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Biocatalysis **Hot Paper**

Radical Ring-Opening Reaction of Non-Activated Oximes Catalyzed by Aldoxime Dehydratases

Shunsuke Kato, Haruka Nishiwaki, Keiji Endo, and Takashi Hayashi**

Abstract: The iminyl radical is a distinctive *N*-centered radical which serves as a versatile synthon in preparation of nitrogen-containing compounds. In principle, iminyl radicals can be directly generated by single electron reduction of oximes through elimination of OH group. However, due to the low reactivity of the oxime N─OH bonds, direct conversion of the oximes does not proceed efficiently, thereby enforcing chemical activation of the oxime OH group which results in the formation of stoichiometric by-products. To overcome this problem, we are developing a new biocatalytic system using aldoxime dehydratases. Through a series of enzyme screenings, we identified an aldoxime dehydratase from *N. simplex* (NsOxd) which is capable of catalyzing iminyl radical-mediated ring-opening reactions. Notably, NsOxd efficiently converts the "non-activated" 2-phenylcyclobutanone oxime within 10 min under ambient conditions and quantitatively produces the corresponding γ-sulfinylated nitrile in >95% yield. This enzyme activity is even faster than that of previously-reported chemo-catalysts. Furthermore, evaluation of the scope of potential substrates indicates that NsOxd has a versatile N─O bond cleaving activity which efficiently generates iminyl radicals from various "non-activated" oximes. These findings highlight the utility of aldoxime dehydratases for managing the reactivity of "non-activated" oximes and for achieving challenging iminyl radical-mediated catalytic reactions.

Nitrogen-centered radicals are highly reactive and useful synthetic intermediates in modern organic chemistry.[1–3] In particular, iminyl radicals undergo various chemical transformations, such as radical addition to C=C bonds,[4–8] β-fragmentation for radical ring-opening reactions,[9–14] and hydrogen atom abstraction for C─H functionalization,[15–17] therefore providing practical synthetic routes to a wide range of nitrogen-containing compounds.[18–20] In general, these iminyl radicals are generated through one-electron oxidation or reduction of oxime derivatives activated by redox auxiliaries (Figure 1a, left), such as *O*-acyloxime esters (R=COAr) and *O*-aryl oxime ethers (R=Ar), with the aid of photoredox catalysts or transition metal catalysts (Figure S1).[19]

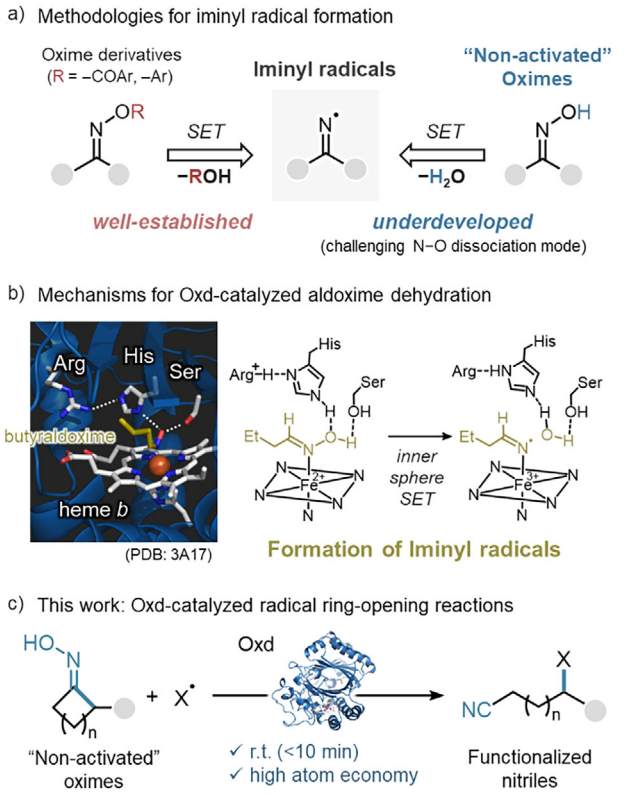

Figure 1. a) Methodologies for the catalytic formation of the iminyl radicals. b) Crystal structure of aldoxime dehydratase with butyraldoxime (PDB: 3A17) and a proposed mechanism for the aldoxime dehydration. c) Biocatalytic radical ring-opening reactions of "non-activated" oximes investigated in this study.

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However, direct generation of the iminyl radicals from "non-activated" oximes (R═H) remains challenging even though they offer significant advantages in terms of atom economy and the simplicity of synthetic procedures (Figure 1a, right). The main reason for this difficulty is due to the fact that the non-activated oximes are less redox active than the oxime derivatives and the facile dissociation mode of the oxime O–H bond competes with the desired N─O bond cleavage pathway. Although a few progressive reaction systems have been recently reported to overcome the low reactivity of non-activated oximes (Figure S2),[21,22] there is still room for improvement in the catalytic efficiency of these reaction systems.

To overcome this challenge, we considered the use of a class of enzymes known as aldoxime dehydratases (Oxds) for the catalytic generation of iminyl radicals. Oxds are members of heme-dependent enzymes which catalyze the dehydration of aldehyde oximes to produce nitriles.[23–26] This enzymatic dehydration is proposed to proceed via a stepwise mechanism involving an inner-sphere single electron transfer (SET) process that generates an iminyl radical intermediate (Figures 1b and S3).[27] Since amino acid residues in the enzyme active site can selectively protonate the OH group of aldehyde oximes, the subsequent inner-sphere SET allows the formation of iminyl radicals from the non-activated aldehyde oximes. Taking advantage of this enzyme mechanism, we are investigating Oxds for abiotic radical ring-opening reactions of "non-activated" cycloketone oximes (Figure 1c). Although aldehyde oximes are the only known substrates for Oxds,[28] we expected that the enzymes could also promote the formation of iminyl radicals from non-activated cycloketone oximes, followed by subsequent β-fragmentation, which leads to a new-to-nature catalytic modes to generate γ- and ε-substituted nitriles depending on the size of cycloketone oxime ring (Figure S4).

Based on this hypothesis, we set out to investigate the radical ring-opening reaction using 2-phenylcyclobutanone oxime (**1a**) as a model substrate for the "non-activated" oxime. Oxd from *Rhodococcus erythropolis* (ReOxd), one of the most well-studied aldoxime dehydratases,[27–30] was first selected as a catalyst for the reaction and recombinantly expressed following the protocol of our previous report (Figure S5).[31] The reaction was performed with 0.17 mol% loading of ReOxd in the presence of $Na_2S_2O_4$ as a reducing and radical terminating reagent.[32,33] Consequently, ReOxd was found to show promising activity for the ring-opening of the non-activated oxime **1a** as we expected, and the γ-sulfinylated nitrile **2a** was generated as a single product as demonstrated by LC-MS measurements (Figure S6). The exact chemical structure of product **2a** was confirmed by a series of NMR experiments (Figures S7–S9). The NMR chemical shifts of enzymatically-produced **2a** are completely consistent with the chemical shifts of the chemically-synthesized authentic sample (Figure S10).[34] The γ-sulfinic acid group of product **2a** is considered to be formed via radical termination by the sulfur dioxide radical anion derived from $Na_2S_2O_4$ (Figure S11).[32,33]

Encouraged by these promising results exhibited by ReOxd, we next performed protein database mining to discover a novel Oxd with higher catalytic activity for this radical ring-opening reaction. A sequence similarity network (SSN) was first generated using the EFI–EST web tool[35,36] to visualize the relationship of bacterial Oxds in the UniProtKB database, and 18 enzymes which are widely distributed across the entire SSN were selected for the activity screening (Figure 2a, Table S1). Interestingly, higher radical ring-opening activity was observed for several specific Oxds located at the same cluster in the SSN. In particular, Oxd from *Nocardioides simplex* (NsOxd) and Oxd from *Pseudomonas parafulva* (PpaOxd), which have 70% and 74% sequence identity to ReOxd, respectively, produce the desired product **2a** in highest yield (Figures 2b and S12). Under modified reaction condition with 0.5 mol% catalyst loading, NsOxd and PpaOxd quantitatively convert "non-activated" substrate **1a** within 1 h, and the ring-opened product **2a** is obtained in >95% yield (>190 TON) (Figures 3 and S13). Chiral HPLC analysis revealed that γ-sulfinylated nitrile **2a** was obtained as a racemic mixture (Figure S14). Since the carbon-centered

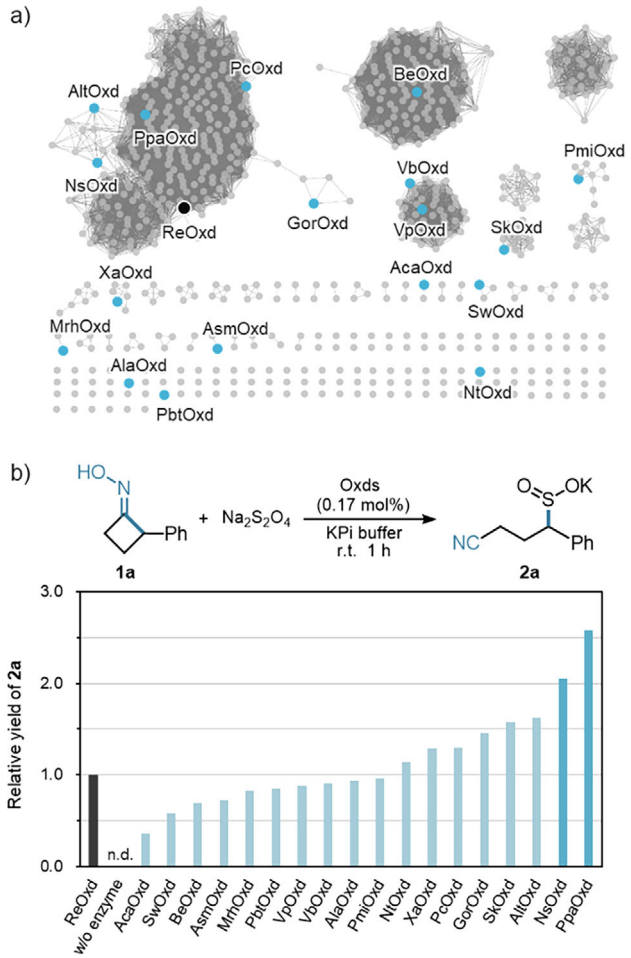


Figure 2. a) SSN of aldoxime dehydratases generated by EFI-EST. Query sequence: ReOxd, database: UniProt, Node: 718, Edge: 18742 (>75% identity). The 18 enzymes selected for the screening are highlighted in cyan circles. b) Screening results of the selected Oxds for the radical ring-opening reaction. Reaction conditions: Oxds (3.3 μM, 0.17 mol%), **1a** (2.0 mM), $Na_2S_2O_4$ (5.0 mM) in KPi buffer (100 mM, pH 7.5) containing DMF (2.0 *v/v*%), 25 °C, 1 h under N_2.

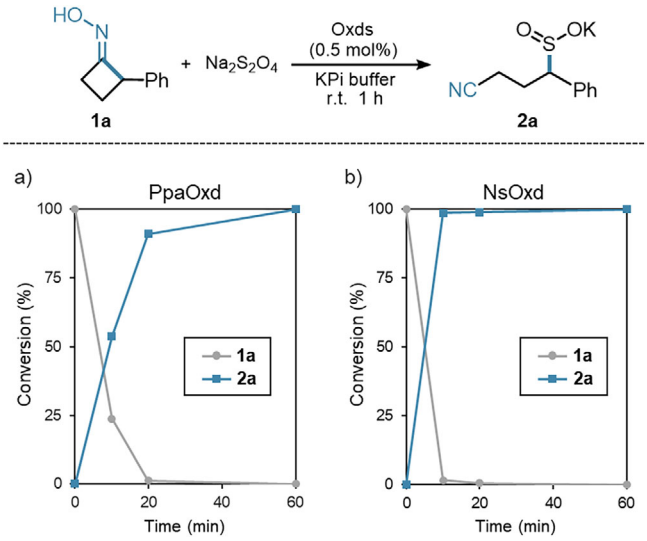
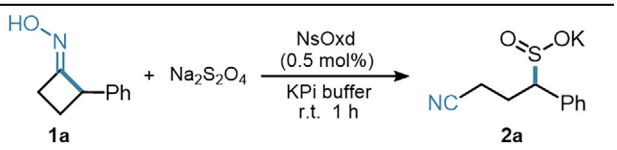


Figure 3. Time course plots for consumption of **1a** (gray) and formation of **2a** (blue) catalyzed by a) PpaOxd and b) NsOxd. Reaction conditions: Oxd (10 μM, 0.50 mol%), **1a** (2.0 mM), $Na_2S_2O_4$ (5.0 mM) in KPi buffer (100 mM, pH 7.5) containing DMF (0.80 *v*/*v*%), 25 °C under N_2. Conversion is determined by LC-MS and ^{1}H NMR.

Table 1: Oxd-catalyzed radical ring-opening reaction.[a]



Entry	Catalyst	Yield (%)[b]
1	NsOxd	>95
2	hemin	Trace[c]
3	myoglobin	Trace[c]
4	NsOxd(R178A)	15
5	NsOxd(S219A)	14
6	NsOxd(H320A)	26
7	NsOxd in the presence of 10 mM TEMPO	n.d.[c]

[a] Standard conditions: catalyst (10 μM, 0.50 mol%), **1a** (2.0 mM), $Na_2S_2O_4$ (5.0 mM) in KPi buffer (100 mM, pH 7.5) containing DMF (0.80 *v*/*v*%), 25 °C under N_2. [b] Determined by ^{1}H NMR. [c] Determined by LC-MS.

radicals are generated at a position distant from the heme iron center, the chiral environment of NsOxd may not effectively contribute to the C–S bond formation process involving radical termination. However, it should be noted that the catalytic efficiencies of these two Oxds are comparable to those of chemo-catalytic systems using photoredox catalysts or transition metal catalysts. In particular, NsOxd completes the reaction within 10 min by manipulating the reactivity of "non-activated" ketoximes. To the best of our knowledge, this is even faster than the reaction rates of previously-reported chemo-catalysts (Figures 3b and S2).[21,22] Furthermore, we performed the ring-opening reaction catalyzed by NsOxd on a larger scale. Although partial oxidation of the sulfinic acid occurred during column chromatography, substrate **1a** (0.10 mmol) was efficiently converted into **2a** in 81% isolated yield (Figure S15). Considering these results, NsOxd was selected as the best-performing catalyst for the radical ring-opening reactions.

We carried out a series of control experiments to investigate the catalytic properties of NsOxd (Table 1). In contrast to the high catalytic activity of NsOxd, only trace amounts of **2a** are produced when hemin is used alone as a catalyst (Table 1, entry 2). Myoglobin, a representative example of other heme proteins with an axial histidine ligand similar to the axial ligand of Oxd, showed little catalytic activity (Table 1, entry 3). These results clearly demonstrate the importance of the well-ordered active site of Oxds for promotion of the ring-opening reaction. In addition, site-directed mutagenesis at the conserved R178, S219, and H320 residues in the NsOxd active site significantly reduces the yields of **2a** (Table 1, entry 4–6). These amino acid residues have been suggested to be involved in the reaction mechanism as general acid–base catalysts to manage the reactivity of "non-activated" oximes (Figure S16).[27] We next performed radical trap experiments with the TEMPO free radical. The formation of γ-sulfinylated product **2a** is completely suppressed in the presence of TEMPO (Table 1, entry 7), and the adduct of TEMPO with **1a** is detected by LC-MS (Figure S17). These results clearly support the envisioned radical mechanism for the ring-opening reaction.

Next, the substrate scope of the radical ring-opening reaction was investigated using NsOxd as a catalyst (Figure 4). We first examined the influence of substituents on the aromatic ring of 2-arylcyclobutanone oximes. Substrates **1b**–**1d**, which have electron-withdrawing groups at the *para*-position, are efficiently converted into the corresponding γ-sulfinylated nitriles **2b**–**2d** in high yields. These substrates with chloro, trifluoromethyl, and ester groups are found to be suitable for the present biocatalytic system, and total TONs of the reaction are greater than 100. In contrast, substrates **1e** and **1f**, which have an electron-donating group, have low TON values. Under increased catalyst loading conditions (up to 1.5 mol%), NsOxd affords **2e** and **2f** in 44% and 64% yields, respectively. Considering the fact that 4-aryl-3-butenenitriles were generated as side-products during the catalysis of **1e** and **1f** (Figure S18), the electron-donating substituents may promote unfavorable side reactions which proceed via the formation of carbocation intermediates (Figure S19). In addition to the *para*-substituents, 2-arylcyclobutanone oximes **1g** and **1h**, which have *meta*- and *ortho*-substituents, are efficiently converted into the desired products **2g** and **2h** in good yields with high TONs, highlighting the broad substrate promiscuity of NsOxd for the ring-opening reaction. Interestingly, 2-benzylcyclobutanone oxime (**1i**) was also found to be compatible with the present biocatalytic system even though the reaction proceeds via a non-benzylic secondary alkyl radical intermediate. Under high catalyst loading conditions, 4-cyano-1-phenylbutane-2-sulfinate (**2i**) was obtained in 43% yield. In the reaction of oxetan-3-one oxime (**1j**) and *N*-Boc-3-azetidinone oxime (**1k**), aminosulfonates **3j** and **3k** were produced in moderate yields instead of the ring-opened products. This suggests that the iminyl radicals derived from

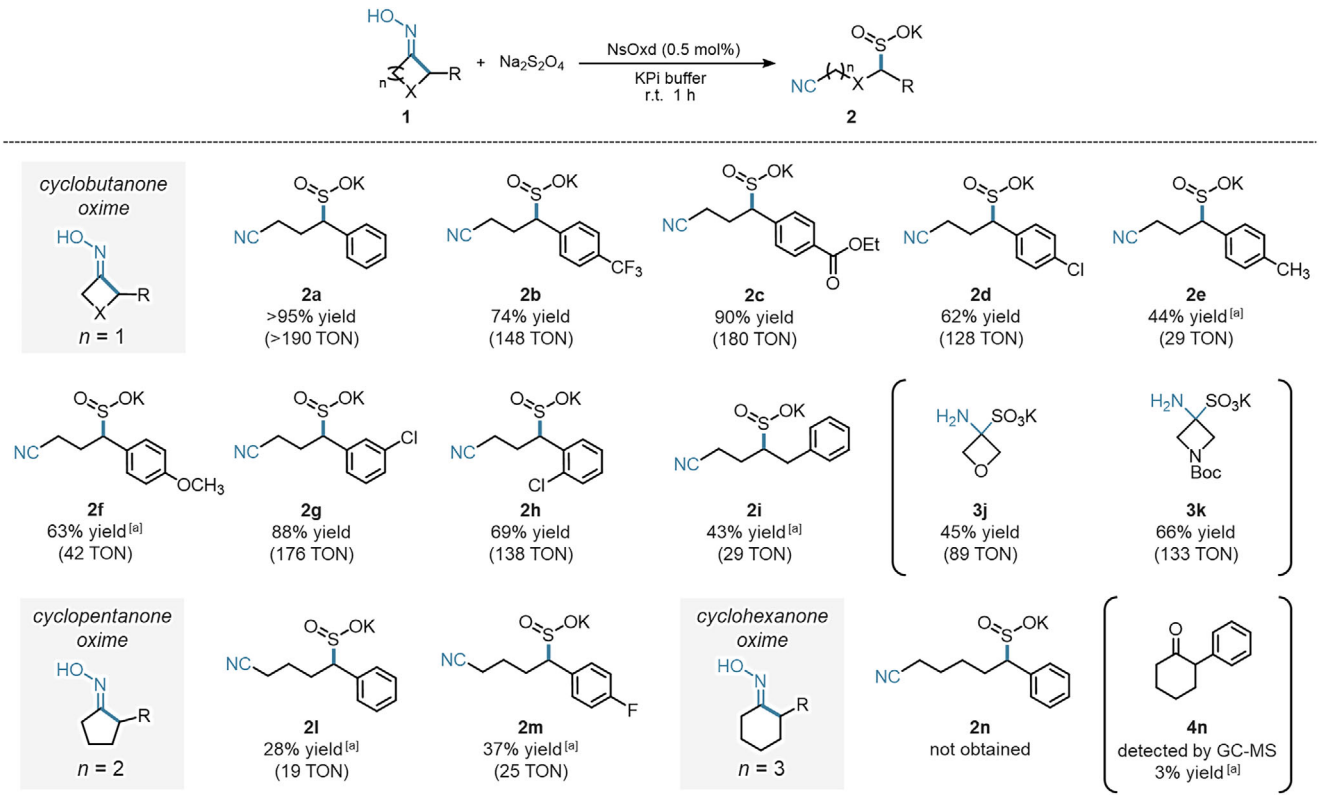


Figure 4. Substrate scope for the NsOxd-catalyzed radical ring-opening reaction. Reaction conditions: NsOxd (10 μM, 0.50 mol%), cyclic oximes 1 (2.0 mM), $Na_2S_2O_4$ (5.0 mM) in KPi buffer (100 mM, pH 7.5) containing DMF (0.80 *v/v*%), 25 °C under N_2. Yields were determined by ^{1}H NMR using sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4 as an internal standard. a) NsOxd (30 μM, 1.5 mol%).

these substrates undergo a one-electron reduction process to form imines rather than following a β-fragmentation process which generates primary alkyl radicals (Figure S20). Since the kinetically unstable *N*-unsubstituted imine intermediates with four-membered ring structures are difficult to synthesize from the corresponding ketones and ammonium salts,[37] the catalytic activity of NsOxd to produce **1j** and **1k** is also noteworthy (Figures S21 and S22). In the scale-up experiment, aminosulfonate **3k** was obtained in 18% isolated yield, with the lower yield partly attributable to difficulties encountered during the purification process (Figure S23).

Moreover, NsOxd exhibits promising catalytic activity toward the ring-opening reactions of 2-arylcyclopentanone oximes **1l** and **1m** with a five-membered ring structure. The ε-sulfinylated nitriles **2l** and **2m** are produced in 28% and 37% yields, respectively. In contrast, NsOxd has no ring-opening activity toward cyclohexanone oxime **1n** (Figure S24). Similarly, 2,2-dimethyl- and 2-carbethoxycyclopentanone oximes did not produce any ring-opened products (Figures S25 and S26). The ring strain energies of cycloketone oximes and the stability of radical intermediates are considered to be crucial for promoting the ring-opening reactions. Interestingly, instead of ring-opened products, NsOxd was found to produce ketone and enamine products from these substrates (Figures S24–S26). Since these products were not formed in the absence of NsOxd, these ketone and enamine products appear to be obtained through the formation of imines which are enzymatically generated via the reduction of the iminyl radical intermediates (Figure S27). Furthermore, similar ketone products were also obtained in the reaction of "acyclic" ketoximes, such as 2-hexanone oxime (Figure S28). Notably, the yield of 2-hexanone reaches 65%. Such high reactivity of acyclic ketoxime could be due to the small steric hindrance around the oxime nitrogen atom which coordinates to the heme iron center (Figure S29). Taken together, these results suggest that NsOxd has a versatile N—O bond cleaving activity to provide the iminyl radicals from a wide range of "non-activated" oxime substrates, regardless of their backbone structures. Considering these versatile activities of NsOxd, the iminyl radical chemistry provided by Oxds has great potential for application in the ring-opening reactions as well as other attractive reactions, such as radical cyclization and C—H functionalization.[18,20]

In summary, we have developed a novel biocatalytic system for the iminyl radical-mediated ring-opening reaction of "non-activated" oximes based on the catalytic mechanism of aldoxime dehydratases. Through enzyme screening using SSN analysis, aldoxime dehydratase from *N. simplex* (NsOxd) was successfully identified as the best-performing catalyst capable of catalyzing the radical ring-opening reaction of non-activated cyclobutanone/cyclopentanone oximes. Notably, the reaction of 2-arylcyclobutanone oxime **1a** is completed within 10 min under ambient conditions, and NsOxd quantitatively provides γ-sulfinylated nitrile **2a** in

>95% yield. This remarkable catalytic activity of NsOxd is even faster than the reaction rate of previously-reported chemo-catalytic systems.[21,22] Furthermore, evaluation of the substrate scope reveals that NsOxd has additional versatile catalytic activities which efficiently generate iminyl radicals from various "non-activated" oxime substrates. Given the significant recent progress in the field of biocatalysis,[38–42] the present NsOxd-based biocatalytic system is expected to provide a powerful synthetic tool to manage the reactivity of "non-activated" oximes and to promote challenging catalytic reactions mediated by the iminyl radicals.

Supporting Information

Supplementary method for sample preparation, experimental details, analytical data, amino acid, DNA sequence, and characterization data. The authors have cited additional references within the Supporting Information.[43–47]

Acknowledgements

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the Supporting Information of this article.

Keywords: Aldoxime dehydratase · Biocatalysis · Hemoproteins · Iminyl radicals · Oximes

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