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Current Opinion in Chemical Biology

The emergence of nitric oxide in the biosynthesis of bacterial natural products

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Nitric oxide (NO) has a broad range of biological utilities including as a biosynthon for bacterial natural products (NPs). The biosyntheses of thaxtomin A and rufomycin require an NO-dependent nitration step where a bacterial NO synthase provides the necessary NO and nitration is catalyzed by a cytochrome P450 homolog. Undiscovered NO-dependent biosynthesis pathways are likely to have similar requirements: a pathway dedicated NO source and a metalloenzyme to catalyze the NO-dependent chemistry. This review discusses the current literature on NO-dependent nitration biosynthesis and relevant enzyme mechanisms. The proposal that NO is important for N–N bond containing NPs is also explored, particularly for the formations of nitrosamine and nitramine NPs.

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Current Opinion in Chemical Biology 2019, **49**:130–138

This review comes from a themed issue on **Bioinorganic chemistry**

Edited by **Kyle M Lancaster**

For a complete overview see the Issue and the Editorial

Available online 11th January 2019

https://doi.org/10.1016/j.cbpa.2018.11.007

Introduction

Nitric oxide (NO) exhibits several physiological roles spanning all domains of life. In human physiology, NO is a signal for vasodilation, neurotransmission, and apoptosis and is generated as a defense molecule during the human immune response [1–5]. Additionally, NO is an important signal in plant physiology and for the formation and dispersion of bacterial biofilms [6–8]. NO is a key component of nitrogen cycle pathways. In fact, biological NO was first observed as an emission from denitrifying bacteria [9] and later identified as an obligate intermediate of denitrification [10], bacterial and archaeal nitrification [11,12], and anammox pathways [13]. A new role for NO has been recently discovered for the biosynthesis of bacterial secondary metabolites, otherwise known as natural products (NPs).

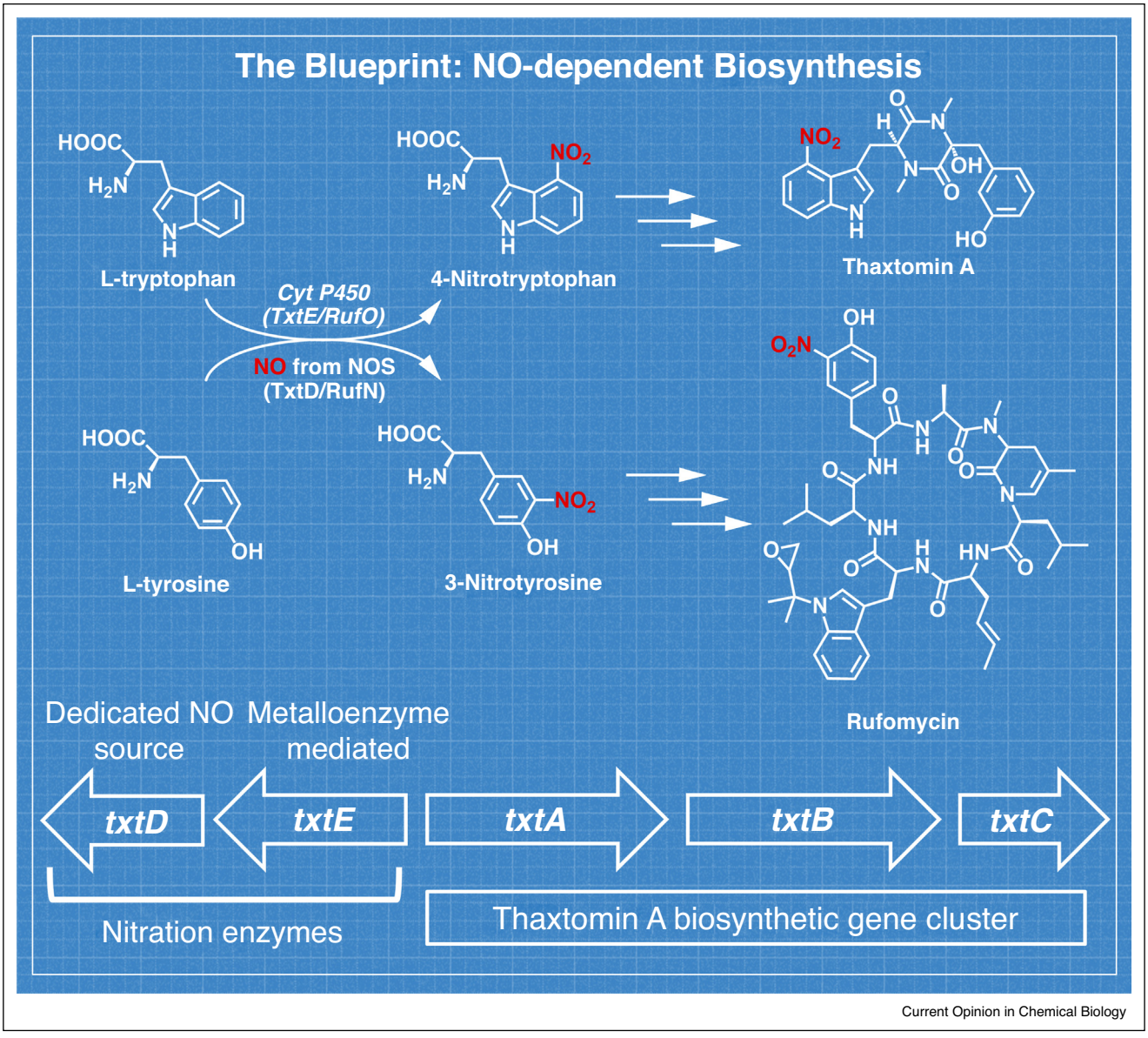
The breadth of NO's biological utility reflects its ability to participate in diverse reaction pathways. This reactivity is well illustrated during nitrosative stress—cellular damage resulting from exposure to NO [14,15]. Cell lesions observed during nitrosative stress include the inhibition of metabolic proteins, nitration [addition of nitro groups ($R–NO_2$)] and nitrosation of biomolecules [addition of nitroso group (R–NO)], and DNA mutagenesis or cleavage. These damages can lead to cell death; in fact, macrophages generate NO and superoxide (O_2^-) during the human immune response to kill invading microorganisms [5]. While NO can directly inhibit or degrade metabolic proteins, reactive nitrogen species (RNS) resulting from NO reacting with O_2 or O_2^- cause the most serious damage. RNS including peroxynitrite ($ONOO^-$), nitrogen dioxide ($^{\bullet}NO_2$), or dinitrogen trioxide (N_2O_3) are nitration or nitrosation agents [14–16]. Nitration of catalytic amino acids can inhibit enzyme activities while nitrosation agents react with thiols or amines to form N-nitrosothiols or nitrosamines, respectively. The latter family of compounds promotes DNA alkylation and mutagenesis [15]. Degradation of $ONOO^-$ can form hydroxyl radicals ($^{\bullet}OH$), which causes DNA hydroxylation and strand cleavage. Nitration and nitrosation reactions initiated by NO appear to be repurposed by bacteria for use in NP biosynthesis.

The resurgence in research on bacterial NP biosynthesis is driven by the need for new drugs and aided by modern DNA sequencing technologies and genome mining strategies [17]. Genes coding for the component enzymes of an NP biosynthetic pathway cluster within the genome, resulting in, so called, biosynthetic gene clusters (BGCs). Genome mining can identify BGCs by searching for clusters of previously characterized NP biosynthetic enzymes. Currently, there are few genetic signatures for NO-dependent pathways. However, pioneering research led by Loria, Crane, and co-workers on thaxtomin A biosynthesis has suggested a blueprint for such pathways (Figure 1): a pathway-dedicated NO source and a metalloenzyme to catalyze the NO-dependent chemistry. The following reviews recent literature on NO-dependent nitration and NP N–N bond formation and discusses evidence that NO is required to form nitrosamine and nitramine N–N bonds.

NO-dependent nitration mediated by cyt P450

Study of thaxtomins revealed the first NO-dependent NP biosynthetic pathway. Thaxtomins were discovered in the extracts of potatoes infected with scab, a disease that

Figure 1

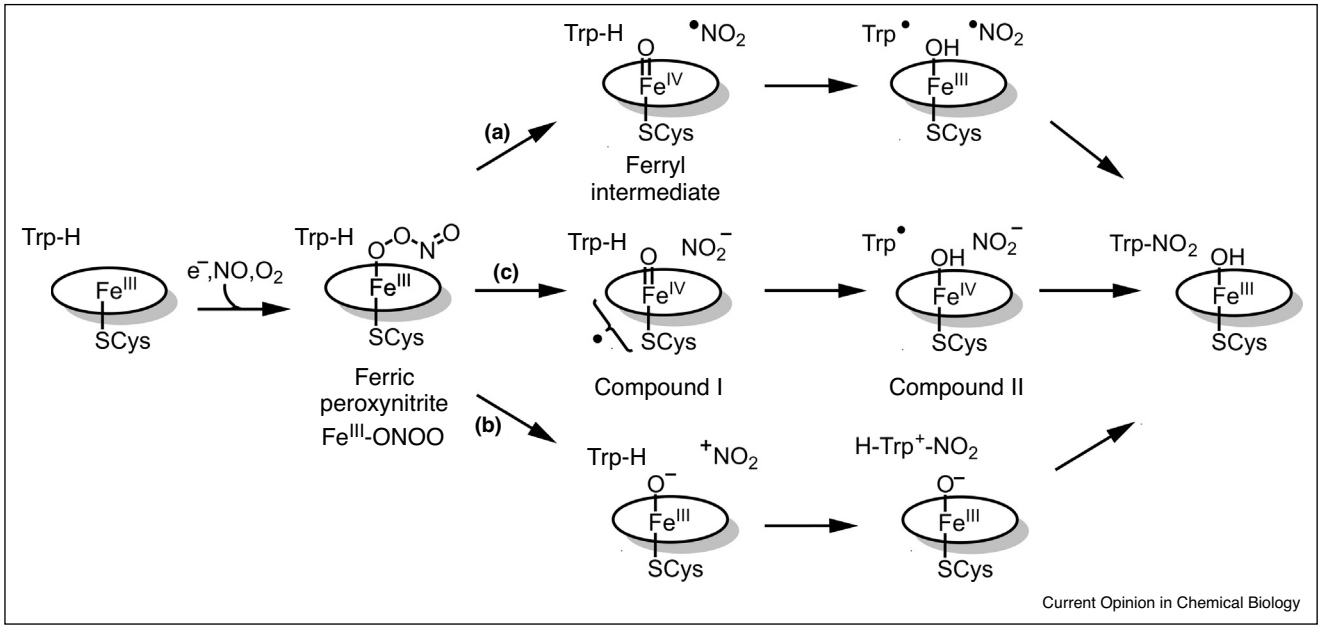


Nitric oxide (NO)-dependent nitration in the biosynthesis of the bacterial natural products (NPs) thaxtomin A and rufomycin. The biosynthetic gene cluster (BGC) for thaxtomin A is shown to illustrate the apparent minimal requirements for NO-dependent nitration: a dedicated NO-source and a metalloenzyme to mediate the nitration. For thaxtomin A and rufomycin biosynthesis these are nitric oxide synthase (NOS) and cytochrome (cyt) P450, respectively.

causes lesions in root vegetables [18,19]. Treatment of immature potato tubers with purified or synthetic thaxtomins induces scab [20–22]; thus, thaxtomins are the chief virulence factors of scab, with thaxtomin A specifically shown to inhibit cellulose production [23]. This disease is onset by plant-pathogenic *Streptomyces* species, with the virulence genes located on a mobile pathogenic island [24]. Genetic studies of this pathogenic island allowed for elucidation of thaxtomin A biosynthesis.

Thaxtomin A is a cyclic dipeptide formed from 4-nitrotryptophan and phenylalanine. The piperazinedione core is constructed by two non-ribosomal peptide synthetases (NRPSs), TxtA and TxtB [25]. A cytochrome (cyt) P450 homolog, TxtC, hydroxylates the cyclized NRPS product to complete thaxtomin A biosynthesis. The stereochemistry of the piperazinedione core and the presence of the nitro group are critical for thaxtomin A's virulence activity [22]. This nitro group is incorporated by an NO-

Figure 2



Proposed intermediates on the TxtE nitration pathway. All pathways initiate via formation of a ferric-peroxynitrite (Fe^{III}-ONOO). In pathway A, the key intermediate is a ferryl intermediate, which mediates hydrogen abstraction and nitration occurs via radical recombination. In pathway B, nitrosonium ($^{+}NO_2$) is formed which adds to tryptophan by classical electrophilic aromatic substitution. In pathway C, the key intermediate is compound I, which mediates hydrogen abstraction.

dependent direct nitration of tryptophan to form the 4-nitrotryptophan precursor. A bacterial nitric oxide synthase (bNOS), TxtD, oxidizes arginine (Arg) to form citrulline (Cit) and the necessary NO [26–28]: $2Arg + 3NADPH + 4O_2 + H^+ \rightarrow 2Cit + 2NO + 3NADP^+ + 4H_2O$. A cyt P450 homolog, TxtE, catalyzes the NO-dependent nitration of tryptophan in the presence of O_2, NADPH, and electron transfer components [29]. This biosynthetic pathway has been targeted for synthetic biology and protein engineering efforts [30–34].

A second NO-dependent nitration was found for the biosynthesis of the antibiotic rufomycin, a nitro-containing cyclic peptide produced by *Streptomyces atratus* (Figure 1) [35•]. The rufomycin BGC was found by searching for NRPS, tryptophan N-dimethylallyltransferase, and nitration genes in *S. atratus*. Rufomycin biosynthesis is largely mediated by an NRPS where one precursor is 3-nitrotyrosine. The presence of adjacent bNOS (RufN) and cyt P450 (RufO) genes suggested their necessity for 3-nitrotyrosine production. Under turnover conditions and in the presence of NO, recombinant RufO catalyzes the production of this precursor. Thus, rufomycin provides the second example of an NO-dependent nitration.

The TxtE/RufO mechanism has not yet been elucidated. Only one nitro oxygen is derived from O_2 based on isotope labelling studies [29]. Figure 2 shows three proposed mechanisms consistent with this result [29]. Tryptophan binding gates reduction of the Fe^{III} heme to Fe^{II}. O_2 binds to Fe^{II} to form a ferric-superoxide, which reacts with NO to form a ferric-peroxynitrite (Fe^{III}–OONO). In one proposed pathway (Figure 2A), homolytic cleavage of the O–O bond forms a ferryl intermediate and nitrogen dioxide ($^{\bullet}NO_2$). The resulting ferryl intermediate abstracts a hydrogen atom to form a tryptophan radical that reacts with $^{\bullet}NO_2$ to form 4-nitrotryptophan. Alternatively (Figure 2B), heterolytic cleavage of the O–O bond results in formation of nitronium (NO_2^+), which reacts with tryptophan via electrophilic aromatic substitution to form 4-nitrotryptophan. A third possibility (Figure 2C) is based on canonical cyt P450 activity, for which compound I promotes hydrogen abstraction [36]. This species could result from heterolytic cleavage of the O–O bond with concomitant formation of NO_2^-. Hydrogen abstraction by compound I forms the tryptophan radical (Trp$^{\bullet}$) and compound II, which oxidizes NO_2– to form $^{\bullet}NO_2$. Radical recombination of $^{\bullet}NO_2$ with the tryptophan radical results in 4-nitrotryptophan. All proposed pathways proceed via the Fe^{III}–ONOO species, which in principle, could also be generated from the reaction of Fe^{III}-TxtE with $ONOO^-$. Notably, myoglobin reacts with $ONOO^-$ to form NO_3^- and ferric myoglobin [37] via formation of a ferryl intermediate and $^{\bullet}NO_2$ as proposed in pathway A

for TxtE. However, cyt P450 reacts with $ONOO^-$ to form an $\{FeNO\}^6$ complex [38]. Thus, it remains to be seen if Fe^{III}–ONOO is a viable intermediate for the TxtE nitration mechanism.

Are there NO-dependent N-nitrosations?

Over 200 N–N bond containing NPs have been identified, including hydrazine, hydrazone, hydrazide, azoxy, diazo, nitrosamine, and nitramine compounds [39]. Balskus and co-workers recently reviewed the known biosynthetic pathways for these NPs [40••]. An earlier review of these compounds by Le Goff and Ouazzani proposed an N–N coupling pathway in which amines couple with electrophilic metal nitrosyls. Such species could include ferric- or cupric-nitrosyls ($\{FeNO\}^6$ or $\{CuNO\}^{10}$ in Enemark-Feltham notation, respectively) [39]. Iron-nitrosyls are commonly observed as intermediates of nitrogen cycle enzymes that form the N–N bond of nitrous oxide (N_2O). An $\{FeNO\}^6$ intermediate was observed for the *c*-heme enzyme, cytochrome P460, a nitrification pathway enzyme that oxidizes hydroxylamine (NH_2OH) to N_2O [41]:

$$2NH_2OH \rightarrow N_2O + H_2O + 4H^+ + 4e^-.$$

Evidence supported a mechanism for which N–N bond formation resulted from nucleophilic attack of $\{FeNO\}^6$ by NH_2OH. The reactive $\{FeNO\}^6$ could be formed independently by reacting Fe^{III} cyt P460 with NO. By analogy, one potential NP N–N bond formation mechanism is reaction of an amine or an organic hydroxylamine with $\{FeNO\}^6$.

Mechanistic studies of NO reductases (NORs) suggest that iron-nitrosyls other than $\{FeNO\}^6$ are competent for N–N bond formation. NORs reduce two molecules of NO to N_2O:

$$2NO + 2H^+ + 2e^- \rightarrow N_2O + H_2O.$$

The bacterial respiratory NORs, fungal cyt P450 NORs, and flavo-diiron protein (FDP) NORs have different active sites and exhibit different metal-nitrosyl intermediates prior to N–N bond formation. Bacterial respiratory NORs have a mixed heme/non-heme active site where the reactive intermediate is a heme/non-heme diferrous-dinitrosyl ($[\{FeNO\}^7]_2$) [42,43]. Fungal cyt P450 NORs have a mononuclear active site. Radical-radical coupling of NO with a ferric-hydroxylamine radical (Fe^{III}-$^{\bullet}NH_2OH$) is proposed to form the N–N bond [44]. FDP NORs have a binuclear non-heme diiron site; a binuclear non-heme $[\{FeNO\}^7]_2$ directly precedes N_2O formation [45]. The accumulated studies of these four N2O-producing enzymes show precedent for N–N bond formation by metalloenzymes with NO as a precursor. Are metalloenzymes and NO also required for NP N–N bond formation?

Recent studies show that metalloenzymes catalyze NP N–N bonds, but NO is not essential. Instead, N-oxygenation of amines, to hydroxylamine or nitroso NPs activate the nitrogen for nucleophilic attack by a second amine [39,40••]. This strategy is observed in the biosynthesis of both L-piperazic acid and hydrazinoacetic acid (Figure 3), precursors for kutzneride and, s56-p1, respectively.

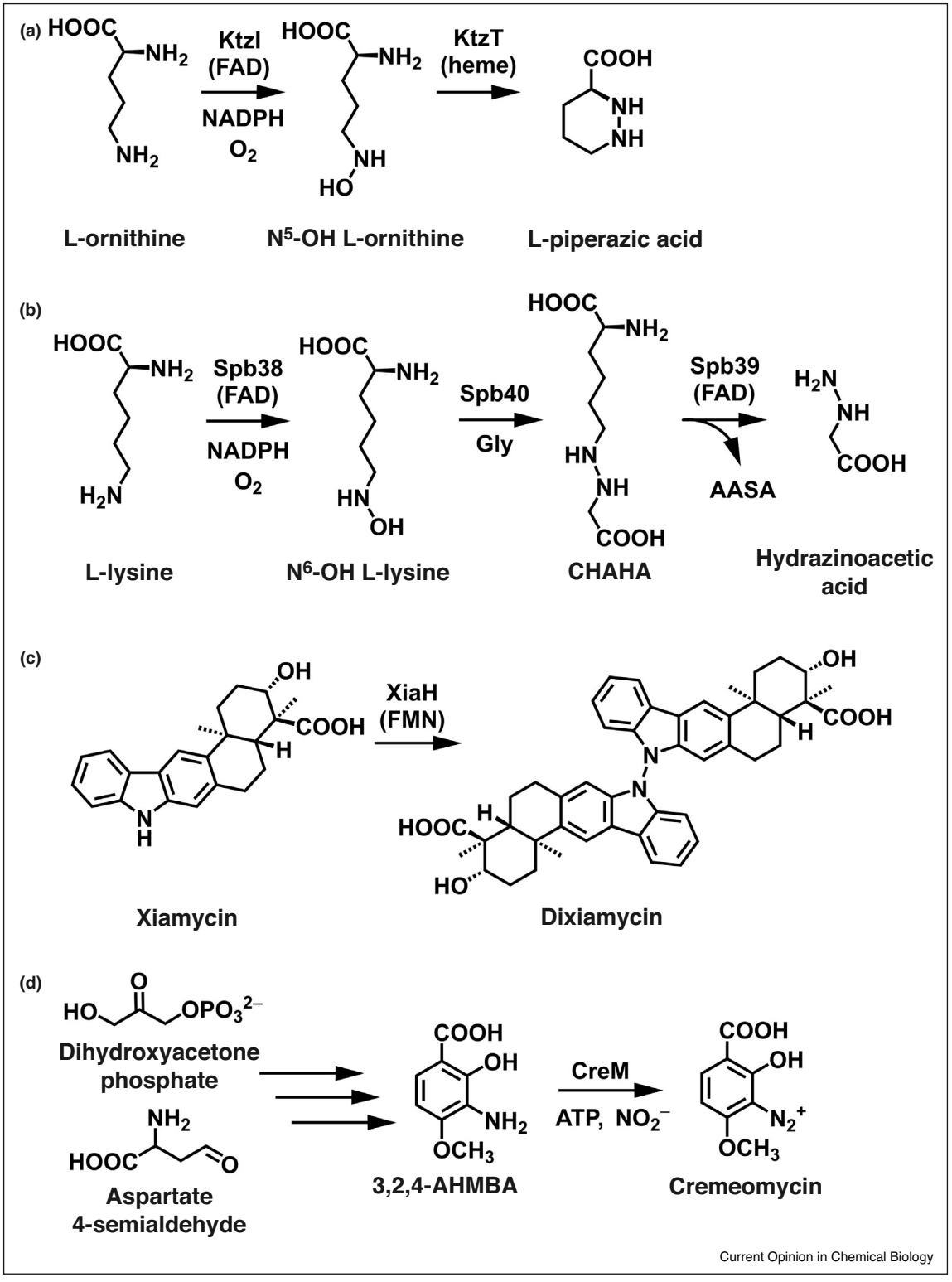
For L-piperazic acid biosynthesis, a flavin-dependent N-hydroxylase, KtzI, oxidizes L-ornithine to N^5–OH L-ornithine [46••]. A heme protein, KtzT, couples N^5–OH with the α-amino group of N^5–OH L-ornithine to form L-piperazic acid (Figure 3). This reaction is not dependent on either the heme oxidation state or the presence of O_2, suggesting against further oxidation of N^5 before N–N bond formation. Furthermore, NH_2OH inhibits L-piperazic acid formation, potentially by binding to the heme. These observations suggested that N^5–OH is activated by binding to the heme cofactor, thereby polarizing the N–O bond for nucleophilic attack by the α-amino group.

Hydrazinoacetic acid biosynthesis is also initiated by flavin-dependent hydroxylation, which catalyzes the conversion of lysine to N^6–OH L-lysine [47•]. N–N bond formation is catalyzed by, Spb40, a fusion protein composed of cupin and methionyl tRNA synthetase domains. This enzyme couples N^6–OH L-lysine with glycine to form N′-carboxymethylhydrazino-2-amino-hexanoic acid (CHAHA). This compound is rearranged by Spb39 to form hydrazinoacetic acid and L-2-aminoadipate 6-semialdehyde (AASA). Mutation of a histidine in a putative metal binding site of Spb40 abolished its activity, suggesting a metallocenter-catalyzed N–N bond formation.

Formation of NP N–N bonds do not require metalloenzymes. Formation of xiamycin dimers, or dixiamycins (Figure 2) occurs by an apparent radical initiation [48••]. A mixture of N–C and N–N-coupled dimers was observed upon incubation of xiamycin with the flavin enzyme, XiaH. This observation suggested that XiaH catalyzes the one-electron oxidation of a xiamycin monomer, monomer release, and non-enzymatic radical recombination of xiamycin monomers to form the mixture of dimers.

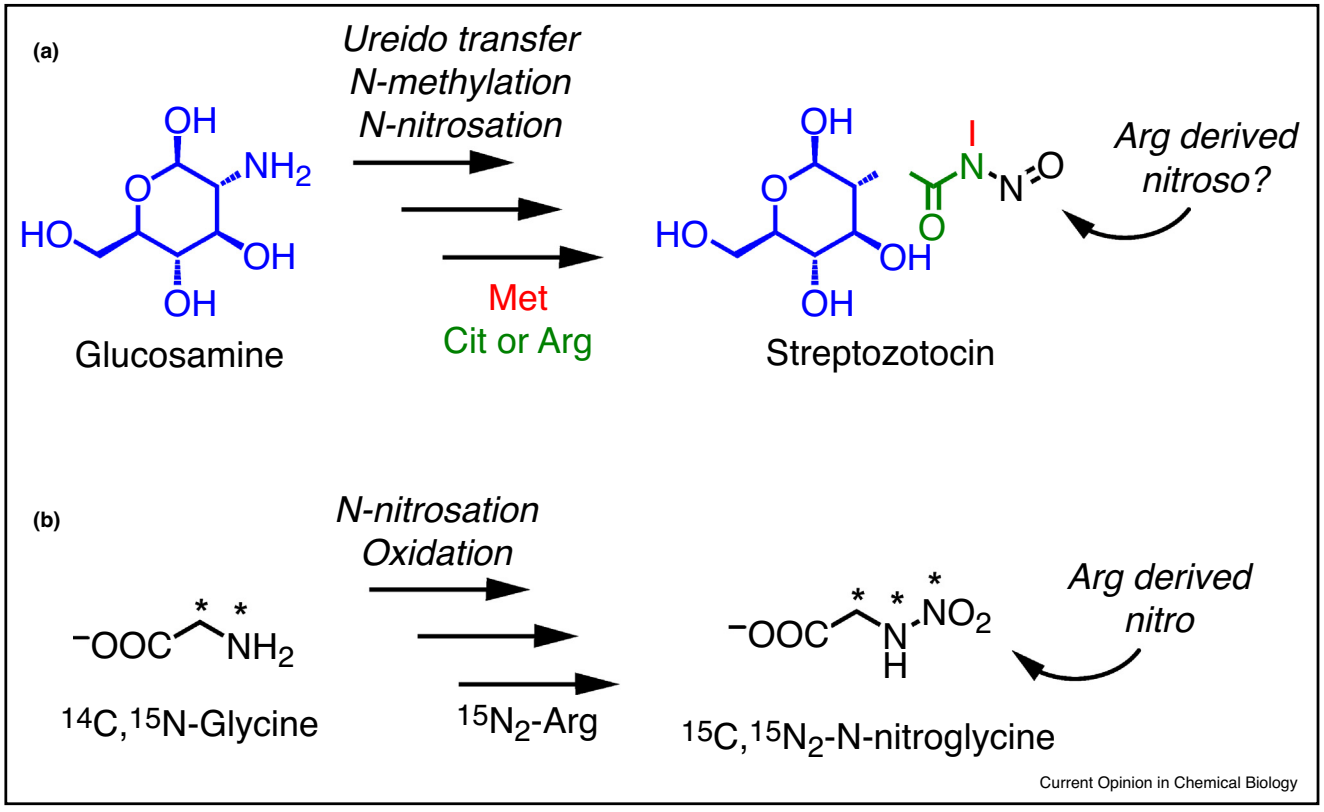
Finally, adenosine triphosphate (ATP) may activate NO_2^- to react with 3-amino-2-hydroxy-4-methoxybenzoic acid (3,2,4-AHMBA) [49••,50••,51•] to form the diazo-NP cremeomycin. Remarkably, the NO_2^- needed for diazotization originates from an aspartate oxidation pathway encoded in the cremeomycin BGC. The flavin enzyme CreE oxidizes aspartate to nitrosuccinate, which is a substrate for CreD, a lyase that produces NO_2– and fumarate [49••]. CreM is an ATP-dependent enzyme; N–N bond formation was proposed to occur via nitrite phosphorylation, which would activate nitrite for diazotization of 3,2,4-AHMBA [50••].

Figure 3



Recently elucidated biosynthetic pathways for the formation of N–N bond containing NPs. **(a)** Biosynthesis of L-piperazic acid from N^5–OH L-ornithine, the former is a precursor for kutzneride biosynthesis. **(b)** Biosynthesis of hydrazinoacetic acid, a precursor for s56-p1 biosynthesis via N^6–OH L-lysine and N′-carboxymethylhydrazino-2-amino-hexanoic acid (CHAHA). **(c)** Dimerization of xiamycin into dixiamycins by a radical

Figure 4



Proposed biosynthetic pathways of the nitrosamine, streptozotocin, and the nitramine, N-nitroglycine. Panel **(a)** shows the origin of the streptozotocin carbon backbone as determined from $^{14}C/^{3}H$ labelling experiments (Ref. [56]). Streptozotocin carbons were derived from glucosamine (blue), methionine (red), and from citrulline (Cit) or arginine (Arg) (green). Panel **(b)** shows the result of $^{13}C/^{15}N$ labelling experiments for N-nitroglycine from Ref [58]. The asterisk (*) denotes ^{13}C or ^{15}N labelled atoms.

While NO is unnecessary for many N–N bond formations, it may be necessary to form nitrosamine or nitramine NPs via N-nitrosation (Figure 4). N-nitrosation is well studied in acidic conditions where NO_2^- is converted to the nitrosating agent N_2O_3 [52]. However, nitrosamines also develop in the lower intestine where the pH is neutral or slightly basic [53]. In this environment, NO has been proposed as a nitrosating agent precursor. Furthermore, N-nitrosation in the gut has been traced to bacterial metalloenzymes [54,55]. Since NP biosynthesis largely occurs at neutral pH, NO and metalloenzymes have a role in NP N-nitrosations. This would follow the strategy for NO-dependent biosynthesis illustrated by the thaxtomin A pathway (Figure 1): a dedicated NO source and a metalloenzyme to mediate the NO-dependent chemistry. So far, bNOS is the only known NO source for NP biosynthesis, providing one gene target to identify new NO-dependent BGCs. Stable isotope labeling can also provide candidates for NO-dependent NP biosynthetic pathways. Since bNOS convert $^{15}N_2$-arginine to ^{15}NO, ^{15}N-incorporation into the nitroso or nitro group of candidate nitrosamines or nitramines, respectively, could suggest the use of NO for their biosynthesis.

One candidate is streptozotocin, a nitrosamine produced by *S. achromogenes subsp. streptozoticus*. The origins of the streptozotocin carbons were determined by $^{14}C/^{3}H$ labelling experiments (Figure 4): glucosamine provides a majority of the carbon backbone, the N-methyl group is derived from L-methionine and the ureido group can originate from either citrulline or arginine [56]. N-nitrosation to form the nitroso group was proposed to result via NO_2^- [56]. Alternatively, N-nitrosation could be NO-dependent, with the NO generated by bNOS. In this case, stable isotope labelling with $^{15}N_2$-arginine would be predicted to result in doubly ^{15}N-labelled streptozotocin;

initiated pathway. **(d)** Diazotization of 3-amino-2-hydroxy-4-methoxybenzoic acid by nitrite. Parenthesis indicate confirmed cofactors in each enzyme. FAD = Flavin adenine dinucleotide; FMN = flavin mononucleotide; ATP = adenosine triphosphate; NADPH = Nicotinamide adenine dinucleotide triphosphate; AASA = L-2-aminoadipate 6-semialdehyde.

bNOS activity would convert arginine to ^{15}NO and ^{15}N-citrulline, which would result in ^{15}N-labelling of the streptozotocin nitroso and ureido moieties, respectively. Reports on streptozotocin biosynthesis are greatly anticipated.

Another candidate for an NO-dependent biosynthetic pathway is the nitramine N-nitroglycine produced by *S. noursei* [57]. This biosynthetic pathway was proposed to occur via N-nitrosation of glycine followed by oxidation of the nitroso group to form the nitramine [39]. Recent studies supplied *S. noursei* cultures with ^{13}C,^{15}N-glycine and $^{15}N_2$-arginine, resulting in triply-labelled ^{13}C,$^{15}N_2$-nitroglycine (Figure 4B) [58]. The labelling of the nitroso group by arginine suggested that NO formed by bNOS is required for N-nitroglycine biosynthesis, however, the genome sequence of *S. noursei* lacked bNOS homologs. It was suggested that this pathway uses a novel NOS, such as an independently evolved cyt P450 homolog or a non-heme iron enzyme.

It is likely that NO sources will vary as new NO-dependent pathways are discovered. Another possible NO source is from enzymes typically found in nitrogen cycle pathways. There is precedent for the involvement of such enzymes in NP biosynthesis [59,60]. Pyrrolomycins are nitro-containing compounds, which evidently require a direct nitration step. Culture feeding studies with ^{15}N,^{18}O-NO_3^- showed incorporation of ^{18}O into both nitro oxygens [61]. Furthermore, the pyrrolomycin BGC contains genes for an assimilatory nitrate reductase [59]. The combined observations suggest NO_3^- or NO_2^- as the nitro donors. Alternative NO sources can also be envisioned in which NO_2^- reductase reduces NO_2^- to NO. Thus, nitrogen cycle enzymes may also prove to be an NO source, providing potential target genes for discovering new BGCs.

Conclusions

Prior work on NO chemistries have shown its participation in numerous reaction pathways that modify biomolecules. Nature has evolved means of harnessing this reactivity for selective biosyntheses. There appears to be at least two requirements for NO-dependent NP biosynthesis: a pathway dedicated NO donor and a metalloenzyme to mediate the NO chemistry. Studies of nitrosamine formation in the gut and of N_2O-producing metalloenzymes suggest a role for NO in the biosyntheses of nitrosamine or nitramine NPs. Until these enzymes are identified, genome mining to identify candidate NO-dependent BGCs will rely on finding dedicated NO sources. One verified NO source is bNOS. A BLAST search using the *S. turgidiscabies txtD* sequence returns over 500 results, many from *Streptomyces* species or other NP producing bacteria. This suggests that many NO-dependent pathways remain to be explored. Nitrogen cycle enzymes, such as nitrate or nitrite reductases are other potential NO sources and thus, target genes for genome mining. Discovering NO-dependent pathways will uncover new metalloenzyme reactivities, pathway intermediates, and targets for protein engineering. We will also expand our understanding of the breadth of physiological utilities for this fascinating molecule, NO.

Conflict of interest statement

Nothing declared.

Acknowledgement

JDC thanks the University of Central Florida College of Sciences and Department of Chemistry for startup funding.

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Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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