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N-oxygenation of amino compounds: early stages in its application to the biocatalyzed preparation of bioactive compounds

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Abstract

Among the compounds that contain unusual functional groups, nitro is perhaps one of the most interesting due to the valuable properties it confers on pharmaceuticals and explosives. Traditional chemistry has for many years used environmentally unfriendly strategies; in contrast, the biocatalyzed production of this type of products offers a promising alternative. The small family of enzymes formed by *N*-oxygenases allows the conversion of an amino group to a nitro through the sequential addition of oxygen. These enzymes also make it possible to obtain other less oxidized N-O functions, such as hydroxylamine or nitroso, present in intermediate or final products. The current substrates on which these enzymes are reported to work encompass a few aromatic molecules and sugars. The unique characteristics of *N*-oxygenases and the great economic value of the products that they could generate, place them in a position of very high scientific and industrial interest. The most important and best studied *N*-oxygenases will be presented here.

Keywords
N-oxygenases; *N*-oxygenation; monooxygenases; nitro compounds.

1. Introduction

A nitro compound can be defined as any organic compound that contains a nitro group (NO_2) in its structure. These compounds can be aliphatic or aromatic, the latter being those of greatest industrial interest. Nitrogen is positively charged due to the union of both oxygens, leaving it with electron deficiency. When attached to an aromatic ring it can delocalize electrons from the π orbitals to satisfy its electronic imbalance, which not only causes the molecule to polarize but also to show unique properties.
Nitro compounds are of great interest due to the high level of bioactivity that they display, being among them antibiotics, antiparasitics, antitumoral, food additives, fragrances and flavorings (Mügge et al., 2020). In addition, nitro group is present in substances with high added value such as explosives,

pigments and pesticides (Winkler and Hertweck, 2007). The +3 oxidation state of nitrogen in nitroarenes allows their self-oxidation, which generates a rapid release of heat in the form of an explosion (Cohen et al., 2007). Trinitrotoluene (TNT), the most widely used explosive in military and mining, is the main example. It was produced in very large quantities during the WWI and WWII, but today its preparation is strongly controlled due to the environmental pollution it generates. Picric acid is used in both explosives and dyes, although their use decreased (Lee, 1998). The most common dyes are anilines, whose synthesis consists in the hydrogenation of nitrobenzene precursors. Some nitrated building blocks stand out in the pesticide industry. Nitrophenol is the starting material for the synthesis of the herbicides fluorodiphene and bifenox, and insecticides like parathion and carbofuran. Nitro groups can also be found in building blocks of many pharmaceuticals. Substituted nitrobenzenes and nitropyridines were used in the production of indole derivatives, which showed important bioactivity as drugs and agrochemicals (Li and Cook, 2005). Antipsychotic drugs such as phenothiazine derivatives can be generated from halonitrobenzenes (Gritsenko, A.N., Ermakova, Z.I. & Zhuravlev, 1970), while chloronitrobenzene is useful in the synthesis of the analgesic anpirtolin (Rádl et al., 1999). Two over-the-counter drugs, paracetamol and lidocaine, use *p*-nitrophenol and 2,6-dimethylnitrobenzene in their synthesis (Bhattacharya et al., 2006).

Chemical synthesis of nitro compounds is dangerous for the environment, expensive and difficult to perform. The classical method of aromatic nitration, which requires the use of excessive amounts of nitric acid and other concentrated acids such as sulfuric acid, is widely used in industrial production, despite releasing large amounts of nitrogen oxides and acids as waste. These compounds are dangerous both for the environment and human health. Nitrogen oxide cause genetic mutations and respiratory diseases. Nitric acid is highly reactive with animal and plant tissues; it produces corrosive burns and serious respiratory problems. On the other hand, sulfuric acid remains in the air and can easily spread in the environment and in water. It generates irritations on the skin and mucosa -especially the lungs-, in addition to being carcinogenic (Ju and Parales, 2010).

This procedure also brings production disadvantages. Mixtures of *para*, *ortho* and *meta* isomers are always generated, the proportions of which can vary widely according to small changes in temperature and the substituent groups on the benzene ring. The characteristics of the substituents also affect the success of nitration.

Less polluting and more regioselective catalytic methods have been sought. A possible strategy involves the use of acids in solid state, since they can be removed with better control and can be reused, such as Nafion resins (Samajdar et al., 2000). To improve regioselectivity in *para* the use of copper nitrate in montmorillonite K-10 has been proposed; it was reported to raise the conversion rate to 70%. β-zeolite is another option with better results (Ono, 2003). Although they are compelling alternatives, the increase in cost and changes in the line of production in order to use them in industrial processes are restrictive. Eco-friendly and less harmful alternatives are still under development or present various practical difficulties such as di or polynitration and low regioselectivity. Biocatalysis emerged as an attractive option to overcome these drawbacks, generating also a low environmental impact and simplified processes.

Besides the nitro group, other *N*-oxygenated functions such as hydroxylamine, isoxazolidine, oxime, nitrone, and nitroso are also found in natural products. The

syntheses of compounds containing these functional groups present experimental problems such as lack of selectivity or over-oxidation, among others, which could also be solved using *N*-oxygenases as biocatalysts. **Figure 1** shows representative natural products containing N-O linkage in their structures.

2. Biocatalyzed synthesis of *N*-oxygenated compounds

Although the biosynthetic pathways of some bioactive nitro compounds are described in the literature, the actual knowledge available regarding the specific enzymes that have the ability to generate hydroxylamine, nitroso and nitro compounds is relatively scarce. Direct nitration has few available examples, although it is no less interesting for that. The enzymes TxtE from *S. scabies* (Barry et al., 2012)and RufO from *S. atratus* (Tomita et al., 2017) are well characterized, and are involved in the biosynthesis of thaxtomin and rufomycin respectively. In contrast, both nitropropionic acid and nitroglycine occurrence is well documented but its biosynthetic pathways are still unknown (Sulzbach and Kunjapur, 2020). Within the compounds generated by direct nitration, the biosynthetic pathway of nitrosuccinic acid has been conveniently elucidated. It is produced by CreE from *Streptomyces cremeus* as part of the biosynthesis of the antibiotic cremomicyn (Sugai et al., 2016). The differential characteristic of the enzymes involved in direct nitration is that they use oxygen and nitric oxide as cosubstrates.

On the other hand, two groups of enzymes, haloperoxidases and *N*-oxygenases, have been reported to produce nitro compounds from amines. Haloperoxidases, which perform the oxygenation through the use of reactive oxygen species, catalyze the oxidation step from an Ar-NH_2 precursor to its nitro derivate (Hollmann et al., 2011). Two examples are the oxidation of aniline to nitrobenzene by a bromoperoxidase from *Pseudomonas putida* and the *N*-oxidation of the amino precursor of the antibiotic pyrrolnitrin by a chloroperoxidase present in *Pseudomonas pyrrocinia* (Kirner and van Pée, 1994). However, these reactions have only been successfully performed under artificial, specific and extreme conditions (in vitro at low pH, excess of H_2O_2 and/or in the absence of halide ions) that induce the formation of reactive oxygen species (Ju and Parales, 2010).

On the other hand, *N*-oxygenases are a group of enzymes whose oxygenation mechanism involves the sequential addition of molecular oxygen to an amino compound (Winkler and Hertweck, 2007). NMR studies of nitropropionate biosynthesis using labeled oxygen (^{18}O) and nitrogen (^{15}N) in the mid-1980s showed that both oxygen atoms came from molecular oxygen (Baxter and Greenwood, 1986).

Probably the best known examples of *N*-oxygenases are AurF from *Streptomyces thioluteus*, involved in aureothin biosynthesis (Chanco et al., 2014; He and Hertweck, 2004), KijD3 form *Actinomadura kijaniata*, which participates in the biosynthetic pathway of the antibiotic kijanimicin (Thoden et al., 2013), and CmlI from *Streptomyces venezuelae*, involved in chloramphenicol biosynthetic pathway (Lu et al., 2012). These enzymes are commonly called genuine, true or authentic *N*-oxygenases (Winkler and Hertweck, 2007).

It is known that the production of many bioactive compounds by *Streptomyces* secondary metabolic pathways is regulated by *Streptomyces* antibiotic regulatory proteins (SARPs) (Spasic et al., 2018). Enzymes associated with these secondary metabolic pathways that involve the sequential addition of

molecular oxygen can also generate partial oxygenations. Such is the case of those involved in the production of valanamycin, an azoxy compound generated by *Streptomyces viridifaciens* that has shown antitumor and antibiotic properties (Garg and Parry, 2010).
The metabolic pathway corresponding to chloramphenicol biosynthesis was reported in full (Lu et al., 2012), where two enzymes carry out *N*-oxygenations independently. CmlA, which is a diiron monooxygenase responsible for a β-hydroxylation of 4-aminophenylalanine (Kaniusaite et al., 2019) and CmlI that is an *N*-oxygenase responsible for the independent six-electron oxidation into nitroaryl group (Makris et al., 2015).
Partial amino oxygenations also occur in the secondary metabolic pathway of calicheamicin biosynthesis in *Micromonospora echinospora*. Through CalE10 oxygenase, a single oxygenation step produces a hydroxylamine group starting from an amine (Kersten and Dorrestein, 2010). Likewise, it has been reported the ability of the flavoenzyme XiaK to produce an *N*-hydroxylated derivative from the indolosesquiterpene Xiamicyn A (Zhang et al., 2017) and the fungal flavin oxygenase OxaD, involved in a nitrone generation from an amine in the biosynthesis of roquefortine L (Teufel, 2017).
In the same sense, other *N*-hydroxylations have been reported in metabolic intermediates of bioactive compounds, mainly siderophore groups associated with virulence factors. Such is the case of ornithine hydroxylases as SidA from *Aspergillus fumigatus* (Chocklett and Sobrado, 2010), PdvA from *Pseudomonas aeruginosa* (Olucha and Lamb, 2011) and MbtC from *Burkholderia mallei* (Franke et al., 2013) as well as the lysine hydroxylases as IucD from *Escherichia coli* (Meneely and Lamb, 2007) and NbtG from *Nocardia farcinica* (Binda et al., 2015). These *N*-oxygenases are members of the class B flavin monooxygenases, are NADPH-dependent and have the ability to hydroxylate the primary amines of L-amino acid side chain, being very specific for their substrates.
An enzyme implicated in another partial *N*-oxygenation is RubN8 from *Streptomyces achromogenes* var. rubradiris, involved in the biosynthesis of rubradyrine and which has shown the ability to carry out the nitration of aliphatic aminosugars. This enzyme generates a nitroso intermediate that is then presumably spontaneously oxidized to nitro (Kersten and Dorrestein, 2010; Lin et al., 2013). An analogous case is that of the enzyme AzoC, which is part of the azomycin biosynthesis cluster in *Streptomyces chattanoogensis*, this enzyme catalyzes the oxidation of the amino group to a nitroso one (Guo et al., 2019).

3. True *N*-Oxygenases: enzymes and whole cells as *N*-oxygenation biocatalysts

As previously mentioned, true *N*-oxygenases could be defined as those enzymes that have the ability to generate nitro groups from amines, making a sequential addition of oxygen atoms.
The large group of enzymes that comprise oxygenases belongs to the oxidoreductases family, and can oxidize molecules by introducing a single oxygen atom (monooxygenases) or two oxygen atoms (dioxygenases). *N*-oxygenases revised in this review are monooxygenases that can be classified according to the cofactor they use to activate oxygen, and include non-heme diiron oxygenases (as AurF, CmlI), Rieske/mononuclear iron oxygenases (as PrnD) and flavin-dependent monooxygenases (as KijD3, DnmZ).
Non-heme diiron oxygenases encompass an enzyme group containing a non-heme di-nuclear iron center that plays a central role in the generation of small

bioactive molecules. They are capable of catalyzing a wide-range of tough oxidative/oxygenative reactions, including hydroxylation of non-activated carbon centers, desaturation of alkanes, *N*-oxygenation and epoxidation of alkenes (Dong et al.,2019). Rieske non-heme mononuclear iron oxygenases is a vast group of oxidative enzymes that are usually involved in degradation reactions of xenobiotic compounds (Bruijnincx et al., 2008), but their biosynthetic activity has also been reported (Barry and Challis, 2013). These enzymes have two domains, one contains a Rieske type center [2Fe-2S] and the other a non-hemic mononuclear iron (Koehntop et al., 2005). Flavin-dependent enzymes have aroused interest as they participate mainly in the biosynthesis of *N*-oxidized aminosugars, including hydroxylamine, nitroso and nitro sugars (Al-Mestarihi et al., 2013), which are key building structures for the synthesis of antibiotics such as kijanimicin and everninomycin (Lin et al., 2013; Waldman et al., 2017). Probably the most studied examples of the true *N*-oxygenation reaction are related to the enzymes AurF and CmlI and involve the *N*-oxygenation of arylamines (Komor et al., 2018). In both cases, the proposed reaction mechanisms include the sequential addition of oxygen, probably through a hydroxylated intermediate (Waldman et al., 2017)

As far as is known, the wide variety of enzymes involved in the production of biomolecules associated with secondary metabolisms (especially nitro compounds) are linked to complex regulatory systems in vivo (Onaka, 2017). The delicate regulation of the production of these metabolites is affected by a large and not always determined number of environmental and nutritional factors and even the presence of other microorganisms. Added to this are specific or global regulatory pathways, which can even be self-regulated, with functions related to hormones (Liu et al., 2013). One consequence of this, particularly for *N*-oxygenases, is that they usually exhibit high substrate specificity (Winkler and Hertweck, 2007). However, studies carried out with CmlI and AurF showed that, although heterologous expression or overexpression in native cells is difficult, its use as biocatalyst is viable (Fryszkowska and Devine, 2020).

In this sense, systems of two or more bacteria have recently been reported in which their interactions enhance the biosynthetic pathways involved in the synthesis of nitro compounds, leaving the bacteria probably in a resting metabolic state, achieving good yields without engineering intervention (Nóbile et al., 2020).

True *N*-oxygenases also make it possible to obtain other less oxidized N-O functions, such as hydroxylamine or nitroso, present in intermediate or final products (Barreiro and Martínez-Castro, 2019; Onaka, 2017; Spasic et al., 2018; Xia et al., 2020). The most important and best studied enzymes to date, summarized in **Table 1**, are described below.

3.1. AurF

AurF is a non-heme diiron monooxygenase (Hedges and Ryan, 2019), being the best studied true *N*-oxygenase of its class to date. In vivo, AurF catalyzes the generation of *p*-nitrobenzoic acid (pNBA), a vital starter unit in the synthesis of aureothin (Winkler and Hertweck, 2007). This antibiotic and antiviral compound was first reported in *Streptomyces thioluteus*, where the enzyme complex that produces it was discovered (He and Hertweck, 2004) . As part of this metabolic pathway AurF provides pNBA, the necessary substrate for aureothin polyketide synthase to proceed towards aureothin production (Komor et al., 2018).

For biocatalytic purposes, this enzyme has been studied in order to optimize its reaction parameters in vitro and has shown to have a remarkable regiospecificity. Chanco and coworkers determined, for AurF from *S. thioluteus,* an unusual optimum pH of 5.5, kinetical parameters as well as its substrate acceptance (Chanco et al., 2014). In this regard, it was observed that substituents in the 2 position of its natural pABA substrate, such as the $-NH_2$, -OH or $-CH_3$ groups, and the replacement of the carboxyl group by carboxymethyl or sulfonate groups were not detrimental to the activity, maintaining a strong preference for the presence of the amino group in position *para* with respect to the acid group (Winkler and Hertweck, 2005). It was also reported that AurF does not accept secondary amines, nor derivatives with substituents at position 3 or with bulky substituents at any position as substrates. Contrary to the finding by the Chanco group that AurF is highly specific towards arylamines in which a negatively charged substituent occupies the *para* position relative to the amino group, Platter and coworkers reported that AurF was capable of carrying out the *N*-oxygenation of 5-substituted aminophenols, expanding the range of available substrates (Platter et al., 2011).

The elucidation of the AurF mechanism and the determination of the intermediates, confirmed that the oxygen atoms of the nitro group derived from molecular oxygen (Cutsail et al., 2020). By the stepwise addition two oxygen atoms from two oxygen molecules, in three successive $2e^-$ steps at the same active site, the amino group of the substrate forms a hydroxylamine intermediate which, after conversion by dehydrogenation into a nitroso group, is then biotransformed into nitro with the addition of another oxygen, as was first demonstrate by Hertweck group (Winkler and Hertweck, 2005). As a consequence of the presence of the nitroso group as an intermediary of this biotransformation, the non-enzymatic formation of azoxybenzene derivatives by the condensation of hydroxylamines with nitroso compounds or by the dimerization of nitrosobenzenes has been observed. This finding has contributed not only to corroborate the proposed mechanism but also to elucidate the biosynthesis of other natural compounds such as malleobactin D (Franke et al., 2013) The complete mechanism for the synthesis of pNBA from pABA is shown in **Figure 2**.

AurF is formed by two subunits, with two asymmetric chains each. There is an active center in each subunit where metal ions form a coordination bridge with the molecular oxygen to participate in the oxidation as an oxygen-activating cofactor. Both iron and manganese have been proposed for the active site. Hertweck group demonstrated, based on X-ray structures, colorimetric assays and radical formation monitored by electron-spin resonance spectroscopy (ESR), that AurF has a 20-fold preference for manganese over iron, showing two manganese ions. However, an iron content of about 15% was detected (Winkler et al., 2007; Zocher et al., 2007). Furthermore, by conducting rational site-directed mutagenesis experiments, they were able to assign essential histidine residues into the active site for manganese coordination and enzyme activity. Nevertheless, the reported activity was much lower than that obtained by the Zhao group (0.148 vs 0.0032 μmol $mg^{-1}min^{-1}$ at pH 7.5), who demonstrated by bioinformatics, inductively coupled plasma (ICP), mass spectrometry (MS), electron paramagnetic resonance (EPR) and x-ray crystallography, that AurF is a carboxylate-bridged diiron enzyme (Chanco et al., 2014; Choi et al., 2008). They proposed that the difference in activity could be related to the Fe content and that the incorporation of this metal from the culture medium could be related to the counterion used, citrate (Hertweck group) instead of sulfate in their works

(Choi et al., 2008). Additionally, they demonstrated that the conformation that the active site acquires is different for both metals, allowing, the one containing Fe, to better accommodate the substrate, improving activity. Chanco and colleagues (Chanco et al., 2014) also suggested that the observed activity differences could be due not to the metal but to the source of the reducting equivalents (H_2O_2 or PMS/NADH) used for AurF activity in vitro, and that this enzyme could be promiscuous concerning the metal. Finally, based on the characteristics found by the mentioned groups, Bollinger's group proposes that AurF would contain a heterodinuclear Mn/Fe redox cofactor as observed in ribonucleotide reductase from *Chlamydia trachomatis* (Krebs et al., 2007). This proposal makes it possible to reconcile apparently contradictory observations. Extensive research was also done on the binding of pABA with the active site and two key factors were demonstrated: the molecular oxygen must bind before pABA, and that both the substrate and the product are captured by the interaction between the carboxylic acid and Glu-196 involved in the active site. In addition, a positively charged guanidinyl residue of an arginine (R96) forms a salt bridge with the negatively charged acidic group of the substrate, which places the *para* amino group in the correct position for the oxygenation. Fries and coworkers (Fries et al., 2010) confirmed the role of this aminoacid by preparing the R96E mutant and showing that this enzyme was unable to transform pABA into pNBA since the carboxyl of the substrate and the enzyme were not able to form hydrogen bonds. Interestingly, *p*-aminophenylguanidine and *p*-aminobenzamidine were oxygenated by both the mutant and the wild type enzyme, exerting a diverse recognition pattern.

3.2. CmlI

CmlI, typically isolated from *S. venezuelae*, is also a non-heme diiron monooxygenase (Lu et al., 2012) that catalyzes the three final steps in chloramphenicol (CLAM) biosynthesis (Sankaralingam et al., 2018), as shown in **Figure 3**.

Although biocatalytic applications of CmlI are limited by its natural low activity, its applicability to arylamines *N*-oxygenation is well characterized and biotransformation capabilities are reported (Trehoux et al., 2016). Related compounds to the initial CLAM synthesis substrate - (1*R*,2*R*)-2-(*N*-dichloroacetyl)-amino-1-(4-aminophenyl)-1,3-propanediol (NH_2-CLAM) - such as pABA, *p*-aminophenol and L-4-aminophenylalanine showed to be substrate of CmlI. In addition, the presence of 7% ethanol increased the enzyme activity by approximately 50%. It has also been reported that the presence of a keto or a hydroxyl group at the benzylic position may be sufficient for recognition as a substrate, suggesting that CmlI has great potential as a biocatalyst (Lu et al., 2012).

Structurally, CmlI resembles inactive dimanganese AurF in that the small and closed active site cannot accommodate the substrate. Although much research was carried out to elucidate the regulation of enzymatic activity at the structural level, it remains an enigma how the conformational changes that cause the opening of the active site are triggered (Komor et al., 2018; Waldman et al., 2017).

CmlI shares 34% amino acid sequence identity with AurF. The similar amino acid sequences and the type of reactions catalyzed by CmlI and AurF would suggest that they can form similar reactive intermediates. However, while the overall reactions stoichiometry is the same, the proposed mechanisms are different.

Similar to the AurF mechanism (the stepwise addition of two oxygen atoms from two oxygen molecules, in three successive $2e^-$ steps), the first step generates the hydroxylamine derivative and a diferric cluster. Nevertheless the CmlI mechanism differs in that hydroxylamine acts as an internal reductant, regenerating the diferrous center for the second activation of O_2 without requiring exogenous electrons, and in the state of the diiron enzymatic group at the end of a single turnover cycle (Komor et al., 2016; Makris et al., 2015; Waldman et al., 2017). CmlI forms a hyperstable unusual binding mode μ-η^1:η^2 peroxo intermediate (P) coordinated by His and acidic active site residues (Jasniewski and Que, 2018; Wang and Chen, 2017).
The mechanism involved in the in vivo synthesis of CLAM requires $CmlI^{ox}$ to be initially reduced by two non-substrate electrons, whose source is an yet unidentified reductase (Komor et al., 2018). The following step is the reaction of $CmlI^{red}$ with O_2 that yields CmlI-P. The amphiphilic nature of this intermediate plays an interesting role. First, as an electrophile, CmlI-P reacts with NH_2-CLAM and forms $CmlI^{ox}$-NHOH-CLAM that oxidizes itself to $CmlI^{red}$-NO-CLAM while reducing the diiron cluster. Finally, $CmlI^{red}$-NO-CLAM binds O_2 to re-form P, which now, as a nucleophile, will attack NO-CLAM. Chloramphenicol and $CmlI^{ox}$ result from the cleavage of the peroxo bond and the concomitant transfer of the oxygen atom (Jasniewski et al., 2017). The high specificity of the enzyme relies on the fact that no intermediate products are dissociated during the multistep arylamine oxygenation (Komor et al., 2018). Since the redox partners required for efficient CmlI turnover have not yet been identified, reconstitution of enzyme activity in vitro is a major challenge. The use of a redox system composed of NADH, the redox mediator phenazine methosulfate (PMS) and H_2O_2 to directly generate reactive peroxo species, have been reported with variable levels of enzymatic activity (Komor et al., 2018).
In order to improve activity, Zhang and coworkers (Zhang et al., 2018) have described for the first time, the immobilization of CmlI on core shell nanoparticles. Polymers such as poly (4-vinylpyridine) (P4VP) exhibit high electron affinity and favorable electron donation properties, which can be exploited to improve the performance of oxidoreductases as biocatalysts (Rochat and Swager, 2013). CmlI was immobilized on core-shell nanoparticles of P4VP coupled to a surrogate redox system. This system showed 5 and 1.6 times higher activity than those of the free enzyme for *p*-aminophenol and pABA, respectively, which was attributed to a more efficient supply of electrons due to the proximity of enzymes and cofactors, opening in this way the path to application on a variety of synthetic platforms.

3.3. PrnD

The Rieske-mononuclear oxygenases are a group of metalloenzymes that have the ability to catalyze the oxidation of a wide spectrum of compounds, whose interest lies especially in the degradation of contaminating xenobiotics (Perry et al., 2018). PrnD is a member of the Rieske non-heme mononuclear iron oxygenase family. Despite the structural differences, its biocatalytic characteristics are similar to those of AurF, taking into account its natural sequential *N*-oxygenation ability to produce Ar-NO_2 from Ar-NH_2, which involves two monooxygenation steps and one dehydrogenation step. This enzyme is related to *prn*ABCD gene cluster, that codes for PrnA, PrnB, PrnC and PrnD enzymes, involved in the last step of the biosynthesis of the bioactive molecule pyrrolnitrin [3-chloro-4-(2'-nitro-3'-chlorophenyl)pyrrole] (Pawar et al., 2019).

Pyrrolnitrin is a metabolite with marked antifungal activity, originally isolated from *Pseudomonas fluorescens* Pf-5 (Lee et al., 2005). It is mainly produced by the secondary metabolism of many *Pseudomonas*, although its presence has also been reported in *Burkholderia* and *Serratia* (Singh et al., 2016) and other Rhizobacteria (Pawar et al., 2019).
PrnD, as a Rieske monooxygenase, typically comprises a terminal oxygenase, containing a domain formed by a Rieske iron-sulfur center [2Fe-2S] and a second domain containing a non-hemic mononuclear Fe, and a flavin reductase, which supplies the reduced flavin required for the activity. This Rieske center transfers electrons, usually from NADH through ferredoxin partners via a network of internal hydrogen bonds, to the mononuclear iron site where molecular oxygen activation actually occurs (Tiwari et al., 2011) (**Figure 4**).
Mechanical studies have shown that the PrnF enzyme (a flavine NADH reductase) is linked to the *N*-oxygenation bioactivity of PrnD in *P. fluorescens*. (Tiwari et al., 2012).
PrnD was successful produced heterologously in *E. coli,* its activity being restored in vitro coupling NADPH and FMN to the flavin reductase system SsuE of the cell (Lee et al., 2005; Platter et al., 2011). Furthermore, the overexpression in *E. coli* of the *isc* operon increased the biocatalytic activity of PrnD four times, consistent with its in vivo participation in the assembly of [2Fe-2S] group (Tiwari et al., 2011).
PrnD revealed high substrate specificity toward physiological substrate aminopyrrolnitrin, but also accepts *p*-aminobenzyl amine, *p*-aminobenzyl alcohol, and *p*-aminophenyl alanine, showing that the position of the functional group may be more important than the size of the substrate. These results suggest that substrate binding occurs at a hydrophobic site large enough to accommodate arylamine substrates such as those containing two aromatic rings like aminopyrrolnitrin (Lee et al., 2005). Docking, modeling and site-directed mutagenesis demonstrated that both substrate specificity and bioactivity of PrnD can be significantly changed by manipulating residues F312 and L277. In particular, the F312S modification stands out, located in the deepest zone of the substrate binding pocket, which showed that eliminating a bulky residue generates more bioactive space (Lee et al., 2006; Pawar et al., 2019).

3.4. KijD3

KijD3 is a flavin-dependent *N*-oxygenase (Huijbers et al., 2014), found in *A. kijaniata,* that naturally biocatalyzes the oxidation of the *C*-3' amino group of dTDP-3-amino-2,3,6-trideoxy-4-keto-3-methyl-D-glucose into its *C*-3' nitro derivative D-kijanose. As biocatalyst, KijD3 adds sequentially molecular oxygen to generate a hydroxylamine intermediate, and then, in a still unclear way, produces the final nitro derivative (**Figure 5**) (Thoden et al., 2013).
This nitro-sugar is part of the spirotetronate antibiotic kijanimicin, which in addition to its antitumor activity, has proven activity against anaerobes, Gram-positive bacteria and malaria's parasite, *Plasmodium falciparum*. Moreover, a recent research work characterized the presence of D-kijanose in new lobophorin analogues with improved bioactivity (Tan et al., 2020).
Because of its overall fold and despite the low aminoacidic sequence homology (17% to 26%) KijD3 belongs to the acyl-CoA dehydrogenase superfamily as a member of the class D flavin dependent monooxygenases which depends on a separate flavin reductase for the supply of reduced flavin (either FAD or FMN) as the cofactor (Singh et al., 2012).

Thoden and coworkers showed that KijD3 catalyzes the two-electron oxidation from the dTDP-aminosugar to its hydroxylamine-containing derivate (Thoden et al., 2013). This step requires a flavin nucleotide to react with O_2 and form a flavin 4a-hydroperoxide (Hu et al., 2008). This electrophilic intermediate is attacked by the sugar amino group on the newly added distal oxygen. Meanwhile, the flavin-bound oxygen receives a hydrogen from the amino moiety and the *N*-hydroxylamine is formed (Lin et al., 2013). Teufel (Teufel, 2017) suggested that this flavin-*C*4a-hydroperoxide may act a second time to oxidize the *N*-hydroxylamine to the nitroso moiety. It remains unclear if KijD3 is the catalyst of the last oxidation step or if the nitro group is the result of a non enzymatic oxidation such as an spontaneously photo-oxidation (Vey et al., 2010; Waldman et al., 2017).
KijD3's X-ray structure showed that the enzyme happens to be formed by four subunits with three distinct regions each: an α-helical bundle, an eight-stranded β-sheet and a second five α-helical bundle (Bruender et al., 2010). Interestingly, each subunit has a dTDP-linked sugar dimensioned cleft. KijD3's active site presents a high proportion of water molecules, but no classic acidic or basic catalytic residues (Thoden et al., 2013). Moreover, structural separation of the flavin nucleotide and the substrate binding elements in the active site have been reported. These features allow a structural variability of the substrate without disturbing the cofactor binding (Kugel et al., 2017).

3.5. DnmZ

ORF36, RubN8 and DnmZ, involved in the biosynthesis of everninomycin, rubradirin and baumycin, respectively, are enzymes closely related to KijD3. They catalyze the conversion of amines from different dTDP-deoxy amino sugars into nitroso groups (Teufel, 2017). Due to the levels of homology in their sequences -in fact, the amino acid identity exceed 60% among them- and to the specific reaction requirements, these enzymes are classified as nitrososynthases and belong to class D of flavin monooxygenases (Hu et al., 2008). This class of monooxygenases is characterized by an acyl-CoA dehydrogenase fold and by the coupling with an individual NADPH dependent flavin reductase to supply reduced flavin for flavin 4a-hydroperoxide formation (Sartor et al., 2015; van Berkel et al., 2006).
DnmZ has been considered as a special section due to the uniqueness of its biocatalytic activity. As ORF36, RubN8 and KijD3, DnmZ carry out the *N*-oxygenation of the exocyclic amine of dTDP-deoxy amino sugars to its nitroso derivative through a hydroxylamine intermediate (Waldman et al., 2017). However, after the nitroso derivative is generated from dTDP-L-epi-vancosamine, DnmZ promotes a single retro oxime-aldol cleavage, and the resulting oxime group is subsequently hydrolyzed, followed by additional reduction steps (Teufel, 2017). **Figure 6** shows the proposed DnmZ mechanism as part of the biosynthesis of baumycin, a powerful anthracycline antibiotic produced by *Streptomyces peucetius* (Al-Mestarihi et al., 2013; Huynh et al., 2020).
Regarding the DnmZ structure, this *N*-oxygenase adopts a homo-tetrameric fatty acyl-CoA dehydrogenase quaternary fold, and its monomer consists of three domains. The *N*-terminal domain is made up exclusively of helices and creates a 'cis-peptide loop', a unique feature of the nitrososynthases that has been observed also in KijD3, ORF36 and RubN8. The similarities in the conformation of the active site that these enzymes present cause that all of them oxidize the

exocyclic amine of 2,3,6-trideoxy-3-amino sugars. Although each enzyme prefers a specific deoxy sugar, all of them show some activity with each other's substrates. Structural and experimental studies are currently being performed in order to clarify the substrate preferences of each enzyme (Sartor et al., 2015).

3.6. NspF

NspF biocatalyzes the aromatic *C*-nitrosation of 3-amino-4-hydroxybenzoic acid (3,4-AHBA) into 4-hydroxy-3-nitrosobenzoic acid (4,3-HNBA) and it was first discovered in *Streptomyces murayamaensis* (**Figure 7**) (Kersten and Dorrestein, 2010). Unlike the previously mentioned enzymes, where nitrosation is an intermediate step towards the formation of nitro groups, this enzyme is involved in the biosynthesis of interesting *C*-nitroso aromatic compounds, in particular *p*-substituted *o*-nitrosophenols, main components of the ferroverdin pigments (Noguchi et al., 2010).
Ginsbach and coworkers discovered strong parallelisms between tyrosinases and this enzyme. While tyrosinases biocatalyze the production of *o*-iminoquinone from *o*-aminophenol, NspF biocatalyzes the formation of *o*-nitrosophenol from this substrate (Ginsbach et al., 2012). Because it has a tyrosinase-like motif, NspF needs copper to function being the first copper-dependent *N*-oxygenase discovered. Tyrosinase-like enzymes are part of the family of coupled binuclear copper proteins, whose characteristic is to have a magnetically coupled binuclear copper active site that binds symmetrically to molecular oxygen. To incorporate the two copper ions involved in its monooxygenase activity, NspF requires a chaperone protein NspE, crucial for the in vivo activity (Noguchi et al., 2010). As in the case of iron enzymes, the activation of oxygen and the generation of the active peroxo-Cu_2II/II complex require a reduced copper site. The proposed mechanism for the biotransformation involves the reduction of the enzyme by the *o*-aminophenol yielding the corresponding *o*-iminoquinone, the formation of the peroxo intermediate with O_2 and an intramolecular oxidation of the coordinated substrate. This generates the release of *o*-nitrosophenol preventing the over-oxidation of nitrogen and the consequent formation of nitro compounds (Noguchi et al., 2010).
Substrate acceptance studies demonstrated that NspF can catalyze nitroso-forming reactions using a variety of *o*-aminophenols such as 3-amino-4-hydroxybenzoic acid, -benzamide, -benzaldehyde and -sulphonic acid. In contrast, neither *o*-aminophenol nor 4-amino-3-hydroxybenzoic acid were accepted as substrates. According to the tested substrates, the functional group at the position *para* in the *o*-aminophenol strongly influences NspF activity(Kersten and Dorrestein, 2010).

3.7. SznF

SznF is a peculiar enzyme, found in *Streptomyces achromogenes*, which is part of the streptozotocin synthesis pathway (McBride et al., 2020b). This enzyme catalyzes the transformation of N^{ω}-methyl-L-arginine (L-NMA) to N^{δ}-hydroxy-$N^{\omega'}$-methyl-N^{ω}-nitroso-L-citrulline, a crucial precursor of streptozotocin (He et al., 2019). This unique biocatalytic ability of SznF has yet to be applied to biotransformations due to its recent identification. The mechanism has been proposed by Ng and coworkers (Ng et al., 2019) and requires three iron and O_2 dependent steps followed by a rearrangement, in two different sites (**Figure 8**). The first two steps are hydroxylations at two nitrogen atoms of the guanidine

group of L-NMA that occur in the central domain, with nitrogen δ the first one to be oxidized. A conformational change is needed in order to target a different nitrogen atom in each step, via the same intermediate. The final step occurs in the cupin domain, where the modified guanidine of the N^{δ},N^{ω}-dihydroxy-$N^{\omega'}$-methyl-L-arginine (L-DHMA) is converted to *N*-methyl-*N*-nitrosourea through oxidative rearrangement and N–N bond formation.

SznF was the first reported *N*-oxygenase to generate the *N*-hydroxylation at two different nitrogen atoms, making it one of the most complex enzymes of this class (McBride et al., 2020b). Regarding the characteristics of the *N*-hydroxylating central domain, it was classified as a heme-oxygenase-like diiron oxygenase (HDO), a new kind of O_2-activating non-heme diiron enzymes (Jasniewski and Que, 2018).

Spectroscopic and kinetic studies showed that the μ-peroxoFe_2 complex is an intermediate in both *N*-hydroxylation steps catalyzed by SznF. Although the production of similar intermediates by other *N*-oxygenases as AurF and CmlI has been reported, different characteristics such as substrate structure, low stability of the coordinated oxidized cofactor, and also the protein structure, places SznF in a different non-heme diiron enzyme superfamily. These features enable SznF to rapidly reconfigure itself to target two different nitrogen atoms of its substrate, instead of sequential oxidations of a single nitrogen atom as the *N*-oxygenases previously mentioned (McBride et al., 2020b).

The emerging HDO family seems to have a unifying characteristic, the instability of the oxidized form of the cofactor. This peculiarity could also promote product release, avoiding further oxidation at the central domain and allowing the transfer to the cupin domain to continue the biotransformation until the final nitrosourea product (McBride et al., 2020a). Besides the iron cofactor, SznF also uses some sort of reductant cofactor to regenerate the iron active sites of the *N*-oxygenase domains. In vitro studies used NADH, but there is no information on whether this cofactor is involved in these transformations in vivo. Interestingly, the cupin domain does not require it: this domain couples the rearrangement with a full 4-electron reduction of one molecule of O_2 (Ng et al., 2019).

The substrate binding site was elucidated by X-ray crystallography. A deep pocket connected to the protein surface was found close to the iron cofactor sites of the HDO domain. This site locates the substrate and the oxygen peroxide at the correct distance to promote a direct nucleophilic attack, necessary for the mechanism of the reaction. On the other hand, the pocket is highly hydrophobic, which explains why unmethylated arginine is not a substrate for the enzyme (McBride et al., 2020a).

3.8. RohS

RohS is other recently discovered *N*-oxygenase classified as member of the new HDO family of non-heme diiron oxygenases. This enzyme is encoded in the five-gene region *rohPQRST,* a gene cluster responsible for the biosynthesis of azomycin (2-nitroimidazole). This cluster was recently reported after fully reconstitution in vitro of the biosynthetic pathway starting from L-arginine (Hedges and Ryan, 2019). RohS is capable of carrying out the last oxygenation step of this pathway by transforming 2-aminoimidazole into its nitro derivative (Nakane et al., 1977) (**Figure 9**). Azomycin was shown to have potent activity against trichomoniasis and is a fundamental building block in the synthesis of benznidazole, the most widely used drug to treat Chagas disease (Ang et al., 2017). Moreover, because of its resemblance to energetic chemicals such as the

propellants 2,4-dinitroimidazole and 3-nitro-1,2,4-triazole-5-butanetriol trinitrate, 2-nitroimidazol plays a potential role in the energetic materials industry (Graham et al., 2018).
Through bioinformatics, Hedges and Ryan were able to identify microorganisms that could likely carry the gene encoding this *N*-oxygenase. All those selected turned out to belong to the genus *Streptomyces*, among which *S. eurocidicus* stood out for having two copies of the gene (Hedges and Ryan, 2019). These results support those obtained in the 1960s by Lancini, using whole cells of *Streptomyces sp.* LE /3342, and those of Graham in 2018 using *Streptomyces eurocidicus*, which demonstrated the ability of these microorganisms to oxidize 2-aminoimidazole to 2-nitroimidazol. (Graham et al., 2018; Lancini et al., 1966).
Although the mechanism of action of RohS has not been elucidated yet, the arylamine to arylnitro transformation takes place, in vitro, in the presence of phenazine methosulfate (PMS), NADH, $FeSO_4$ and exogenous iron as cofactors. (Hedges and Ryan, 2019). The fact that research on RohS as an enzyme itself has started in 2018 must be highlighted, consequently the information collected here is very recent and limited. Very little is known about the structure and therefore the active site of RohS. The enzyme has not been crystallized yet, but through multiple sequence alignment it was found that its putative iron binding sites show high similarity to those of SznF. Although a strict iron-dependence was observed, kinetic studies revealed a low iron-binding capacity, even in the presence of excess iron, characteristic of this HDO family of enzymes.
The specificity of the biocatalytic site was analyzed by performing the oxidation of alkyl 2-aminoimidazoles into their nitro derivates. The enzyme was able to oxygenate substrates carrying alkyl groups in positions 4 or 5, decreasing its efficiency with increasing chain length. Oxygenation of the 4,5-dimethyl derivative was also observed, but in lower yields, while in substrates with a methyl group in position 1 or a phenyl group in positions 4 or 5 no conversion to the nitro-derivates was detected (Lancini et al., 1968).

4. *N*-oxygenases for biotechnological processes.

Nitro compounds synthesis are of great interest due to the presence of nitro groups in many bioactive products that show antibiotic, antibacterial, antifungal or antitumor activities. While considerable effort has been put into synthesizing nitro compounds over the years, several areas remain unexplored. Many issues such as regioselectivity, complex methodologies, low yields and presence of by-products as well as the use of corrosive and toxic reagents and waste disposal problems that affect life and human health and the environment, continue unsolved(Badgujar et al., 2016).
The enzymes that are the subject of this review constitute a set of enzyme tools that provide biocatalyzed routes to produce nitro compounds from their corresponding amines that could be used in greener and more sustainable processes. Among them, *N*-oxygenases such as RohS are promising alternatives. The efficacy of Nifurtimox and Benznidazole, drugs often used to treat Chagas disease caused by *Trypanosoma cruzi*, has attracted interest in the use of nitro heteroaromatic compounds for the treatment of infectious diseases, such as tuberculosis and hepatitis C (Zhou et al., 2013).
However, the application of redox enzymes in biotechnological processes is conditioned by the regeneration of their cofactors. In particular, *N*-oxygenases

require the restoration of the O_2 binding-dimetal cofactor at the active site, which is carried out in vivo by the appropriate enzymes.
To restore the native enzymatic activity of AurF, only the addition of H_2O_2 was required as an electron donor and as a source of O atoms, via the so-called "peroxide shunt catalysis"(Bailey and Fox, 2009). This approach, proposed by Winkler and colleagues (Winkler et al., 2006), was based on the behavior of another family of binuclear enzyme such as toluene monooxygenase (Sono et al., 1996). Using this strategy, Hertweck's group carried out, for the first time, chemo- and regioselective oxygenation of aromatic amines to nitro compounds in a fixed-bed tube reactor containing immobilized AurF (Winkler et al., 2006). Subsequently, Zhao's group reported the in vitro reconstitution of the enzymatic activity of AurF, using PMS and NADH based on the protocols developed for methane monooxygenase (Chanco et al., 2014). In this work, they showed that the reaction profile and product yield were different from those obtained with peroxide shunt catalysis. They rationalized the observed substrate specificity of the enzyme in terms of binding and affinity at the active site, which is important for future optimizations of AurF to be applied in biotransformations.
In view of potential biotechnological applications of these enzymes, biocatalyst immobilization provides multiple benefits in large-scale processes. This kind of techniques protects the biocatalysts from the environment and from possible microbiological contaminations in non-sterile processes, stabilizing the biocatalysts and favoring the retention of their activity during long-term storage. Immobilization approaches facilitate serial reuses in continuous processes, with the consequent improvement in productivity and reduction of production costs (Nóbile et al., 2019). Regarding their application to processes biocatalyzed by redox enzymes, as is the case of *N*-oxygenases, immobilization improves activity due to a more efficient electron delivery process caused by the proximity of enzymes and cofactors. In this regard, Zhang and colleagues reported the preparation of coated core-shell nanoparticles by the co-assembly of poly (4-vinylpyridine) and CmlI, which could be potentially useful in bioprocesses (Zhang et al., 2018). In this case, the reducing system composed of NADH and PMS led to about 2 times more activity of the immobilized enzyme compared to the peroxide shunt method.
In contrast to non-heme dimetal monooxygenases, the reconstitution of Rieske-mononuclear oxygenases such as PrnD requires additional coenzymes for in vitro activity. In this sense, PrnD showed significant activity when a flavin reductase, FMN, and NADPH were used for reconstitution (Lee et al., 2005). PrnD can be used complementary to AurF, for larger substrates with nonpolar groups in the *para* position to the amino group.
AurF homologs, which are often associated with non-ribosomal peptide synthetases or polyketide synthases, have substrate binding pockets with different amino acid compositions that cause changes in substrate specificities. These systems are responsible for synthesizing natural products with potential relevant pharmacological activities. Unlike AurF, the homolog from *Pseudomonas syringae pv. phaseolicola* showed higher activity with substituted *o*-aminophenols at pH 9 in buffer solutions containing 40% methanol (Platter et al., 2011). Similarly, the *Rhodococcus jostii RHAI* homolog showed activity mainly with aminophenols and only a few PABA derivatives, and did not exhibit strong *para* regioselectivity (Indest et al., 2015; Platter et al., 2011). These new sources of arylamino *N*-oxygenases with high tolerance to solvents and expanded substrate capacities, may prove useful for their application as industrial biocatalysts. Additionally, subjecting enzymes to protein engineering

and directed evolution methods has the potential to customize substrate specificity, ultimately leading to new green synthetic pathways for nitroaromatics.

On the other hand, glycosylation of bacterial secondary metabolites complements and expands the chemical diversity of the natural product. These sugar attachments dramatically influence their properties, such as pharmacokinetics and pharmacology. Therefore, differential glycosylation of natural products has emerged as a viable strategy to produce bioactive compounds with enhanced activity (Singh et al., 2012). In this context, efforts are being made to find biocatalyzed pathways that allow the synthesis of modified glycosides. As mentioned above, compounds such as everninomicin (Vey et al., 2010), that contain nitrosugars, have important biological activities and therefore, the preparation of nitrosugars mediated by *N*-oxygenases is an option to consider for biotechnological processes. In this sense, ORF36 and RubN8 were able to oxidize in vitro the amino sugar L-TDP epi-vancosamine to the corresponding nitroso derivative when NADPH, FAD and a flavin reductase from *Vibrio fisheri* were supplied (Hu et al., 2008).

Advancing the complete understanding of the biochemistry of *N*-oxygenases is essential to provide efficient biocatalysts with broad substrate specificity that will be required for the biocatalytic synthesis of nitro compounds in scale processes. Although the possible future applications of these enzymes have been indicated, it is necessary to emphasize that all the techniques and technologies described in this section are still in an early stages and there is still a wide range of obstacles to be solved (Graham et al., 2018; Sulzbach and Kunjapur, 2020).

5. Perspectives on cryptic genes, enzymes and secondary metabolites

In recent years, novel tools and techniques have been developed and applied to the discovery of new secondary metabolites synthetic pathways and their involved enzymes. The expansion of genome sequencing over the past two decades has provided initially unsuspected information, particularly for *actinomycetes*. It has been shown that, on average, each strain can contain 30, 40 and even more genetically encoded secondary metabolic pathways carrying the potential to produce unsuspected metabolites that are not usually produced under normal laboratory conditions (Nett et al., 2009). These groups of genes, which usually remain "dormant", have been called cryptic gene clusters and to activate them, chemical signal probes, ribosome engineering, regulation unblocking, and heterologous expression have been employed (McCranie and Bachmann, 2014; Xia et al., 2020). It has been hypothesized for most *Streptomyces* strains that their genome contains between 20 and 60 cryptic biosynthetic gene clusters (Devine et al., 2017; Onaka, 2017). An emblematic case is that of *S. coelicolor*, of which 5 secondary metabolites were known since the 60s, but after analyzing the results of the sequencing of its genome in 2002, it was revealed that it contained 32 cryptic gene clusters coding a multiplicity of enzymes, with the potential to produce several bioactive natural products (Challis, 2014).

In the better known *N*-oxygenases like PrnD, CmlI and AurF, their corresponding gene clusters contain at least 5 encoded enzymes, and at least one is an *N*-oxygenase, so it can be presumed the existence of numerous *N*-oxygenases not yet discovered.Taking this into account, it is not unreasonable to think that

several of these unknown and potentially bioactive compounds contain a nitro group in their molecule, and consequently, there must be new *N*-oxygenases that catalyze their synthesis. Zhao and coworkers have identified and activated in the *Penicillium rivulum* genome, a biosynthetic cryptic cluster capable of producing the α-nitro containing cyclic tripeptide psychrophilin (Zhao et al., 2016), thus demonstrating that regulatory genetic manipulation can successfully replace heterologous expression (Tong et al., 2019).
Probably the simplest way to obtain an improved catalytic activity is the development of joint cultures of strains that can be competitors in their natural habitat. This has been proven successfully by activating secondary metabolism in *Streptomyces* (Nóbile et al., 2020; Onaka, 2017). This phenomenon probably occurs due to physical strain interactions, communication mediated by small molecules or even mediated by a soluble metabolite precursor (Trottmann et al., 2019; Xia et al., 2020).
In summary, *N*-oxygenases are useful enzymes for synthesizing a variety of nitro compounds from the corresponding amines, thus adding a new activity to the existing enzyme toolbox potentially applicable in industrial biocatalysis. However, the limitations that arise in terms of commercial availability and limited acceptance of substrates should not be ignored. Crystal structure studies and enzymatic promiscuity evidences also show that there is still a long way to go, but the critical advances reported in the last years suggest a promising future for the generation of bioactive compounds using *N*-oxygenases.

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Figure captions

Figure 1: Bioactive compounds carrying N-O functional groups.

Figure 2: AurF biocatalyzed conversion from pABA to pNBA. pABA: *p*-aminobenzoic acid; pHABA: hydroxylamine-pABA derivative; pNOBA: nitroso-pABA derivative; pNBA: *p*-nitrobenzoic acid.

Figure 3: CmlI *N*-oxygenation involved in Chloramphenicol biosynthesis. NH_2-CLAM: (1*R*,2*R*)-2-(*N*-dichloroacetyl)-amino-1-(4-aminophenyl)-1,3-propanediol; NHOH-CLAM: (1*R*,2*R*)-2-(*N*-dichloroacetyl)-amino-1-(4-hydroxylaminophenyl)-1,3-propanediol; NO-CLAM: (1*R*,2*R*)-2-(*N*-dichloroacetyl)-amino-1-(4-nitrosophenyl)-1,3-propanediol.

Figure 4: Pyrrolnitrin obtained via *N*-oxygenation of aminopyrrolnitrin by PrnD. FR: flavin reductase.

Figure 5: Biocatalyzed nitrosugar D-kijanose production via KijD3 *N*-oxygenation. FN: flavin nucleotide.

Figure 6: DnmZ sequential *N*-oxygenation of dTDP-L-epi-vancosamine, via hydroxylamine and nitroso derivatives. FR: flavin reductase.

Figure 7: *N*-oxygenation of diverse substrates by NspF. 3,4-AHBA: 3-amino-4-hydroxybenzoic acid; 4,3-HNBA: 4-hydroxy-3-nitrosobenzoic acid.

Figure 8: SznF *N*-oxygenation of N^{ω}-methyl-L-arginine (L-NMA) to N^{δ},N^{ω}-dihydroxy-$N^{\omega'}$-methyl-L-arginine (L-DHMA), via double independent hydroxylamine conversion steps.

Figure 9 – 2-Nitroimidazole (azomycin) produced via RohS *N*-oxygenation.

Table 1 – Enzymes studied in the present review.

Enzyme	Source	Related bioactive compound	N-O derivative formed	Reference
AurF	*Streptomyces thioluteus*	Aureothin	Nitro	(Chanco et al., 2014)
CmlI	*Streptomyces venezuelae*	Chloramphenicol	Nitro	(Komor et al., 2018; Lu et al., 2012)
DnmZ	*Streptomyces peucetius*	Baumycin	Nitroso	(Sartor et al., 2015)
KijD3	- *Actinomadura kijaniata* -*Micromonospora carbonacea*	Kijanimicin	Hydroxylamine	(Thoden et al., 2013; Zhang et al., 2007)
NspF	*Streptomyces murayamaensis*	Nitrobenzamide	Nitroso	(Noguchi et al., 2010)
PrnD	*Pseudomonas fluorescens*	Chloramphenicol	Nitro	(Al-Mestarihi et al., 2013; Lee et al., 2006)
SznF	*Streptomyces achromogenes*	Streptozotocin	Hydroxylamine	(Ng et al., 2019)
RohS	- *Streptomyces eurocidicus* -*Streptomyces sp.* LE/3342 - *Streptomyces cattleya*	Azomycin	Nitro	(Hedges and Ryan, 2019; Lancini et al., 1966)

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: