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Sodium borohydride and thiol mediated nitrite release from nitroaromatic antibiotics

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ABSTRACT

Nitroaromatic antibiotics are used to treat a variety of bacterial and parasitic infections. These prodrugs require reductive bioactivation for activity, which provides a pathway for the release of nitrogen oxide species such as nitric oxide, nitrite, and/or nitroxyl. Using sodium borohydride and 2-aminoethanol as model reductants, this work examines release of nitrogen oxide species from various nitroaromatic compounds through several characterization methods. Specifically, 4- and 5-nitroimidazoles reproducibly generate higher amounts of nitrite (not nitric oxide or nitroxyl) than 2-nitroimidazoles during the reaction of model hydride donors or thiols. Mass spectrometric analysis shows clean formation of products resulting from nucleophile addition and nitro group loss. 2-Nitrofurans generate nitrite upon addition of sodium borohydride or 2-aminoethanethiol, but these complex reactions do not produce clean organic products. A mechanism that includes nucleophile addition to the carbon βto the nitro group to generate a nitronate anion followed by protonation and nitrous acid elimination explains the observed products and labeling studies. These systematic studies give a better understanding of the release mechanisms of nitrogen oxide species from these compounds allowing for the design of more efficient therapeutics.

Nitroaromatic antibiotics, possess a long history of therapeutic use for the treatment of anaerobic bacterial and parasitic infections.[1–4] Metronidazole, a well-known and cost effective nitroaromatic antibiotic on the list of the World Health Organizations (WHO) 50 Essential Medicines, has been used since the 1950′s to treat a variety of ailments including *Heliobacter pylori* infections, trichomoniasis, giardiasis, and amoebiasis.[5–7] PA-824 (Pretomanid) was recently approved by the Food and Drug Administration (FDA) as a combination treatment of highly resistant TB.[8,9] PA-824 also shows activity against various parasites and these nitro-containing drugs hold promise as treatments for many neglected tropical diseases, such as leishmaniasis.[3,10,11] However, the absence of clear and complete molecular mechanisms of action and their associated toxicities have limited development of nitroaromatics as drug candidates despite their biological activity.

Nitroaromatic antibiotics act as prodrugs and require reductive bioactivation for activity. Nitroreductase (NTR, Type I, oxygen insensitive and Type II, oxygen sensitive) catalyzed nitro group reduction results in the formation of reduced nitrogen species including nitro radical anions, nitroso compounds, hydroxylamines or amines depending on the substrate, organism, and specific NTR. The formation of these products may also generate reactive oxygen species (ROS) or trigger the unraveling of the aromatic ring to yield reactive organic compounds.[12–15] These reactive species likely play important roles in the observed biological activity of these antibiotics.[12,16] Reductive bioactivation also provides the opportunity for the conversion of the organic nitro group (N oxidation state = +3) to reactive nitrogen species (RNS) including nitrite (NO_2^-), nitric oxide (NO), and nitroxyl (HNO). Nitric oxide, in particular, demonstrates activity against a number of micro-organisms and many NO-based approaches towards infectious disease treatment have been explored.[2,3,17–19] Isolated examples of nitroaromatic antibiotics generating RNS upon reduction exist and have been recently reviewed.[3,16] For example, metronidazole directly reacts with thiols to release NO_2^- through nitro group substitution by the thiol.[20] Reaction of metronidazole with thiols and iron generates NO as judged by electron paramagnetic resonance (EPR) spectroscopy.[21] Patients taking metronidazole metabolize it to acetamide and an oxamic acid derivative accounting for only two of the initial three N atoms of the drug.[3,16] The biological activity of PA-824 depends upon its ability to release NO or other RNS upon deazaflavin-dependent nitroreductase ($F_{420}H_2$-dependent Ddn) catalyzed hydride transfer.[22]

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These nitroaromatic antibiotics clearly release biologically active RNS upon reduction under some conditions.[16] Given the known biological activity of NO_2^-, NO, and HNO,[23–27] this chemistry may provide a powerful design element toward new therapeutics for bacterial and parasitic infections. As such, we have examined the ability of nitroaromatic antibiotics including metronidazole, PA-824, nitrofurantoin, furaltadone, benznidazole, and nitrofurazone, as well as isoniazid (a non-nitroaromatic control antibiotic) (Figure 1) to release RNS during model reduction with a hydride donor or thiol.

In this report, we systematically examine and compare these clinically important agents as RNS sources upon reaction with hydride donors and thiols using various spectroscopic and analytical techniques that allow for the identification of the RNS and organic product. Such results give insight toward the potential mechanisms of and the structural features that support RNS release in these nitroaromatic antibiotics.

Results/Discussion

For studies of the $NaBH_4$ and thiol mediated nitrogen oxide release from various nitroaromatic antibiotics, commercially available metronidazole, nitrofurantoin, furaltadone, benznidazole, nitrofurazone, and isoniazid were used as purchased (Figures S1-S7). PA-824 was synthesized according to a literature procedure (Figure S8-S10).[28] Determination of NO_2^- was accomplished using the spectrophotometric Griess assay and a commercial Nitric Oxide Analyzer (NOA), which relies on highly sensitive ozone-chemiluminescence technology, with a reaction chamber solution of KI/acetic acid.[29,30] Nitric oxide detection was accomplished using the NOA and a non-reducing buffer solution (*e.g.* PBS buffer, 100 mM, pH = 7.4) in the reaction chamber. Gas chromatographic (GC) headspace detection of nitrous oxide (N_2O) provides evidence for the formation of HNO, which rapidly dimerizes and dehydrates to yield N_2O.[29] Electrospray ionization mass spectrometric (ESI-MS) analysis of the reaction mixtures reveal the presence of organic based derivatives of the nitroaromatics (Figures S11-S23). The Supporting Information provides further details for these procedures.

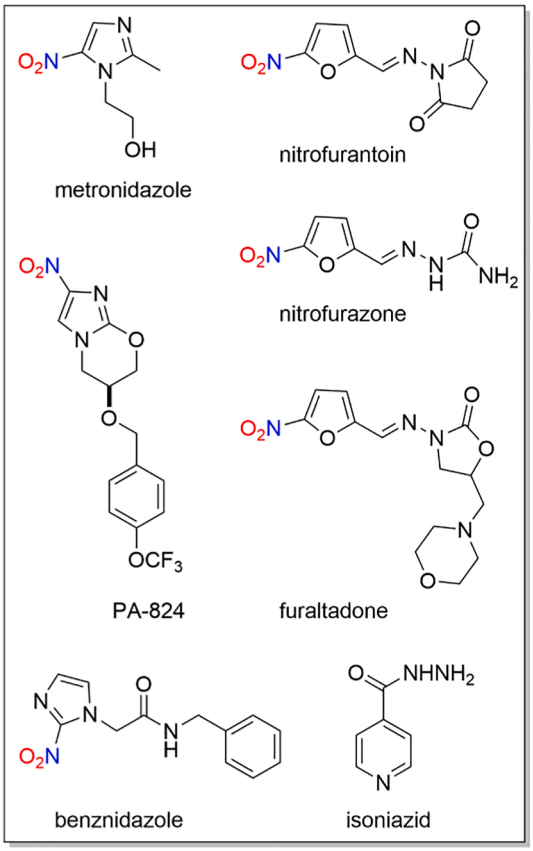


Figure 1. Nitroaromatic antibiotics used for sodium borohydride ($NaBH_4$) and thiol mediated RNS release. Isoniazid was used as a non-nitroaromatic control antibiotic.

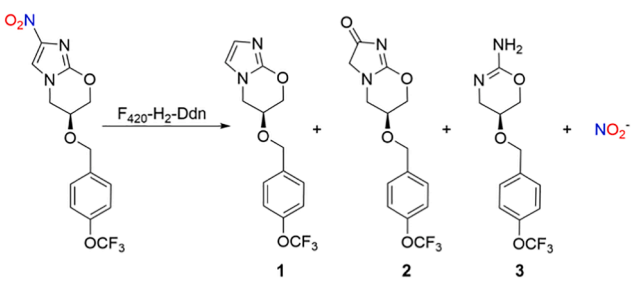
Reactions with sodium borohydride ($NaBH_4$)

The $F_{420}H_2$-dependent Ddn catalyzed reduction of PA-824 generates three organic products; the des-nitro derivative (**1**), the cyclic lactam (**2**), and **3** that may form from further reduction of an unstable hydroxylamine (Scheme 1).[22] These products form from the apparent transfer of hydride ($^-$H) to the antibiotic that also results in the release of NO_2^- and/or NO.[22] $NaBH_4$ thus provides a model hydride source to mimic this enzymatic reduction and treatment of PA-824 with $NaBH_4$ generates NO_2^- and **2** as the major organic product.[22]

Table 1 summarizes the formation of NO_2^- and nitrous oxide upon treatment of the above nitroaromatic antibiotics with $NaBH_4$ in a mixture of buffer (*e.g.* PBS buffer, 100 mM, pH = 7.4)/ethanol at 37 °C for one day. Metronidazole, a 5-nitroimidazole, shows the highest amount (~45%) of NO_2^- formation consistent with its ability to release NO_2^- upon reaction with thiols (*vide infra*).[20] In general, measurement of NO_2^- using the Griess assay or the NOA gave similar results for all compounds except PA-824, which gave lower amounts in the Griess assay suggesting the electron-rich aromatic ring of PA-824 may interfere with the assay (Table 1). For the Griess assay experiments, a 96 well plate and a UV–vis plate reader was used to rapidly screen multiple antibiotics and conditions at one time for NO_2^- release. PA-824, a 4-nitroimidazole, generates NO_2^- (28%), but benznidazole, a 2-nitroimidazole, produced less than 5% NO_2^-. The lack of NO_2^- formation from benznidazole appears consistent with previous work showing the reduction of 2-nitroimidazoles yields glyoxal and guanidine derivatives.[14] The 2-nitrofuran derivatives all gave similar amounts of NO_2^- (10–15%) upon $NaBH_4$ reduction (Table 1). Headspace NOA detection of NO (using buffer (*e.g.* PBS buffer, 100 mM, pH = 7.4) in the NOA reaction chamber) showed no significant NO formation during these reactions. GC measurements were used to probe N_2O formation as evidence of HNO release, as HNO dimerizes and dehydrates to give N_2O. While the 2-nitrofuran derivatives formed the most N_2O, none of these reductions produced more than 10% N_2O.

Control experiments showed no NO_2^- or N_2O release by the Griess Assay, NOA, or GC from these nitroaromatic antibiotics in the absence of $NaBH_4$. Further GC controls show the headspace of a solution of $NaNO_2$ and $NaBH_4$ in EtOH does not contain N_2O eliminating the borohydride reduction of NO_2^- as an HNO source. Treatment of isoniazid, a non-nitroaromatic containing antibiotic as a control, with $NaBH_4$ does not produce NO_2^-, NO or N_2O.

In addition, after reduction with $NaBH_4$, ESI-MS data was obtained for each sample to assess the loss of the nitro group and the formation of a reduced product. The reaction of metronidazole with $NaBH_4$ showed a prominent peak with a mass/charge (m/z) ratio = 127 (Figure S11), indicative of the loss of the nitro group and the formation of 2-(2-



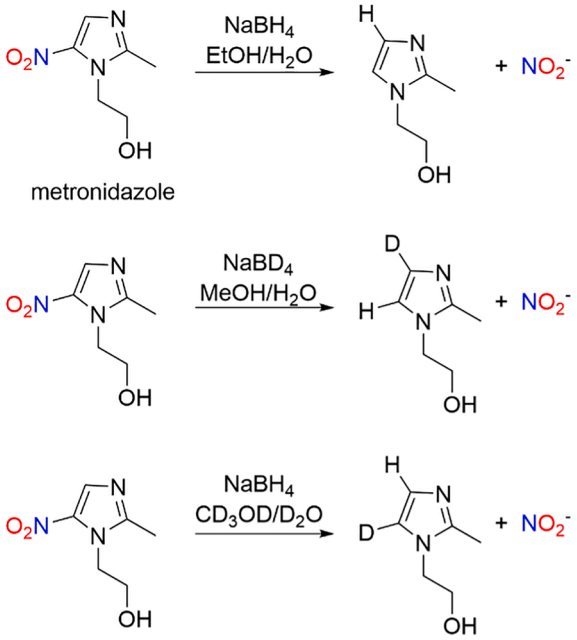
Scheme 1. PA-824 reduction products 1–3 with F_{420}-H_2-Ddn.[22.]

Table 1
Nitrogen oxide release results for nitroaromatic antibiotics treated with $NaBH_4$.

Nitroaromatic Antibiotics	Nitro Group Position	Griess Assay (NO_2^-)	NOA (NO_2^-)	GC (HNO)
Metronidazole	5	45%	43%	3%
PA-824	4	10%	28%	2%
Nitrofurantoin	2	8%	9%	8%
Furaltadone	2	14%	9%	2%
Nitrofurazone	2	13%	12%	6%
Benznidazole	2	4%	1%	3%
Isoniazid	—	—	—	—

methyl-1*H*-imidazol-1-yl)ethan-1-ol, and only a small peak for metronidazole itself (m/z = 172). Given this clean reaction profile and the large amount of NO_2^- released during the $NaBH_4$ reduction of metronidazole, further mechanistic studies on this reaction were performed. The use of deuterated reducing agents and deuterated solvents identified the position of hydride addition (Figures S24-S30). Reduction of metronidazole on a preparative scale gave 2-(2-methyl-1*H*-imidazol-1-yl)ethan-1-ol in 62% yield, which was characterized by ^{1}H NMR and ^{13}C NMR spectroscopy and through comparison to a commercial standard (Scheme 2 and Figures S26 and S27).

The ^{1}H NMR spectra of this material shows two aromatic resonances at δ = 6.7 and 6.9 that have been assigned to the protons attached to carbons 4 and 5, respectively (Figure S26). Treatment of metronidazole with sodium borodeuteride ($NaBD_4$) in ethanol led to the formation of 2-(2-methyl-1*H*-imidazol-1-yl-4-*d*)ethan-1-ol (18%) (Scheme 2) and unreacted metronidazole (82%) as confirmed by ^{1}H NMR spectroscopy (Figure S28). The ^{1}H NMR spectrum showed the intensity of the more upfield aromatic signal (δ = 6.7 ppm) to be dramatically decreased (Figure S28) suggesting deuterium incorporation at C-4 of the imidazole ring system. Treatment of metronidazole with $NaBH_4$ in deuterated methanol (CD_3OD) gave the 2-(2-methyl-1H-imidazol-1-yl-5-*d*)ethan-1-ol with a dramatic reduction in the intensity of the most deshielded aromatic signal (δ = 6.9 ppm, Figure S29, S30). The decreased proton signal implies deuterium incorporation into the imidazole, however at C-5 (Scheme 2). These results are consistent with and similar to previous results for $NaBH_4$/$NaBD_4$ reduction of PA-824, which forms **1**, **2** (major product) and NO_2^-.[22] The major peak from the reduction of PA-824 with



Scheme 2. Metronidazole reactions with $NaBH_4$ and $NaBD_4$.

$NaBH_4$ shows a m/z = 331, which suggests the formation of the lactam (**2**, Scheme 1 and Figure S12) and is consistent with the reported $NaBH_4$ reduction of PA-824 under similar conditions.[22] This ESI-MS analysis shows some remaining PA-824, but no evidence of the des-nitro product (**1**) (m/z = 315) (Figure S12).[22] The $NaBH_4$ reduction of the 2- nitrofuran, nitrofurantoin, yields a mixture of the des-nitro product (m/z = 194), starting material (m/z = 239), and evidence of a diazo dimer (m/z = 413) (Figure S13). The mass spectrum of the $NaBH_4$ reduction of nitrofurantoin is much less defined as those of the reactions of metronidazole and PA-824. Treatment of nitrofurantoin with $NaBH_4$ on a preparative scale also gave the diazo dimer as evidenced by ESI-MS (m/z = 413) (Scheme 3). Direct two-electron reduction of the nitro group to form the nitroso compound, which could dimerize and undergo further reduction, provides a possible mechanism for diazo dimer formation (Scheme 3).

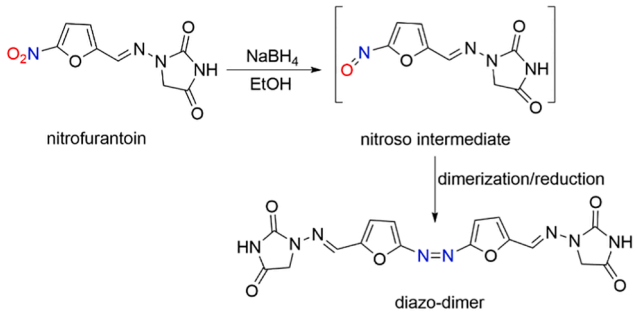
The other nitrofurans (*e.g.* furaltadone and nitrofurazone) also demonstrated poorly defined ESI-MS that showed mostly starting material (Figures S14-S15). ESI-MS analysis of the reduction of benznidazole with $NaBH_4$ did not show the formation of any products, with the starting material (m/z = 361) being the prominent species (Figure S16).

Reactions with thiols

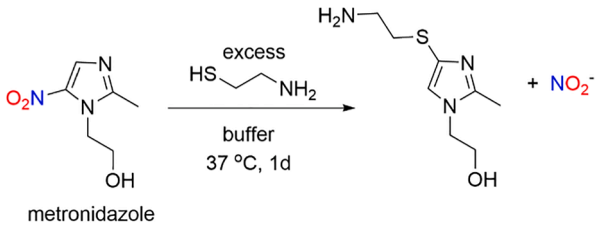
Similar to the reaction of PA-824 with $NaBH_4$, the treatment of metronidazole with 2-aminoethanethiol yields 4-[(2-aminoethyl)thio]-2-methylimidazole-1-ethanol and nitrous acid (nitrite) (Scheme 4).[20]

This study confirmed the release of nitrogen oxides from metronidazole and structurally similar compounds upon nucleophilic substitution of the nitro group of 5-nitroimidazoles by thiols.[20] Under more basic conditions, an isomeric imidazole product forms and a plausible mechanism may be direct thiol addition to the nitroimidazole forming a Meisenheimer-type complex that generates nitrous acid and restores aromaticity upon protonation.[20] These results clearly show direct nitrogen oxide release from metronidazole and supports the use of 2-aminoethanethiol as a model reductant for nitrogen oxide release from nitroaromatic antibiotics. In these experiments, the aforementioned nitroaromatic antibiotics were treated with excess 2-aminoethanethiol in buffer (*e.g.* PBS buffer, 100 mM, pH = 7.4) at 37 °C for one day (Scheme 4), followed by nitrogen oxide release measurements utilizing the Griess Assay, NOA, and GC.

Table 2 displays the results for the release of nitrogen oxides upon thiol addition to the nitroaromatic antibiotics. As expected, metronidazole shows robust amounts (36–48%) of NO_2^- formation.[20] Interestingly, the addition of thiol to PA-824, a 4-nitroimidazole, liberates smaller but reproducible amounts of NO_2^- indicating a potentially new mechanism for nitrogen oxide release from this drug. As before, benznidazole only produced 1% NO_2^- under these conditions, consistent with its poor RNS release profile.[14] The 2-nitrofuran derivatives, nitrofurantoin and nitrofurazone both generated significant amounts of NO_2^- (~45%) similar to that seen with metronidazole, but furaltadone only gave ~ 10% NO_2. Headspace NOA detection of NO (using buffer (*e.g.* PBS buffer, 100 mM, pH = 7.4) in the NOA reaction chamber) showed no



Scheme 3. Reduction of nitrofurantoin leading to a diazo-dimer product.



Scheme 4. Metronidazole reaction with 2-aminoethanethiol.

Table 2
Nitrogen oxide release results for nitroaromatic antibiotics treated with 2-aminoethanethiol.

Nitroaromatic Antibiotics	Nitro Group Position	Griess Assay (NO_2^-)	NOA (NO_2^-)	GC (HNO)
Metronidazole	5	36%	48%	2%
PA-824	4	7%	17%	2%
Nitrofurantoin	2	48%	41%	2%
Furaltadone	2	9%	9%	2%
Nitrofurazone	2	48%	44%	3%
Benznidazole	2	1%	1%	2%
Isoniazid	—	—	—	—

significant NO formation during these reactions. GC measurements were used to probe N_2O formation as evidence of HNO release as before. None of these compounds under these conditions produced more than 3% N_2O. Low N_2O amounts in these experiments could indicate the lack of HNO formation or result from the reaction of HNO with excess thiol to generate the corresponding disulfide and hydroxylamine.[31] Control experiments showed no NO_2^- or N_2O release by the Griess Assay, NOA, or GC from these nitroaromatic antibiotics in the absence of thiol. Treatment of isoniazid with 2-aminoethanethiol does not produce NO_2^-, NO or N_2O.

ESI-MS measurements provides evidence that 2-aminoethanethiol adds to each of the nitroaromatic antibiotics with the loss of the nitro group, however some reactions provided much cleaner more efficiently ionized spectra than others (Figures S17-S23). Metronidazole showed a prominent peak at $m/z = 202$, with a small peak for metronidazole itself ($m/z = 172$), indicative of a thiol adduct (98%) and nitro group loss, as expected (Figure S17).[20] While some PA-824 remains upon treatment with 2-aminoethanethiol as judged by ESI-MS ($m/z = 360$), this reaction mixture also showed a thiol adduct with loss of the nitro group at $m/z = 390$, supporting the notion of thiol-mediated NO_2^- release (Figure S19). ESI-MS experiments with nitrofurantoin revealed a prominent peak at $m/z = 283$ indicative of thiol addition/nitro group loss, as well as starting material ($m/z = 239$) (Figure S20). Although these results are promising for nitrofurantoin, the known bioactivation of nitrofurantoin remains poorly understood and speculation remains whether it produces RNS rather than ROS.[32,33] The reaction of furaltadone with 2-aminoethanethiol, which was poorly ionized, also showed starting material ($m/z = 325$), with very little evidence of the thiol adduct ($m/z = 355$), which corresponds to the small amounts of NO_2^- measured (Figure S21). While nitrofurazone did not show strong evidence of the thiol adduct ($m/z = 229$), the starting material peak ($m/z = 199$) is not pronounced by ESI-MS, which corresponds with the significant NO_2^- release measured (Figure S22). For benznidazole, the starting material ($m/z = 361$) is the most prominent peak in the ESI-MS, which supports the lack of NO_2^- release as evident in both the Griess Assay and NOA experiments (Figure S23).

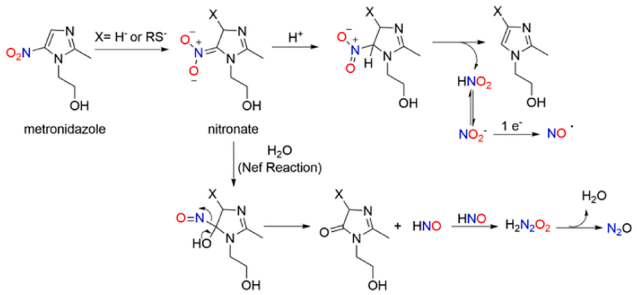
Metronidazole was also treated with the more biologically relevant thiol, glutathione (GSH) using the same reaction conditions developed with 2-aminoethanethiol (*vide supra*). ESI-MS formation of a thiol adduct for metronidazole-GSH showed the best evidence of a reaction, with a prominent peak ($m/z = 432$) corresponding to a metronidazole-GSH adduct with loss of the nitro group (Figure S18). These results contrast older work that indicates metronidazole does not generate NO_2^- or GSH adducts in rat and human liver homongenates.[34] Despite the promising ESI-MS results with GSH and metronidazole, difficulties arose during the assessment of NO_2^- release from these reactions using the Griess assay or NOA as GSH appeared to interfere with NO_2^- detection giving low and irreproducible results. This interference may be related the differences in pKa's for 2-aminoethanethiol and GSH, the buffer composition and strength and the expected decrease in pH upon release of HNO_2. The reactivity of GSH with RNS (NO, NO_2^-, HNO) is complex and may complicate these results.[35,36] For example, the formation of NO_2^- under slightly acidic conditions in the presence of excess GSH could generate S-nitrosoglutathione (GSNO) thus masking NO_2^- detection.[37] Further work will clarify NO_2^-/NO release from the GSH reactions as well as GSNO production, but the ESI-MS clearly shows the ability of this important biological thiol to directly react with metronidazole.

Scheme 5 (with metronidazole) depicts a possible mechanism for NO_2^- release in these reactions where hydride ion or thiol adds to the carbon β to the nitro group to generate a nitronate anion that is protonated by the solvent. Elimination of nitrite/nitrous acid from this species forms the observed products and a reactive nitrogen species (HNO_2/NO_2^-) and the isotopic labeling experiments support this pathway. Nucleophile addition to the carbon β to the nitro group as opposed to other sites allows for resonance stabilization of the resulting anion. Addition at other sites, forming a Meisenheimer-type complex that generates nitrous acid and restores aromaticity upon protonation as previously proposed,[20] would account for isomeric addition products. 4- and 5-Substituted imidazoles appear particularly suited to this reactivity compared to 2-substituted imidazoles or 2-nitrofurans. Scheme 5 provides a mechanistic framework for consideration in the design of 4- and 5-nitroimidazoles capable of NO_2^- release.

While not observed as predominant products in the true reaction, Scheme 5 also outlines the potential mechanism of NO and HNO formation. Further one electron reduction of nitrite, especially under acidic conditions, would yield NO.[16] Hydrolysis of the nitronate (Nef Reaction)[38] gives a dihydroxy nitroso compound (Scheme 5) that would decompose to lactam and HNO. While the lactam product 2 was observed during the $NaBH_4$ reduction of PA-824 (Scheme 1), N_2O as evidence of HNO, was not detected. Given the reactivity of HNO, with the absence of N_2O formation, could indicate HNO trapping by another species (RSH, $NaBH_4$). In these simple studies, NO_2^- appears to be the major RNS formed.

Conclusion

These systematic studies show release of nitrogen oxides species from various nitroaromatic antibiotics upon reaction with $NaBH_4$ or thiols. Specifically, 4- and 5-nitroimidazoles reproducibly generate higher amounts of NO_2^- (not NO or HNO) during the reaction of model hydride donors or thiols. Mass spectrometric analysis shows clean formation of products resulting from nucleophile addition and nitro group loss. Of particular interest is the formation of NO_2^- from the $NaBH_4$



Scheme 5. Possible mechanism for NO_2^- release from metronidazole.

reduction of metronidazole and the reaction of 2-aminoethanethiol with PA-824, which reveal new pathways of RNS release from these clinically used drugs. Scheme 5 illustrates a simple nucleophilic addition-nitrous acid (nitrite) elimination that accounts for these reactions. While 2-nitrofurans generate NO_2^- upon addition of $NaBH_4$ or 2-aminoethane thiol, these reactions appear much more complex and do not produce a clean organic product. In future studies, other nitroaromatic compounds can be examined as well as other reductants, including nitroreductases or other reducing enzymes. The reaction of GSH with metronidazole signals that GSH and other biologically relevant low molecular weight (LMW) thiols, such as bacillithiol (BSH) and mycothiol (MSH),[39–41] may react with these compounds. The release of NO_2^-, NO, and HNO could incite the continued development and attention to the potential for controlled bioactivation through the design of nitroaromatic antibiotics by organism-specific reducing systems, which can lead to the delivery of biologically active nitrogen oxide agents with high activity and minimal toxicity.

Declaration of Competing Interest

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.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2021.128245.

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