe/2OG enzymes that catalyze vinyl isonitrile or isocyanoacrylate formation (Supplementary Fig. 5). To evaluate the potential role of this capping loop, we mutated this loop to the corresponding loop in PvcB (I159R_N160S_K161A) and characterized the product profile using LC-MS. Under current experimental conditions, while this variant is substantially less active (~20% of 3 was produced compared to the wild-type enzyme), the product distribution is not altered (Supplementary Fig. 6). Therefore, the capping loop likely provides a hydrophobic environment to maintain effective catalysis but does not affect the selectivity.
reconstituted enzyme), 4, 5, and 6 were consumed -0.96, 0.80, and 0.45 mM in the PIsnB-catalyzed reactions. In PvcB, -0.82, 1.0, and 0.74 mM of 4, 5, and 6 consumption were detected. To further support the LC-MS results, structures of 11 and 12 are confirmed by 13C-NMR using [2-13C]6 as the substrate. In the PIsnB-catalyzed reaction, three peaks with chemical shifts of 119, 120, and 124 ppm were observed (Fig. 6). Similar to [2-13C]6, that exhibits two peaks (δ = 56.8 and 61.2 ppm) representing two tautomers in the solution state, 12 shows two peaks at 120 and 124 ppm11. The third peak (δ = 119 ppm) most likely originates from the other stereoisomer with the cis-geometry of the double bond. On the other hand, in the PvcB-catalyzed reaction, two peaks at 132 and 133 ppm are consistent with formation of 11 (Fig. 6). A minor peak at 120 ppm was also detected. While the configuration, i.e., cis vs. trans-isomer, of the newly installed olefin of 11 and 12 remains to be determined, LC-MS and NMR results demonstrated that PIsnB and PvcB can accommodate substrate analogs and affect chemically divergent desaturations. Notably, formation of 3 using 4 and 5 implies that reaction pathway involves the second hydrogen atom abstraction (Supplementary Fig. 23) is less likely wherein changing the chirality from L to D, i.e., 4 to 5, likely inverts the Cα-H position in the active site (Supplementary Fig. 7).

Fluorinated substrate analog and hydroxylated probes reveal plausible pathway of the PIsnB- and PvcB-catalyzed desaturation

In the PIsnB-catalyzed reaction, alternation of the para-substituent from an electron-donating group, e.g., hydroxyl group, to an electron-withdrawing group, e.g., fluoride, changes the reactivity from decarboxylation-assisted desaturation to hydroxylation, thus weighing against the pathway includes hydroxylation as an intermediate10. If a carboxylation species is deployed by PvcB, due to electron-withdrawing property of fluoride that destabilizes the carbonation, one would expect 7 to impede isocyanoacrylate formation, thus directing the reaction outcome. In contrast, if desaturation undergoes hydroxylation followed by dehydration as proposed previously or other pathways involving an electron-transfer promoted C-C bond cleavage (Fig. 2 and Supplementary Fig. 23), 7 could decrease product formation, but should not influence product distribution. To test this hypothesis, substrate analog with a fluoride appended at the para-position (7) was synthesized. The enzymatic reactions using 7 was carried out. As shown in Fig. 3C and Supplementary Fig. 8A, under the similar conditions, while similar level of the substrate consumption was detected (-0.96/0.60 and 0.82/0.50 mM substrate (4/7) consumption in PIsnB and PvcB, Supplementary Fig. 8A), only the peak with an m/z value corresponds to hydroxylation was detected in both PIsnB and PvcB (Fig. 3C). Furthermore, we compared the active sites of PIsnB and PvcB16,34 by superimposing the two protein structures and modeled 4 into the PvcB structure (Fig. 3C, D). Residues interacting with the substrate (4) and the iron center are highly conserved in PIsnB and PvcB (Figs. 4D and 5D). All interactions required for ligand binding are maintained, including the histidine imidazole sidechain stacking with the substrate’s aromatic ring and the hydrophilic interactions anchoring the carboxylate group (Fig. 5D). In addition, modeling of 7 in the active site of PIsnB reveals that 7 and 4 bind analogously (Supplementary Fig. 7). While formation of a hydroxylated product using 7 is consistent with the mechanism involves the intermediacy of cation, it could also be caused by dissociation of that species from the active site prior to dehydration or the fluoride-substitution may induces the departure of the hydroxylated compound (10). To further elucidate the reaction pathway, analogs (9 and 10 with the para-substituent being H or F) were prepared and investigated. Under the condition of enzyme to substrate ratio of 1.20 with the final enzyme concentration of 100 µM, no obvious new peak can be detected (Supplementary Fig. 13A). Another possibility is that 9 and 10 cannot enter the active site. We carried out the competition experiment by incubating the substrate analog with the opposite chirality (5), may also fit into the active site. Indeed, the N-formyl moiety has been identified in several natural products, including fumiformamide and melanocin E10,11. To validate this observation, we modeled 6 in the active site of PIsnB. The N-formyl group does not give rise to an obvious steric clash (Fig. 5B). Furthermore, the molecular modeling using 5, 7 and 8 indicates that all hydrophobic interactions at the phenyl ring and the hydrophilic interaction at the carboxylate group are well-preserved (Supplementary Fig. 7). When the para-substituent is replaced by fluoride (7), PvcB and PIsnB only catalyze hydroxylation (m/z: 192.1 + 208.1). Products associated with desaturations (m/z: 144.1 or 146.1) were not detected.

In the PIsnB reaction, alternation of the substituent is replaced by fluoride (7), PvcB and PIsnB only catalyze hydroxylation (m/z: 192.1 + 208.1). Products associated with desaturations (m/z: 144.1 or 146.1) were not detected.

Fig. 3 | PIsnB and PvcB catalyze chemically divergent desaturations. LC-MS chromatograms of PIsnB- and PvcB-catalyzed reactions. A In the PIsnB reaction, conversion of 4 to vinyl isonitrile 3 was identified (m/z 190.1–144.1, purple). B Isocyanoacrylate 2 was detected as the dominant product in the PvcB-catalyzed reaction (m/z 190.1–188.1, red). In both PIsnB- or PvcB-catalyzed reactions, a minor hydroxylation product (m/z 206.1, yellow) was detected. The intensity of the hydroxylated peak is magnified 10-fold for clear visualization. C When the para-substituent is replaced by fluoride (7), PvcB and PIsnB only catalyze hydroxylation (m/z: 192.1 + 208.1). Products associated with desaturations (m/z: 190.1 + 146.1 or 190.1) were not detected.
native substrate \((4)\) with 9 or 10 to assess later possibility. In comparison with the reaction without adding 9 or 10 (Supplementary Fig. 8A), the reduced activity with -1/2 and 1/7 of substrate \((4)\) consumption was detected in PIsnB. Analogously, -1/3 and 1/9 of substrate \((4)\) consumption was observed in PvcB, thus suggesting 9 and 10 can enter the enzyme active site of PIsnB and PvcB (Supplementary Fig. 13B). Taken together, while we cannot completely rule out the pathway includes a hydroxylated intermediate, these results support the intermediacy of a benzylic carbocation in PIsnB and PvcB-catalyzed desaturation. While pathways include oxygen-rebound and two sequential hydrogen atom abstraction processes have been included in several Fe/2OG enzymes catalyzed desaturation through in vitro as well as computational studies\(^{34,42-50}\), our results imply that a benzylic cation is likely utilized to affect decarboxylation and deprotonation in the PIsnB- and PvcB-catalyzed chemically divergent desaturations (Fig. 2).

**Sequence comparison helps forecast the reaction selectivity**

Highly similar active sites but different reactivities deployed by PIsnB and PvcB suggest that other residues not identified by structural comparison might be important for the observed selectivity. We carried out a sequence alignment analysis of the Fe/2OG enzymes that have been reported to catalyze vinyl isonitrile and isocynoacrylate production (Supplementary Fig. 11). The comparison revealed that these enzymes contain divergent sequences at conserved positions. Herein, we annotate enzymes catalyze vinyl isonitrile formation as PIsnB-type and those trigger isocynoacrylate formation as PvcB-type for clarification. Specifically, in PIsnB-type enzymes, a positively charged residue including lysine or arginine is conserved at the K107 position of PIsnB. In contrast, PvcB-type enzymes have a leucine at this position. Moreover, PIsnB-type enzymes occupy a conserved residue including a nitrogen-containing side chain such as asparagine or histidine at the N188 position of PIsnB in the downstream region. In contrast, PvcB-type enzymes have a cysteine at this position. This observation provides a simple method to forecast the reaction selectivity, and can be used for the product prediction. To test this hypothesis, a P IsnB-type orthologue from *Aeromonas hydrophila*, referred to PIsnB-Ah, with an unidentified function was purified and investigated. This enzyme shows a similar activity and selectivity as of PIsnB and can accommodate analogs 5 and 6 (Supplementary Fig. 8, 9, and 12). While this analysis provides a simple method to predict reaction selectivity, single-point mutation, including K113L and N194C of PIsnB-Ah, does not alter the product profile (Supplementary Fig. 6), and indicates other residues are also involved in reaction selectivity. Further studies are currently ongoing.

In this work, reaction pathways leading to vinyl isonitrile and isocynoacrylate formation are revealed using protein structure, molecular modeling, and in vitro assays with substrate and analogs. Our findings suggest that a carbocation species may be deployed to enable decarboxylation and deprotonation. Furthermore, a sequence alignment of Fe/2OG desaturases reveals that divergent sequences at conserved positions are associated with the reaction selectivity. This observation is validated via reconstitution of an uncharacterized enzyme, PIsnB-Ah, activity in vitro. As suggested by the substrate-bound protein structure and the substrate modeling, the ample space used for isonitrile group can occupy other groups. Indeed, all investigated enzymes, i.e., PvcB, PIsnB, and PIsnB-Ah, can accommodate analogs with the opposite chirality and a formyl group, while maintaining reaction selectivity. Taken together, these findings not only uncover the strategies of Fe/2OG enzymes to enable divergent desaturations, but also delineate the diverse chemistries catalyzed by Fe/2OG enzymes in natural product biosynthesis.

**Fig. 4 | Structure of PIsnB-Mn**

A. 2FoFc map of the metal center of PIsnB-Mn-4. Dash lines show the coordination of the metal with the distances labeled. The 2FoFc composite map is shown by blue mesh contoured to 1.5σ. B FoFc omit map of the PIsnB active site upon incorporating the native substrate \((4)\) in co-crystallization experiment, contoured to 3σ and shown in green mesh. The omit map was calculated with no ligand information to avoid phase bias. The chemical structure of the substrate was then superimposed onto the density for consistency comparison. C The hydrophobic pocket close to the aromatic side chain of 4 is shown as semitransparent surface. D Hydrophilic interactions of the carboxylate group of 4 with Arg265, Gly104, and a water molecule. Arg265 has a cation-n interaction with Trp74.
Methods

Expression and purification of PIsnB, PvcB, and PIsnB-Ah

The plasmid encoding pisnB, pvcB, or pisnB-Ah gene was transformed into E. coli BL21 (DE3) cells (New England Biolabs, MA). A single colony was picked and incubated with 100 mL Luria-Bertani (LB) and 100 µL kanamycin at 37 °C for ~16 h. The cells were used as starting culture for large-scale expression with a volumetric ratio of 1:80 of starting culture to growth media. After inoculation, the cells were growing at 37 °C. Upon optical density at 600 nm (OD600) reached of ~0.6, IPTG with final concentration of 1.0 mM was added to the culture. The cells were growing at 18 °C for 15 h before harvesting by centrifugation at 8 °C. To obtain the protein, the cells were suspended in an ice-chilled buffer (100 mM Tris, pH 7.5), and lysed by sonication. The resulting lysate was subjected to centrifugation for 30 min at 22,000 rpm at 4 °C, and the supernatant was loaded onto a Ni-NTA agarose column. The column was washed with six volumes of buffer containing 10 mM imidazole (100 mM Tris, pH 7.5). Subsequently, the desired protein was eluted using buffer containing 250 mM imidazole (100 mM Tris,
Reaction samples were quenched using 200 µL of 200 mM Tris (pH 7.68) were prepared. Reactions were carried out at 4 °C. Reaction mixtures containing protein (PIsnB or PvcB), Fe(II), [2-13C]-2OG, and ascorbate with the final concentration of 0.12 mM enzyme, 0.1 mM Fe(II), 3 mM 2OG, and 0.5 mM ascorbate, were performed as described below. Reaction mixtures including enzyme, Fe(II), substrate, 2OG, and ascorbate with the final concentration of 0.12 mM enzyme, 0.1 mM Fe(II), 3 mM 2OG, 0.5 mM substrate, and 2 mM ascorbate with final volume of 200 µL in 50 mM Tris (pH 7.68) were prepared. Reactions were carried out at 4 °C. Reaction samples were quenched using 200 µL of acetonitrile at 10 mins. Prior to LC-MS analysis, all samples were centrifuged at 12,000 g for 30 min to remove the protein.

In vitro assays of PvcB, PlsnB, and PlsnB-Ah catalyzed reactions

LC-MS was carried out on an Agilent Technologies (Santa Clara, CA) 1200 system coupled to an Agilent Technologies 6120 quadrupole mass spectrometer. The associated Agilent MassHunter and OpenLAB software package were used for data collection and analysis. Detection was performed under electrospray ionization in negative mode (ESI−). The drying gas temperature was 350 °C with a nebulizer pressure of 35 psi and flow rate of 12 L/min. The capillary voltage is set to 3000 V. Reaction mixtures were separated on a Merck SeQuant C18 column (150 × 2.1 mm, 3.0 µm particle size). A gradient elution using solvent A (20 mM ammonium acetate aqueous solution) and solvent B (acetonitrile) with a flow rate of 0.35 mL/min was applied. Starting with 90% solvent A and 10% solvent B, followed by a gradient of 90–60% solvent B from 4 to 8 min. The system was then kept isocratic with 60% solvent B from 8 to 13 min, and then a gradient from 60–90% solvent B was applied from 13 to 17 min. The column was allowed to re-equilibrate for 9 min under initial conditions before subsequent sample injections.

Reactions associated with Fig. 3 are plotted using GraphPad Prism and were performed as described below. Reaction mixtures including enzyme, Fe(II), substrate, 2OG, and ascorbate with the final concentration of 0.12 mM enzyme, 0.1 mM Fe(II), 3 mM 2OG, 0.5 mM substrate, and 2 mM ascorbate with final volume of 200 µL in 50 mM Tris (pH 7.68) were prepared. Reactions were carried out at 4 °C. Reaction samples were quenched using 200 µL of acetonitrile at 10 mins. Prior to LC-MS analysis, all samples were centrifuged at 12,000 g for 30 min to remove the protein.

Using 13C-NMR spectroscopy to follow the enzymatic reactions

Reaction mixtures containing protein (PlsnB or PvcB), Fe(II), [2-13C]-6, 20G, and ascorbate with the final concentration of 0.48 mM protein (PlsnB or PvcB), 0.4 mM Fe(II), 12.0 mM 2OG, 2.0 mM [2-13C]-6, and 8.0 mM ascorbate with final volume of 600 µL in 50 mM Tris (pH 7.68) were prepared. The reaction mixtures were shaken with a speed of 220 rpm for 17 h at 18 °C. Prior to NMR measurement, 30 µL of DMSO-d₄ was added to the reaction followed by centrifugation at 12,000 g for 30 min. The supernatant was then transferred to the NMR tubes. The 13C-NMR spectra were recorded using Bruker NEO 700 MHz. The NMR spectra plotted using MestReNova are shown in Fig. 6.

Crystallization and X-ray structure determination

In order to identify crystallization conditions for PlsnB, 12 mg/mL of purified PlsnB protein sample was incubated overnight with 2.5 mM Mn²⁺ ion before the screening in sparse matrix with a Phoenix crystallization robotic system (Art Robbins Instruments). After 2-weeks incubation of screening trays at 4 °C, rod shaped crystals appeared in a condition that contains 0.1M MES (pH 6.5), 0.2M ammonium sulfate, and 30% PEG 5000MME. This crystallization condition was further optimized by manually setting sitting-drop vapor diffusion experiments with varying pH and precipitant concentration, resulting in diffraction-quality crystals. For co-crystallization of 4 with PlsnB protein, 12 mg/mL of PlsnB protein was incubated overnight with 2.5 mM Mn²⁺ ion and 2 mM 4 prior to crystallization setup in an identical condition. Individual crystals were flash-frozen directly in liquid nitrogen after brief incubation with a reservoir solution supplemented with 30% (v/v) glycerol. X-ray diffraction data were collected at 23-ID-B beamline in Advance Photon Source (Lemont, IL). By using HKL2000, X-ray diffraction pattern was processed to 1.98 Å resolution for PlsnB-Mn complexed with 4. In Phenix software, phases were obtained by molecular replacement using a previously obtained PvCB structure as the initial search model (PDB code 4YLM). The molecular replacement solution for PlsnB structure were iteratively built using Coot⁴⁵ and Phenix refinement package. The omit map was first calculated, revealing a strong positive density close to the metal binding center. Different chemical compounds used during the expression and purification was manually built inside the density with Coot⁴⁵ to calculate the density. The 2FoFc and FoFc maps were with the most likely candidates, as shown in Supplementary Fig. 4C–F. The quality of the finalized crystal structure was evaluated by MolProbity. The final statistics for data collection and structural determination are shown in Supplementary Table 1.

Atomic coordinates and structure factors for the reported crystal structures in this work have been deposited to the Protein Data Bank (PDB) under accession number 71CL for PlsnB complex with compound 4.

Substrate docking

The binding models for PlsnB with different ligands (5–8) were obtained by using the complex structure of PlsnB and 4 as an initial reference. The binding mode was then optimized with Maestro (Schrödinger, LLC)⁴⁶ which features a minimization routine based on OPLS-2005 Forcefield⁴⁷. A substrate binding model of PvcB was conducted in a similar method using apo PvcB structure (PDB code: 4YLM). The initial position of the substrate were obtained by the superimposition of apo PvcB and PlsnB with substrate complex. The model is then subject to energy minimization in Maestro.

Data availability

Data that support this study are available at the supplementary information. The coordinates are deposited in the Protein Data Bank with PDB accession code 71CL for PlsnB complex with compound 4. The DNA sequences encoding PlsnB, PvcB and PlsnB-Ah are from *Photorhabdus luminescens* (WP_011470371.1), *Pseudomonas aeruginosa* PA0I (AAC21672.1) and *Aeromonas hydrophila* (WP_017765143). Data is available from the corresponding authors upon request.

References

1. Moore, R. E., Cheuk, C. & Patterson, G. M. L. Hapalindoles: new alkaloids from the blue-green alga Hapalosiphon fontinalis. *J. Am. Chem. Soc.* **106**, 6456–6457 (1984).
2. Moore, R. E. et al. Hapalindoles, antibacterial and antimycotic alkaloids from the cyanophyte Hapalosiphon fontinalis. *J. Org. Chem.* **52**, 1036–1043 (1987).
3. Park, A., Moore, R. E. & Patterson, G. M. L. Fischerindole-L, a new isonitrile from the terrestrial blue-green-alga *Fischerella musiciola*. *Tetrahedron Lett.* **33**, 3257–3260 (1992).
4. Smitka, T. A. et al. Ambiguine isonitriles, fungicidal hapalindole-type alkaloids from three genera of blue-green algae belonging to the stigonemataceae. *J. Org. Chem.* **57**, 857–861 (1992).
5. Stratmann, K. et al. Welwitindolinones, Unusual alkaloids from the blue-green algae *Hapalosiphon olsowskii* and westella intrica ta. *J. Am. Chem. Soc.* **116**, 9935–9942 (1994).
6. Bhat, V., Dave, A., MacKay, J. A. & Rawal, V. H. The chemistry of hapalindoles, fischerindoles, ambiguines, and welwitindolinones. Alkaloids *Chim. Biol.* **73**, 65–160 (2014).
7. Takahashi, S. et al. Byelyankacin: a novel melanogenesis inhibitor produced by *-enterobacter* sp. *B20*. *J. Antibiot.* **60**, 717–720 (2007).
27. Duewel, S. et al. Directed evolution of an Fe-II-dependent halo-
25. Islam, M. S., Leissing, T. M., Chowdhury, R., Hopkinson, R. J. &
26. Hayashi, T. et al. Evolved aliphatic halogenases enable regiocom-
24. Ga o ,S .S ., N a o w a r o j n a ,N ., C h e n g ,R ., L i u ,X .&L i u ,P .R e c e n t
14. Micallef, M. L. et al. Comparative analysis of hapalindole, ambiguine
13. Hillwig, M. L. et al. Identi
11. Raffa, N. et al. Dual-purpose isocyanides produced by Aspergillus
22. Bollinger Jr, J. M. et al.
20. Hegg, E. L. Jr L. Q. The 2-His-1-carboxylate facial triad An emerging
15. Hohlman, R. M. & Sherman, D. H. Recent advances in hapalindole-
16. Drake, E. J. & Gulick, A. M. Three-dimensional structures of Pseu-
18. Chang, W.-c et al. In vitro stepwise reconstitution of amino acid
19. Dunham, N. P. et al. Two distinct mechanisms for C-C desaturation
30. Renata, H. Exploration of iron- and a-ketoglutarate-dependent
32. Martinez, S. & Hausinger, R. P. Catalytic mechanisms of Fe(ii) and 2-
33. Huang, X. & Groves, J. T. Beyond ferryl-mediated hydroxylation: 40
28. Cha, L. et al. Alternative reactivity of leucine 5-hydroxylase using an
29. Zwick, C. R. & Renata, H. Harnessing the biocatalytic potential of iron-
30. Renata, H. Exploration of iron- and a-ketoglutarate-dependent
31. Price, J. C., Barr, E. W., Tirupati, B., Bollinger, J. M. Jr. & Krebs, C. The first direct characterization of a high-valent iron intermediate in the reaction of an alpha-ketoglutarate-dependent dioxygenase: a high-spin FeIV complex in taurine/alpha-ketoglutarate dioxygenase (Taub) from Escherichia coli. Biochemistry 42, 7497–7508 (2003).
32. Martinez, S. & Hausinger, R. P. Catalytic mechanisms of Fe(II)- and 2-
33. Li, G. L. et al. Coupling of trifluoromethyl isocyanide ligands in binuclear iron carbonyl complexes. J. Fluor. Chem. 166, 34–44 (2014).
34. Duncan Lyngdoh, R. H., Schaefer, H. F. 3rd & King, R. B. Metal-metal (MM) bond distances and bond orders in binuclear metal complexes of the first row transition metals titanium through zinc. Chem. Rev. 118, 11626–11706 (2018).
35. Till, M. et al. Synthesis and characterization of bidentate isonitrile iron complexes. Organometallics 40, 1042–1052 (2021).
36. Yu, C. P. et al. Elucidating the reaction pathway of decarboxylation-assisted olefination catalyzed by a mononuclear non-heme iron enzyme. J. Am. Chem. Soc. 140, 15190–15193 (2018).
37. Khan, I. et al. Identification and bioactivity evaluation of secondary metabolites from Antarctic-derived Penicillium chrysogenum CCTCC M 2020019. RSC Adv. 10, 20738–20744 (2020).
38. Borowski, T., Brolclawik, E., Schofield, C. J. & Siegbahn, P. E. M. Epimerization and desaturation by carbanepen synthase (CarC). A hybrid DFT study. J. Comput. Chem. 27, 740–748 (2006).
39. Mader, S. L., Brauer, A., Groll, M. & Kaila, V. R. I. Catalytic mechanism and molecular engineering of quinolone biosynthesis in dioxygenase AsqJ. Nat. Commun. 9, 1168 (2018).
40. Wojdyłła, Z. & Borowski, T. On how the binding cavity of AsqJ dioxygenase controls the desaturation reaction regioelectivity: a QM/MM study. J. Biol. Inorg. Chem. 23, 795–808 (2018).
41. Dunham, N. P. et al. Two distinct mechanisms for C-C desaturation by Iron(II)- and 2-Oxo)glutamate-dependent oxygenases: importance of alpha-heterolomato assistance. J. Am. Chem. Soc. 140, 7116–7126 (2018).
42. Liao, H. J. et al. Insights into the desaturation of cyclopeptin and its C3 epimer catalyzed by a non-heme iron enzyme: structural characterization and mechanism elucidation. Angew. Chem. Int. Ed. 57, 1831–1835 (2018).
43. Ghafoor, S., Mansha, A. & de Visser, S. P. Selective hydrogen atom abstraction from dihydroflavonol by a nonheme iron center is the key step in the enzymatic flavonol synthesis and avoids byproducts. J. Am. Chem. Soc. 141, 20278–20292 (2019).
48. Liu, Y., Shi, J. & Liu, Y. Mechanistic insights into the oxidative ring expansion from penicillin n to deacetoxycephalosporin c catalyzed by a nonheme iron(II) and α-KG-dependent oxygenase. Inorg. Chem. 59, 12218–12231 (2020).

49. Ali, H. S., Henchman, R. H., Warwicker, J. & de Visscher, S. P. How do electrostatic perturbations of the protein affect the bifurcation pathways of substrate hydroxylation versus desaturation in the nonheme iron-dependent viomycin biosynthesis enzyme? J. Phys. Chem. A 125, 1720–1737 (2021).

50. Wojdyła, Z. & Borowski, T. Properties of the reactants and their interactions within and with the enzyme binding cavity determine reaction selectivities. The case of Fe(II)/2-oxoglutarate dependent enzymes. Chem. Eur. J. 28, e202104106 (2022).

51. Otwinowski, Z. & Minor, W. Processing of X-ray diffraction data collected in oscillation mode. Methods Enzymol. 276, 307–326 (1997).

52. Liebschner, D. et al. Macromolecular structure determination using X-rays, neutrons and electrons: recent developments in Phenix. Acta Crystallogr. D. Struct. Biol. 75, 861–877 (2019).

53. Emsley, P. & Cowtan, K. Coot: model-building tools for molecular graphics. Acta Crystallogr. D. Biol. Crystallogr. 60, 2126–2132 (2004).

54. Schrödinger. Release 2020-1: Maestro. (Schrödinger, LLC, 2020).

55. Shivakumar, D. et al. Prediction of absolute solvation free energies using molecular dynamics free energy perturbation and the OPLS force field. J. Chem. Theory Comput. 6, 1509–1519 (2010).

56. Kaminski, G. A., Friesner, R. A., Tirado-Rives, J. & Jorgensen, W. L. Evaluation and reparametrization of the OPLS-AA force field for proteins via comparison with accurate quantum chemical calculations on peptides. J. Phys. Chem. B 105, 6474–6487 (2001).

Acknowledgements
This work was supported by the grant from the National Institutes of Health (GM127588 to W.-c.C. and GM104896 and 125882 to Y.Z.) and the Goodnight Early Career Innovator. The authors thank R.Y. Moreno for helpful edits. Crystallographic data collections were conducted at advanced photon sources (BL23-ID-B), Department of Energy (DOE) national user facility.

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W.K., G.Z., and K.X. performed crystallization and X-ray diffraction studies, T.-Y.C., L.C., and N.K.C. performed the biochemical assays and prepared substrate analogs and product standards. Y.Z. and W.-c.C. wrote the manuscript with input from all coauthors.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-022-32870-4.

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Peer review information Nature Communications thanks Yongjun Liu and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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