Bioorganic Chemistry 93 (2019) 103318

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Synthesis of novel nitroreductase enzyme-activated nitric oxide prodrugs to site-specifically kill bacteria

Hailey A.J. Hibbard[a], Melissa M. Reynolds[a,b,*]

[a] Department of Chemistry, Colorado State University, Fort Collins, CO 80523, USA
[b] School of Biomedical Engineering, Colorado State University, Fort Collins, CO 80523, USA

ARTICLE INFO

Keywords:
Nitric oxide
Antibacterial
Nitroreductase
Diazeniumdiolate
Prodrug
Antimicrobial

ABSTRACT

The developing antibiotic resistance crisis creates a serious need for new antimicrobial agents. In this work, novel nitroaromatic-protected piperazine diazeniumdiolate (nitric oxide donor) prodrugs are synthesized to release nitric oxide upon enzyme activation to kill bacteria. Antibacterial prodrugs could help reduce side effects due to antibiotics, only releasing the therapeutic where infections are concentrated. The nitroreductase enzyme, which is found almost exclusively in bacteria, reduces the nitroaromatic-protecting group of the synthesized compounds and catalyzes the release of nitric oxide. This paper shows that nitric oxide release from the synthesized compounds only occurs in the presence of a bacteria-derived nitroreductase enzyme, demonstrating the possibility of site-specific delivery of an antibacterial therapeutic. The amount of nitric oxide release is measured at concentrations of 0.01, 0.1, and 1 mM, and is well within antibacterial levels at concentrations of 0.1 and 1 mM. The antibacterial activity of the compounds is demonstrated after exposure of the compounds to *Escherichia coli*, a nitroreductase-producing bacterial species, leading to up to a 94% reduction in the number of viable bacteria after 24 h at 1 mM concentrations of the prodrug. This study is the first example of an antibacterial diazeniumdiolate prodrug activated by a nitroreductase enzyme, and further demonstrates the possibilities of antibacterial prodrugs.

1. Introduction

The alarming trend of increasing antibiotic resistance in bacteria illustrates the dire need for new antibiotics. One major cause of the rise in antibiotic-resistant strains of bacteria is the overuse of antibiotics [1]. Enzyme-activated antibacterial agents, antibiotic prodrugs that only release the active antibiotic when bacteria are present, can help overcome this problem [2,3]. In this work, we show that the bacterial enzyme nitroreductase causes the release of nitric oxide, a known antimicrobial agent, from a novel prodrug to kill bacteria.

Nitroreductases are a well-studied class of enzymes that reduce nitro groups to hydroxylamine or amine moieties [4,5]. They have been used in biocatalysis applications to reduce nitro groups [6–8]. These enzymes have also been used in prodrug applications in ADEPT, VDEPT, and GDEPT for cancer treatments to activate the release of anti-cancer therapeutics [9–14]. Nitroreductases are found in bacteria and very few eukaryotes, but not found in humans [15]. For this reason, nitroreductases could be used to catalyze the release of an antibiotic site-specifically in humans, concentrating the active drug compound in the location of an infection. The compound would only release the therapeutic in large amounts if an infection develops, preventing the overuse of antibiotics, addressing the issue of bacteria developing antibiotic resistance, and potentially reducing or eliminating side effects from antibiotics. Nitroreductase enzymes have previously been used to reduce nitro-containing antibiotics, such as nitrofurazone and nitrofurantoin, demonstrating the viability of this study [16]. In this paper, we use the *Escherichia coli*-derived nitroreductase NfsB, an oxygen-insensitive enzyme, to reduce the nitro group of a prodrug, causing the release of a therapeutic, nitric oxide.

Nitric oxide (NO) has been shown to have potent antibacterial effects. It kills bacteria via multiple mechanisms by reacting with oxygen and superoxide species in the body, forming reactive nitrogen and oxygen species, which can alkylate DNA and inhibit enzyme function in bacteria, among other detrimental effects [17,18]. Studies have shown that bacteria are unable to develop resistance to exogenously-applied NO after 21 days [19]. In addition, NO has a very short half-life, which could minimize side effects from the antibiotic [20]. For these reasons, NO is of interest to be used in new antibiotics.

Enzyme-activated nitric oxide-releasing prodrugs have previously been synthesized, typically employing diazeniumdiolates as the NO

* Corresponding author at: Department of Chemistry, Colorado State University, Fort Collins, CO 80523, USA.
E-mail address: Melissa.Reynolds@colostate.edu (M.M. Reynolds).

https://doi.org/10.1016/j.bioorg.2019.103318
Received 11 July 2019; Received in revised form 18 September 2019; Accepted 26 September 2019
Available online 26 September 2019
0045-2068/

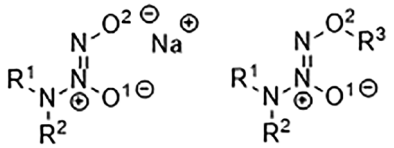


Fig. 1. General structure of unprotected (left) and protected (right) diazeniumdiolate functional group.

donor [21]. Diazeniumdiolates are a type of NO-releasing functional group (left in Fig. 1), releasing two equivalents of NO per one equivalent of diazeniumdiolate spontaneously in biological medium and characteristically have predictable half-lives for NO release [22]. A few antimicrobial diazeniumdiolates have been published which release NO spontaneously, but due to the multiple roles NO plays in the body, spontaneous NO release can have serious side effects [23–25]. By attaching a group to the O^2 position of a diazeniumdiolate (R^3, right in Fig. 1), spontaneous NO release can be slowed or even prevented entirely [21]. Examples include cytochrome p450 enzymes, glycosidases, or esterases to activate the release of NO from O^2-protected diazeniumdiolates [26–28]. However, there are very few examples of antimicrobial diazeniumdiolate prodrugs, with just two examples in which Collins, Allan, and coworkers use transpeptidases or β-lactamases to activate NO release for anti-biofilm applications [29,30].

Precedence for nitroreductase-activated diazeniumdiolates is demonstrated by the Chakrapani group, where they use nitroaromatic-protected diazeniumdiolates for applications in cancer therapeutics [31]. In this study, we synthesize antibacterial nitroaromatic-protected piperazine diazeniumdiolates. Some piperazines have antibacterial activity, depending on substituents, which could further enhance the antibacterial activity of our compounds [32,33]. We demonstrate in this work that a nitroreductase enzyme derived from *Escherichia coli* catalyzes the release of NO. In the absence of the enzyme, no NO is released, demonstrating that NO release is specific to the presence of the nitroreductase enzyme. Excitingly, when *E. coli* is exposed to our synthesized diazeniumdiolates, significant reductions in bacteria are observed. This is the first report of antibacterial nitroreductase-activated diazeniumdiolates, demonstrating the potential use of antibiotic prodrugs for site-specific delivery of therapeutics to treat bacterial infections.

2. Materials and methods

2.1. Chemicals

Ethyl 1-piperazinecarboxylate (99%), 4-nitrobenzyl bromide (99%), 15-crown-5 (98%), nitroreductase from *Escherichia coli* (90%), and β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH, 97%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 1-(*tert*-Butoxycarbonyl)piperazine (98%) and 1-Carbobenzoxypiperazine (95%) were purchased from TCI (Cambridge, MA, USA). Nitric oxide (99%) was purchased from Matheson Tri-gas (Montgomeryville, PA, USA). Sodium methoxide (30% in methanol) was purchased from Acros Organics (Geel, Belgium). Oxoid nutrient broth (OXCM0001B) and Oxoid nutrient agar (OXCM0003B) were purchased from Fisher Scientific (Hampton, NH, USA). *Escherichia coli* (ATCC 25922) was obtained from American Type Culture Collection (ATCC, USA). Argon (ultrahigh purity), nitrogen (ultrahigh purity), and oxygen were purchased from Airgas (Denver, CO, USA). Deionized water (18.2 MΩ·cm) was obtained from a Millipore Direct-Q water purification system (EMD Millipore, Billerica, MA, USA).

2.2. Abbreviations

The following abbreviations are used throughout: rt = room temperature, h = hour, min = minute, THF = tetrahydrofuran, 15-crown-5 = 1,4,7,10,13-Pentaoxacyclopentadecane, NaOH = sodium hydroxide, DCM = dichloromethane, Na_2SO_4 = sodium sulfate, EtOAc = ethyl acetate, SiO_2 = silica, PBS = phosphate-buffered saline, DMSO = dimethyl sulfoxide, Boc = *tert*-Butyloxycarbonyl, Cbz = carboxybenzyl.

2.3. Instruments

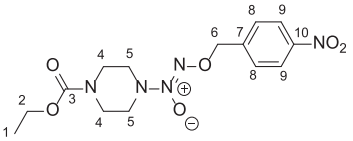
All nuclear magnetic resonance (NMR) spectra were obtained on Varian Inova 400 (400 MHz for ^{1}H; 101 MHz for ^{13}C) and/or Bruker US400 (400 MHz for ^{1}H; 101 MHz for ^{13}C) at room temperature unless noted otherwise. Chemicals shifts were reported in parts per million (δ scale) and referenced according the following standards: chloroform residual signal (δ 7.16) for ^{1}H signals; deuterated chloroform carbon resonances (middle peak is δ 77.1) for ^{13}C signals. Coupling constants were reported in Hertz (Hz) and multiplicities were reported as follows: singlet (s), doublet (d), doublet of doublets (dd), triplet (t), quartet (q), and multiplet (m). IR spectra were obtained on a Thermo Scientific Nicolet 6700 FT-IR spectrometer. Electrospray ionization mass spectrometry data were obtained on an Agilent 6224 TOF mass spectrometer equipped with a dual electrospray ion source operated in positive and negative mode. Melting points were obtained with an Electrothermal Mel-Temp apparatus. UV–Vis measurements were obtained with a Thermo Scientific Nicolet Evolution 300 spectrometer. Reactions were analyzed by thin layer chromatography (TLC) on aluminum sheets that were pre-coated with silica gel 60 F_{254}, and the reactions were purified by column chromatography using Alfa Aesar silica gel 60 (0.06–0.2 mm).

2.4. Experimental procedures

Diazeniumdiolates were synthesized according to previously reported procedures [34]. In brief, the piperazine starting material (**1a-c**, 1 equiv.) was stirred to dissolve in anhydrous methanol (2.1 M) in a high pressure reaction vessel. Sodium methoxide (30% in methanol, 1 equiv.) was added. The reaction vessel was attached to a high-pressure stainless-steel nitric oxide reactor system, purged with high purity argon, then charged with nitric oxide gas (80 psi, 99%). After stirring under pressure for 24 h, the white precipitate that formed was washed with methanol, then diethyl ether and dried under vacuum for 1 h. The products **2a-c** were stored at −20 °C until further use. Spectra matched the previously reported values.

2.4.1. General procedure for synthesis of O^2–(4-nitrobenzyl) diazeniumdiolates (2a-c)

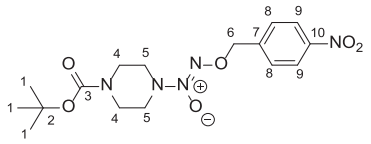
To a 0 °C solution of diazeniumdiolate (**2a-c**, 0.75 mmol, 1.5 eq.) in anhydrous THF (0.1 M), 15-crown-5 (1 mmol, 2 eq.) was added. After 30 min stirring at 0 °C, 4-nitrobenzyl bromide (0.5 mmol, 1 eq.) was added and the reaction was allowed to warm to rt. After stirring for 2–4 h, the reaction mixture was concentrated *in vacuo* to remove THF. The crude product was purified by flash column chromatography, affording the title compound.



2.4.2. O^2-(4-Nitrobenzyl) 1-(4-ethoxycarbonylpiperazin-1-yl)diazen-1-ium-1,2-diolate, 3a

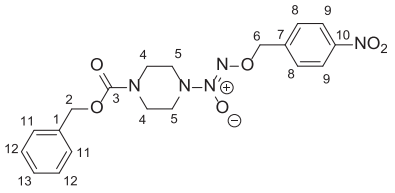
Following the general procedure, stirring for 4 h, prior to column chromatography purification, the crude mixture was dissolved in DCM (10 mL) and NaOH (1 M, 10 mL) was added. The aqueous layer was extracted with DCM (3 × 10 mL), then the combined organic layers were washed sequentially with deionized water (10 mL) and brine (15 mL), then dried over Na_2SO_4 and concentrated *in vacuo* to afford a yellow oil. After purification by column chromatography (SiO_2, 10%,

30% EtOAc in hexanes gradient), **3a** is collected as a pure pale yellow crystalline solid (128–154 mg, 79 ± 7%, n = 4). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.15 (d, J = 8.5 Hz, 2H, H9), 7.46 (d, J = 9.5 Hz, 2H, H8), 5.24 (s, 2H, H6), 4.07 (q, J = 7.1 Hz, 2H, H2), 3.56 (t, J = 5.2 Hz, 4H, H4), 3.30 (t, J = 5.1 Hz, 4H, H5), 1.19 (t, J = 7.1 Hz, 3H, H1). ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.04 (C3), 147.98 (C10), 142.81 (C7), 128.66 (C8), 123.87 (C9), 73.94 (C6), 61.87 (*C*2), 51.01 (C5), 42.33 (C4), 14.59 (C1). HRMS (+ESI) Found 354.1408 (Calcd. 354.1414 for $C_{14}H_{19}N_5O_6$; $[M+H]^+$). UV (DCM) λ_{max} (ε) 241 nm (30.4 $mM^{-1}cm^{-1}$); 263 nm (15.8 $mM^{-1}cm^{-1}$). m.p. 105.5–108 °C. ν_{max}/cm^{-1} 3110 w, 3081 w, 2876 br, 1683 s, 1602 m, 1519 s, 1423 m, 1343 s, 1221 s, 1121 s, 1031 s, 956 s, 848 s, 739 s.



2.4.3. O^2-(4-Nitrobenzyl) 1-(4-tert-butyoxycarbonylpiperazin-1-yl)diazen-1-ium-1,2-diolate, ***3b***

Following the general procedure stirring for 2 h, after purification by column chromatography (SiO_2, 5%, 15%, 40% EtOAc in hexanes gradient), **3b** is collected as a pure pale yellow crystalline solid (46–123 mg, 42 ± 21%, n = 3). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.24 (d, J = 8.7 Hz, 2H, H9), 7.55 (d, J = 8.7 Hz, 2H, H8), 5.31 (s, 2H, H6), 3.58 (t, J = 6 Hz, 4H, H4), 3.35 (t, J = 6 Hz, 4H, H5), 1.46 (s, 9H, H1). ^{13}C NMR (101 MHz, $CDCl_3$) δ 154.17 (C3), 147.98 (C10), 142.85 (C7), 128.65 (C8), 123.85 (C9), 80.57 (*C*2), 73.91 (C6), 51.07 (C5), 42.32 (C4), 28.31 (C1). HRMS (+ESI) Found 382.1721 (Calcd. 382.1727 for $C_{16}H_{23}N_5O_6$; $[M+H]^+$). UV (DCM) λ_{max} (ε) 240 nm (32.8 $mM^{-1}cm^{-1}$); 264 nm (12.6 $mM^{-1}cm^{-1}$). m.p. 132–135 °C. ν_{max}/cm^{-1} 2980 br, 2932 br, 1677 s, 1604 w, 1521 s, 1406 s, 1343 s, 1224 m, 1124 s, 1033 s, 845 s, 738 s.



2.4.4. O^2-(4-Nitrobenzyl) 1-(4-benzyloxycarbonylpiperazin-1-yl)diazen-1-ium-1,2-diolate, ***3c***

Following the general procedure stirring for 2 h, after purification by column chromatography (SiO_2, 15%, 40% EtOAc in hexanes gradient), **3c** is collected as a pure pale yellow crystalline solid (159–184 mg, 81 ± 7%, n = 3). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.16 (d, J = 8.7 Hz, 2H, H9), 7.47 (d, J = 8.8 Hz, 2H, H8), 7.28 (m, 5H, *H*11, H12, H13), 5.24 (s, 2H, H6), 5.06 (s, 2H, H2), 3.60 (m, 4H, H4), 3.30 (m, 4H, H5). ^{13}C NMR (101 MHz, $CDCl_3$) δ 154.80 (C3), 147.98 (C10), 142.80 (C7), 136.17 (C1), 128.67 (C8), 128.58 (C12), 128.29 (C13), 128.07 (C11), 123.86 (C9), 73.95 (C6), 67.62 (*C*2), 50.97 (C5), 42.45 (C4). HRMS (+ESI) Found 416.1564 (Calcd. 416.1570 for $C_{19}H_{21}N_5O_6$; $[M+H]^+$). UV (DCM) λ_{max} (ε) 240 nm (32.8 $mM^{-1}cm^{-1}$); 264 nm (13.0 $mM^{-1}cm^{-1}$). m.p. 74–77 °C. ν_{max}/cm^{-1} 2935 w, 2850 br, 1704 s, 1605 w, 1513 s, 1439 s, 1348 s, 1215 s, 1126 s, 1013 s, 848 m, 744 s, 698 s.

2.5. Nitric oxide release measurements

Nitric oxide generation was recorded in real time using a chemiluminescence-based GE Nitric Oxide Analyzer (NOA). Before each use, the NOA was calibrated using a 43.83 ppm NO calibration gas cylinder (Airgas). For the experiments to determine NO release at different concentrations, solutions of **3a-c** in DMSO were made (1, 10 and 100 mM). PBS (1480 μL, 10 mM in Millipore water), NADPH (400 μL, 10 mM in PBS), and the corresponding **3a-c** solution (20 μL, 1, 10, or 100 mM in DMSO) to a final concentration of 0.01, 0.1, and 1 mM are added to NOA cell in a 37 °C water bath. The solution is purged continuously with ultrapure nitrogen gas for 5–10 min to collect a baseline release. Nitroreductase enzyme (50 μL, 1 mg/mL in PBS) is then added through the side injection port of the NOA vessel. The solution is purged continuously with ultrapure nitrogen gas with bubbling rate of 55 mL/min directly into the solution and flow gas introduced into the remaining headspace for the duration of the experiment. NO release is recorded in 15 s intervals as parts per billion. NO release data is presented as the average of at least 3 trials ± standard deviation.

2.6. NADPH consumption Studies

A baseline spectrum is collected of a solution of 100 μM **3a** (10 μL, 10 mM solution in DMSO) in PBS (970 μL, 10 mM in Millipore water). NADPH (10 μL, 20 mM in PBS) is added to a concentration of 200 μM, and a time zero spectrum from 200 to 400 nm was collected, as well as a spectrum after 5 min as a control to ensure that NADPH absorbance does not decrease over time without enzyme present. Nitroreductase enzyme (10 μL, 1 mg/mL in PBS) is added and data is collected in cycles in intervals (approximately every 5 s for 60 cycles) from 335 to 345 nm at rt. Absorbances are collected at 340 nm and plotted over time. The data is presented as the average of 3 trials ± standard deviation.

2.7. Bacteria Studies

2.7.1. Escherichia coli bacteria culture

Initial stock culture of *Escherichia coli* was made by reconstituting lyophilized bacteria in warm nutrient broth media (NBM, 13 g nutrient broth/1 L Millipore water) and grown overnight at 37 °C and 150 rpm until an optical density at 600 nm (OD_{600nm}) ≈ 1.0 was reached. This bacterial solution was combined with glycerol (30% v/v) in a 1:1 fashion to obtain a final glycerol concentration of 15% (v/v). These solutions were stored at −80 °C until use. Prior to each bacterial experiment, a frozen culture was thawed at rt and then centrifuged at 4700 rpm for 10 min. The supernatant was discarded, and the pellet was resuspended in NBM. This was transferred to additional NBM and allowed to grow overnight with shaking at 120 rpm until the OD_{600nm} ≈ 1.0. The culture was diluted to an OD_{600nm} ≈ 0.3–0.35 using warmed NBM prior to beginning experiments.

2.7.2. Bacterial viability

The previously made **3a-c** solutions in DMSO (10 μL; 1, 10 or 100 mM) were added to *E. coli* culture (10^7 CFU/mL) in NBM (990 μL, OD_{600nm} ≈ 0.3–0.35) in 1.5 mL Eppendorf microcentrifuge tubes to final concentrations of 0.01, 0.1, and 1 mM. The positive control was *E. coli* culture in NBM (1 mL, OD_{600nm} ≈ 0.3–0.35). The solutions were placed in a static 37 °C incubator for 24 h. Agar plates were made by adding 15 mL of sterilized nutrient agar [powder base (14 g) in Millipore water (500 mL)] to sterile Petri dishes and allowed to set for at least 2 h. After 24 h exposure, the *E. coli* solutions underwent serial tenfold dilution with sterile NBM to reach 10^4 or 10^5 dilution factors, and the agar plates were inoculated with 20 μL of the diluted *E. coli* solutions using a sterile plastic or glass spreader. The agar plates were placed in a static 37 °C incubator overnight and colony-forming units (CFUs) were counted after 24 h (n = 9). The number of CFUs were calculated using the equation:

$$\frac{CFU}{mL} = \frac{(\#CFU\ \ counted)(Dilution\ \ factor)}{Volume\ \ plated(mL)}$$

The percent reduction in the number of viable bacteria was calculated as follows:

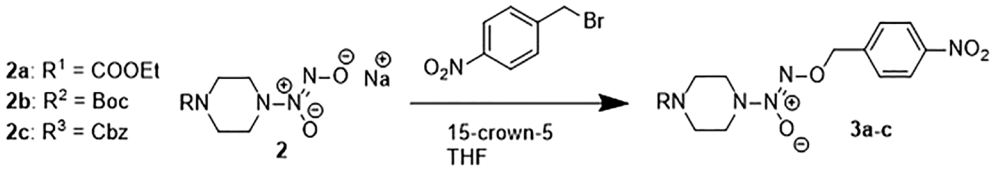


Fig. 2. Reaction scheme showing protection of the piperazine diazeniumdiolate **2** with a nitroaromatic group. Boc = *tert*-Butyloxycarbonyl, Cbz = carboxybenzyl, THF = tetrahydrofuran, 15-crown-5 = 1,4,7,10,13-Pentaoxacyclopentadecane.

$$\%\ reduction = \frac{CFU\ \ after\ \ exposure/ml}{CFU\ positive\ control/ml}$$

2.7.3. Statistical analysis

All biological experiments were performed using at least nine samples. Viability assays are reported as the mean and 95% confidence interval. All data were evaluated for potential outliers using the Grubbs Test. The statistical differences in data were evaluated using the Student's *t* test ($p < 0.05$) at the 95% confidence level.

3. Results and discussion

3.1. Synthesis of O^2-(4-Nitrobenzyl) diazeniumdiolates

We designed the antibacterial diazeniumdiolate prodrugs with specific function and requirements in mind, beginning with known diazeniumdiolates that would be stable under the nitroaromatic protection conditions, to make molecules that would display nitroreductase-activated NO release. Piperazine diazeniumdiolates are known to have half-lives ranging from two to five minutes, a mid-range half-life for diazeniumdiolates, which can range from less than one second to several hours, and this would be ideal for initial testing with our intended application [20,34]. Some piperazines also have antibacterial activity on their own [32,33], and piperazines are similar to the piperidine derivative previously published, providing precedence that protection conditions would not decompose the diazeniumdiolate [31].

To synthesize these novel compounds, several piperazine diazeniumdiolates were synthesized based on a known synthetic procedure [34] by reacting the piperazine starting material (**1a-c**) under pressurized NO gas in the presence of sodium methoxide in methanol, forming **2a-c** (Fig. 2).

After the successful synthesis of the piperazine diazeniumdiolates, we developed a procedure to O^2-protect the diazeniumdiolates (Fig. 1) with a nitroaromatic group, based on a previous literature report [31]. Fig. 2 shows that the diazeniumdiolate was mixed with 15-crown-5 to trap the sodium counterion, then 4-nitrobenzyl bromide was added to form the nitroaromatic-protected diazeniumdiolates **3a-c** in an S_N2-style reaction. We developed a basic work-up procedure to purify the ethyl carboxylate derivative **3a**, followed by column chromatography, significantly improving the yield of **3a** to 79 ± 7% (mean ± standard deviation, $n \geq 3$). Boc- (**3b**, 42 ± 21%) and Cbz- (**3c**, 81 ± 7%) protected piperazine derivatives were also synthesized to evaluate if the group on the piperazine leads to significant differences in the functionality of the compound.

The different piperazine protecting groups also allow for varied deprotecting conditions if the piperazine functional handle is desired. The nitroaromatic protecting group on the diazeniumdiolate should improve the stability to various deprotection conditions of the piperazine. With the piperazine functional handle, the compound could be incorporated into a polymer and used to make antibacterial medical devices or coatings.

3.2. Nitroreductase NfsB enzyme-activated NO release

Once the nitroaromatic-protected diazeniumdiolates were synthesized, we evaluated them for their ability to release NO in the presence of a nitroreductase (NTR) enzyme using a nitric oxide analyzer (NOA). An NOA uses chemiluminescence to directly quantify the amount of NO released by oxidizing NO with ozone, forming nitrogen dioxide in an excited state. Upon relaxation, excited state NO_2 emits a photon, which is measured and related to the moles of NO released via a previously determined calibration constant. This instrument provides direct measurement of NO release as a function of time. For these experiments, the diazeniumdiolate compounds were added to a solution of PBS and NADPH, a necessary reducing agent for the NTR enzyme to function. A baseline release was collected to determine if the compounds release NO spontaneously. The NTR enzyme was then added to the solution to induce NO release, which was quantified until the baseline returned to 0 ppb. The NTR enzyme used in these experiments was the commercially available nitroreductase NfsB, isolated from *Escherichia coli*, a well-studied enzyme that has previously been shown to reduce various nitro groups [16]. The NO release experiments were repeated three times for each compound at each concentration to demonstrate reproducible results.

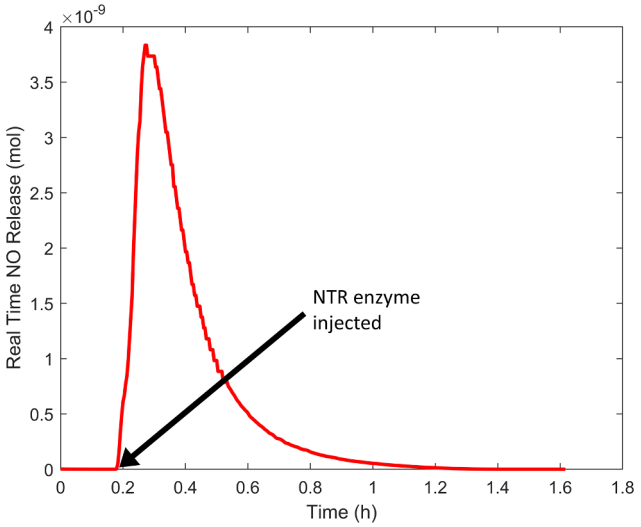


Fig. 3. A representative NO release plot of COOEt **3a** showing that NO release is dependent upon the injection of *E. coli*-derived NfsB nitroreductase (NTR) enzyme (0.1 mM in PBS). Experiments were performed with at least 3 trials, additional plots found in Supporting Information (Figs. S7–S11).

The diazeniumdiolate compounds **3a-c** do not release NO without the NTR enzyme present (Fig. 3). When the diazeniumdiolate compounds **3a-c** are in solution, the baseline release of NO is below the limit of detection, indicating that there is no spontaneous release of NO. Excitingly, upon injection of a solution of the NTR enzyme in PBS, there was an immediate response shown by a steep release of NO (Fig. 3). NO release quickly reaches a maximum, then more slowly releases NO until all of the diazeniumdiolate groups decompose. The hypothesis for how NO release occurs in the presence of nitroreductase is that the enzyme reduces the nitro group first to a hydroxylamine, then to a primary amine [31]. The amine then tautomerizes to the imine, resulting in the loss of the group from the diazeniumdiolate. The amine piece of the diazeniumdiolate is then protonated, causing two molecules of NO to be released from the compound, which displays first order kinetics [35].

Table 1
NO release data showing the normalized amount and the percent of total NO released at different concentrations for **3a-c.**

Entry	R group	Conc. (mM)	Total moles of NO released[a]	% of theoretical NO[a]	Highest instantaneous NO release (μM)
1	COOEt (**3a**)	0.01	$9.7 \pm 1.2 \times 10^{-9}$	24 ± 3	0.2
2	COOEt (**3a**)	0.1	$2.1 \pm 0.1 \times 10^{-7}$	52 ± 3	2.7
3	COOEt (**3a**)	1	$5.3 \pm 0.5 \times 10^{-7}$	13 ± 1	4.8
4	Boc (**3b**)	0.1	$4.4 \pm 0.4 \times 10^{-8}$	11 ± 1	1.1
5	Cbz (**3c**)	0.1	$6.7 \pm 0.6 \times 10^{-8}$	17 ± 2	0.7

[a] Data is presented as mean ± standard deviation, n = 3.

The NO release data shown in Fig. 3 provides strong support that the prodrug is activated by the NTR enzyme.

To provide further direct evidence that the prodrug is enzyme activated, the consumption of NADPH by the nitroreductase enzyme is measured. In this experiment, UV–vis absorbance is measured at 340 nm, where NADPH is known to have a peak, but disappears as it is converted to $NADP^+$ by the enzyme [36]. The NTR enzyme is added to a solution of **3a** and NADPH in PBS, then absorbance is measured over time. The data (Fig. S13) shows that NADPH concentration decreases in the presence of the substrate **3a** after the NTR enzyme is added, as expected based on literature reports [37]. The structure of the reducing agent NADPH is changed in the presence of the NTR enzyme, showing that the enzyme is consuming NADPH in solution. This experiment demonstrates that the enzyme is activated during the experimental conditions and corroborates the results observed in the NO release experiments.

Total NO release is measured for **3a** at concentrations of 0.01, 0.1, and 1 mM (Table 1, entries 1–3), and for **3b-c** at a concentration of 0.1 mM (Table 1, entries 4–5). Table 1 shows that the total amount of NO release does not match the theoretical yield, but literature reports indicate that diazeniumdiolates do not always release 100% of the theoretical NO available, based on their structure or due to decomposition or other factors [38–40]. However, our hypothesis for the observed less than 100% recovery of NO is based on the aqueous experimental conditions. Based on our observations, the synthesized compounds **3a-c** have limited solubility in aqueous solutions at higher concentrations. The NTR enzyme is soluble in water and is unable to access the prodrugs **3a-c** when they are not dissolved in solution, resulting in lower NO recovery. These solubility observations are supported in Table 1, as the COOEt derivative **3a** releases the highest percentage of NO (entry 2) and has the least hydrophobic protecting group. At the highest concentration of the COOEt derivative **3a** (entry 3), less of the theoretical NO is released, as less of it dissolves in the aqueous solution. The Boc- **3b** and Cbz-protected derivatives **3c** are even more hydrophobic, and less of the theoretical NO is released at the same concentration as the ethyl carboxylate derivative **3a** (entries 2, 4–5), further demonstrating that solubility plays a role in NO release.

3.3. Antibacterial activity of diazeniumdiolates against E. coli

To investigate the antibacterial activity of the synthesized prodrugs, *E. coli* was chosen as the initial species of interest. *E. coli* is well-known to contain a nitroreductase enzyme, which is commercially available and was used for the NO release experiments (section 3.2). *E. coli* is a very common infection-forming species and worryingly, is growing increasingly resistant to common antibiotics, indicating a pressing need for new antibacterial agents to treat *E. coli* infections [41,42].

To determine the antibacterial activity of the synthesized nitroreductase-activated diazeniumdiolates **3a-c**, *E. coli* was exposed to varying concentrations of the compounds. The positive control in these experiments was *E. coli* with no synthesized compound present. The initial hypothesis was that *E. coli* would metabolize the synthesized compounds *via* the nitroreductase enzyme it produces, inducing NO release and killing the bacteria.

Fig. 4a shows a decrease in viability of *E. coli* cells when exposed to higher concentrations of the COOEt derivative **3a**, indicating that the compound effectively kills *E. coli.* There is no statistically significant difference between the positive control and the COOEt derivative at a 0.01 mM concentration (Fig. 4a). Excitingly, however, there is a 56% reduction in the number of viable bacteria at concentrations of 0.1 mM **3a**, from $4.6 \pm 0.7 \times 10^8$ CFU/mL to $2.0 \pm 0.7 \times 10^8$ CFU/mL. At a dose of 1 mM **3a**, a 94% reduction in bacterial viability was observed, from $4.6 \pm 0.7 \times 10^8$ CFU/mL to $3.1 \pm 0.6 \times 10^7$ CFU/mL. Likely, the correlation between improved antibacterial activity and **3a** concentration is due to the higher amount of NO release (Table 1, entries 1–3). This observation is supported by Wink and Mitchell, as well as Schairer and co-workers, who show that concentrations of NO in the low micromolar range (> 1 μM) have antibacterial effects [17,43]. At the peak of NO release, concentrations of NO reach micromolar levels for COOEt **3a**, 2.7 μM (0.1 mM, Table 1, entry 2) and 4.8 μM (1 mM, Table 1, entry 3), which could explain the significant antibacterial activity observed at these concentrations.

Fig. 4b shows that the Boc and Cbz-protected derivatives, **3b** and **3c**, also provoke a reduction in the amount of viable *E. coli*. However, the amount of reduction does not match the antibacterial activity of the COOEt derivative **3a**. At a concentration of 0.1 mM, **3b** induces a 26% decrease in the number of viable bacteria, from $3.7 \pm 0.8 \times 10^8$ CFU/mL to $2.8 \pm 0.3 \times 10^8$ CFU/mL. The Cbz derivative **3c** induces a more significant decrease in the amount of viable *E. coli*, 41%, to $2.2 \pm 0.3 \times 10^8$ CFU/mL. At the same concentration (0.1 mM), the COOEt derivative **3a** causes a higher reduction in the number of bacteria (56%). These experimental results are consistent with the literature showing that micromolar concentrations of NO have antibacterial effects; the Boc-protected derivative **3b** peaks at concentrations of 1.1 μM of NO, and the Cbz derivative **3c** peaks at 0.7 μM, almost 1 μM (Table 1, entries 4–5). Because these values are less than the instantaneous release concentration (2.7 μM) for 0.1 mM COOEt **3a**, however, we observe a lower reduction in the number of bacteria. In contrast to the experiments performed with **3a**, preliminary experiments exposing *E. coli* to increased concentrations of the Boc **3b** and Cbz derivatives **3c** does not lead to a further reduction in the number of bacteria (not published). This could be due to the solubility issues observed the NO release experiments discussed in Section 3.2. Due to the more hydrophobic nature of the Boc and Cbz protecting groups compared to the COOEt group, **3b** and **3c** do not dissolve as well in aqueous solutions at higher concentrations, and therefore do not kill more bacteria at higher concentrations.

To further support the claim that NO is responsible for the observed antibacterial effects, *E. coli* are exposed to the non-NO releasing structural parts of the compound in a control experiment. These structures are shown in Fig. 5, 4-nitrotoluene and the corresponding piperazine derivative to the diazeniumdiolate (**1a-c**). For these control experiments, *E. coli* was exposed to both 4-nitrotoluene and the corresponding piperazine, either COOEt (**1a**), Boc (**1b**), or Cbz piperazine (**1c**) at the relevant concentrations (0.01, 0.1, or 1 mM, Fig. S12). Based on these experiments, the COOEt piperazine **1a** and 4-nitrotoluene combination shows no significant reduction compared to the positive control at concentrations of 0.01, 0.1 and 1 mM. At 0.01 mM COOEt **1a** and 4-nitrotoluene, there is an increase in bacterial growth, 120% of the positive control. At a concentration of 0.1 mM, the Boc **1b** and Cbz **1c**

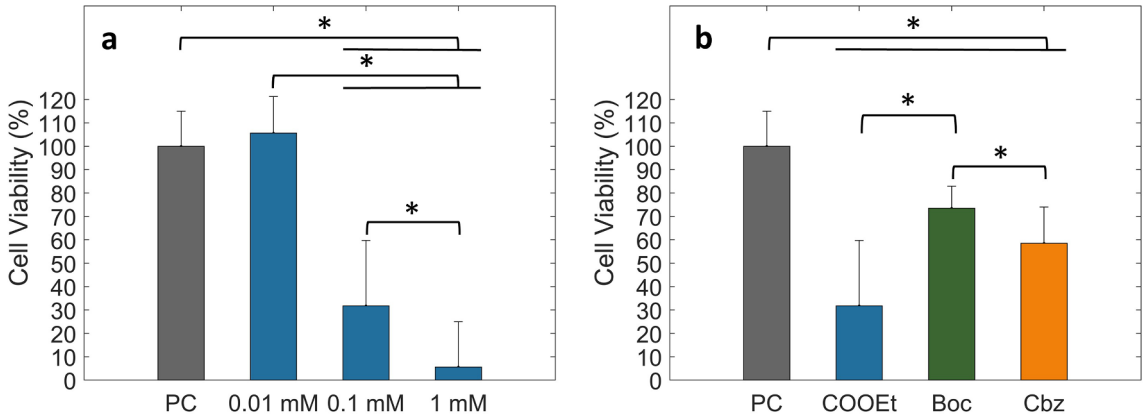


Fig. 4. (a) Percent viability of *E. coli* after exposure to **3a** at concentrations of 0.01 mM, 0.1 mM, and 1 mM. (b) Percent viability of *E. coli* after exposure to **3a, 3b,** or **3c** at a concentration of 0.1 mM. Average and 95% confidence interval displayed, n ≥ 9. Statistically significant differences between cellular viabilities are indicated (*) as determined by a student's *t* test. PC = positive control.

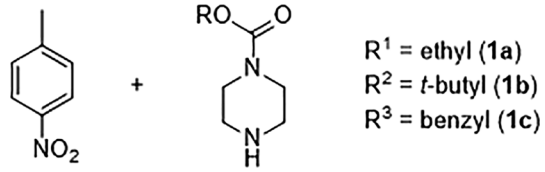


Fig. 5. Control compounds exposed to bacteria, 4-nitrotoluene on the left and piperazines **1a-c** on the right.

piperazine and 4-nitrotoluene combination does not cause a significant reduction in *E. coli*. This experiment also showed a small increase in bacterial viability, to 124% (**3b**) and 126% (**3c**). These control experiments demonstrate that the antibacterial activity of compounds **3a-c** is due to the NO-releasing diazeniumdiolate functional group in the compound, not due to the possible antibacterial activity of the rest of the structures.

After testing the antibacterial activity of our compounds with *E. coli*, we were interested in exploring possible antibacterial activity with other nitroreductase-producing bacteria species. *Pseudomonas aeruginosa* contains a nitroreductase enzyme of the same nitroreductase subgroup as *E. coli* NfsB, indicating the possibility that similar antibacterial activity would be observed with *P. aeruginosa* upon exposure to our compounds [44]. *P. aeruginosa* is a deadly bacterial species, growing increasingly resistant to most common antibiotics, illustrating a critical need to develop new antibacterial agents to kill it [45]. Using the previously stated method, during preliminary testing we observed a decrease in the number of viable bacteria after exposure to **3a** (not published). However, the same controls previously discussed in which the bacteria are exposed to the compounds in Fig. 5 showed the same or greater reduction in the number of viable bacteria. Unfortunately, we are not able to conclude that NO release from **3a-c** induces the same antibacterial activity against *P. aeruginosa* as with *E. coli*. This could be due to possible lower catalytic activity of the *P. aeruginosa* nitroreductase enzyme, or lack of access to the enzyme in *P. aeruginosa*. In the future, this issue could be addressed by simultaneous application of the compounds and isolated NfsB nitroreductase enzyme to kill *P. aeruginosa* infections.

4. Conclusion

To the best of our knowledge, this paper is the first example of an antibacterial enzyme-activated diazeniumdiolate prodrug, activated in the presence of a nitroreductase enzyme. Novel nitroaromatic-protected piperazine diazeniumdiolates were synthesized, COOEt, Boc, and Cbz piperazine derivatives. Analysis of NO release from the compounds demonstrates that NO release occurs only in the presence of a nitroreductase enzyme, releasing up to $5.3 \pm 0.5 \times 10^{-7}$ mol of NO. The antibacterial activity of the compounds against *E. coli* was analyzed, causing up to a 94% reduction in the number of bacteria. This study demonstrates the possibility of antibiotic nitric oxide prodrugs, which could allow targeted delivery of NO to infections. In the future, the compounds could be derivatized and tested against other bacterial species containing nitroreductase enzymes to achieve broad spectrum activity.

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.

Acknowledgements

This work was supported by grants from the Monfort Foundation and the National Institutes of Health (5R21EB016838-02).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.103318.

References

[1] J. O'Neill, Antimicrobial resistance: tackling a crisis for the health and wealth of nations, Rev. Antimicrob. Resist. (2014).
[2] L.E. Fleck, E.J. North, R.E. Lee, L.R. Mulcahy, G. Casadei, K. Lewis, A screen for and validation of prodrug antimicrobials, Antimicrob. Agents Chemother. 58 (2014) 1410–1419.
[3] K. Lewis, Platforms for antibiotic discovery, Nat. Rev. Drug Discov. 12 (2013) 371–387.
[4] M.D. Roldán, E. Pérez-Reinado, F. Castillo, C. Moreno-Vivián, Reduction of polynitroaromatic compounds: the bacterial nitroreductases, FEMS Microbiol. Rev. 32 (2008) 474–500.
[5] J. Whiteway, P. Koziarz, J. Veall, N. Sandhu, P. Kumar, B. Hoecher, I.B. Lambert, Oxygen-insensitive nitroreductases: analysis of the roles of NfsA and NfsB in development of resistance to 5-nitrofuran derivatives in *Escherichia coli*, J. Bacteriol. 180 (1998) 5529.
[6] Y. Yanto, M. Hall, A.S. Bommarius, Nitroreductase from *Salmonella typhimurium*: characterization and catalytic activity, Org. Biomol. Chem. 2010 (1826) 8.
[7] H.-H. Nguyen-Tran, G.-W. Zheng, X.-H. Qian, J.-H. Xu, Highly selective and controllable synthesis of arylhydroxylamines by the reduction of nitroarenes with an electron-withdrawing group using a new nitroreductase BaNTR1, Chem. Commun. 50 (2014) 2861.
[8] M.E. de la Calle, G. Cabrera, D. Cantero, A. Valle, J. Bolivar, Overexpression of the nitroreductase NfsB in an *E. coli* strain as a whole-cell biocatalyst for the production of chlorinated analogues of the natural herbicide DIBOA, New Biotechnol. 50 (2019) 9–19.

[9] A.B. Mauger, P.J. Burke, H.H. Somani, F. Friedlos, R.J. Knox, Self-immolative prodrugs: candidates for antibody-directed enzyme prodrug therapy in conjunction with a nitroreductase enzyme, J. Med. Chem. 37 (1994) 3452–3458.

[10] W. Denny, Nitroreductase-based GDEPT, Curr. Pharm. Des. 8 (2002) 1349–1361.

[11] I. Niculescu-Duvaz, C.J. Springer, Antibody-Directed Enzyme Prodrug Therapy (ADEPT): A Review, Adv. Drug Deliv. Rev. 26 (1997) 151–172.

[12] O. Greco, G.U. Dachs, Gene directed enzyme/prodrug therapy of cancer: historical appraisal and future prospectives, J. Cell. Physiol. 187 (2001) 22–36.

[13] M.H. Rich, A.V. Sharrock, K.R. Hall, D.F. Ackerley, J.K. MacKichan, Evaluation of NfsA-like nitroreductases from *Neisseria meningitidis* and *Bartonella henselae* for enzyme-prodrug therapy, targeted cellular ablation, and dinitrotoluene bioremediation, Biotechnol. Lett. 40 (2018) 359–367.

[14] C. Jin, S. Wen, Q. Zhang, Q. Zhu, J. Yu, W. Lu, Synthesis and biological evaluation of paclitaxel and camptothecin prodrugs on the basis of 2-nitroimidazole, ACS Med. Chem. Lett. 8 (2017) 762–765.

[15] I.M. de Oliveira, J.A.P. Henriques, D. Bonatto, In silico identification of a new group of specific bacterial and fungal nitroreductases-like proteins, Biochem. Biophys. Res. Commun. 355 (2007) 919–925.

[16] P.R. Race, A.L. Lovering, R.M. Green, A. Ossor, S.A. White, P.F. Searle, C.J. Wrighton, E.I. Hyde, Structural and mechanistic studies of *Escherichia coli* nitroreductase with the antibiotic nitrofurazone, J. Biol. Chem. 280 (2005) 13256–13264.

[17] D.O. Schairer, J.S. Chouake, J.D. Nosanchuk, A.J. Friedman, The potential of nitric oxide releasing therapies as antimicrobial agents, Virulence 3 (2012) 271–279.

[18] S. Moncada, E.A. Higgs, The discovery of nitric oxide and its role in vascular biology, Br. J. Pharmacol. 147 (2006) S193–S201.

[19] B.J. Privett, A.D. Broadnax, S.J. Bauman, D.A. Riccio, M.H. Schoenfisch, Examination of bacterial resistance to exogenous nitric oxide, Nitric Oxide 26 (2012) 169–173.

[20] J.A. Hrabie, L.K. Keefer, Chemistry of the nitric oxide-releasing diazeniumdiolate ("Nitrosohydroxylamine") functional group and its oxygen-substituted derivatives, Chem. Rev. 102 (2002) 1135–1154.

[21] L.K. Keefer, Progress toward clinical application of the nitric oxide-releasing diazeniumdiolates, Annu. Rev. Pharmacol. Toxicol. 43 (2003) 585–607.

[22] L.K. Keefer, R.W. Nims, K.M. Davies, D.A. Wink, "NONOates" (1-Substituted Diazen-1-Ium-1,2-Diolates) as Nitric Oxide Donors: Convenient Nitric Oxide Dosage Forms, Methods in Enzymology, Vol. 268 Elsevier, 1996, pp. 281–293.

[23] D. Park, J. Kim, Y.M. Lee, J. Park, W.J. Kim, Polydopamine hollow nanoparticle functionalized with *N*-diazeniumdiolates as a nitric oxide delivery carrier for antibacterial therapy, Adv. Healthc. Mater. 5 (2016) 2019–2024.

[24] Z. Sadrearhami, T.-K. Nguyen, R. Namivandi-Zangeneh, K. Jung, E.H.H. Wong, C. Boyer, Recent advances in nitric oxide delivery for antimicrobial applications using polymer-based systems, J. Mater. Chem. B 6 (2018) 2945–2959.

[25] K.P. Reighard, M.H. Schoenfisch, Antibacterial action of nitric oxide-releasing chitosan oligosaccharides against *Pseudomonas aeruginosa* under aerobic and anaerobic conditions, Antimicrob. Agents Chemother. 59 (2015) 6506–6513.

[26] C.A. Valdez, J.E. Saavedra, B.M. Showalter, K.M. Davies, T.C. Wilde, M.L. Citro, J.J. Barchi, J.R. Deschamps, D. Parrish, S. El-Gayar, U. Schleicher, C. Bogdan, L.K. Keefer, Hydrolytic reactivity trends among potential prodrugs of the O^2-glycosylated diazeniumdiolate family. Targeting nitric oxide to macrophages for antileishmanial activity, J. Med. Chem. 51 (2008) 3961–3970.

[27] S.Y. Hong, J.E. Saavedra, L.K. Keefer, H. Chakrapani, Improved synthesis of V-PYRRO/NO, a liver-selective nitric oxide prodrug, and analogues, Tetrahedron Lett. 50 (2009) 2069–2071.

[28] J.E. Saavedra, P.J. Shami, L.Y. Wang, K.M. Davies, M.N. Booth, M.L. Citro, L.K. Keefer, Esterase-sensitive nitric oxide donors of the diazeniumdiolate family: In Vitro Antileukemic activity, J. Med. Chem. 43 (2000) 261–269.

[29] S.A. Collins, M.J. Kelso, A. Rineh, N.R. Yepuri, J. Coles, C.L. Jackson, G.D. Halladay, W.T. Walker, J.S. Webb, L. Hall-Stoodley, G.J. Connett, M. Feelisch, S.N. Faust, J.S.A. Lucas, R.N. Allan, Cephalosporin-3' -diazeniumdiolate NO-Donor prodrug PYRRO-C3D enhances azithromycin susceptibility of non-typeable *Haemophilus influenzae* biofilms, Antimicrob. Agents Chemother. (2016) AAC.02086-16.

[30] R.N. Allan, M.J. Kelso, A. Rineh, N.R. Yepuri, M. Feelisch, O. Soren, S. Brito-Mutunayagam, R.J. Salib, P. Stoodley, S.C. Clarke, J.S. Webb, L. Hall-Stoodley, S.N. Faust, Cephalosporin-NO-donor prodrug PYRRO-C3D shows β-Lactam - mediated activity against *Streptococcus pneumoniae* biofilms, Nitric Oxide 65 (2017) 43–49.

[31] K. Sharma, K. Sengupta, H. Chakrapani, Nitroreductase-Activated Nitric Oxide (NO) prodrugs, Bioorg. Med. Chem. Lett. 23 (2013) 5964–5967.

[32] A.S. Nayar, T.J. Dougherty, K.E. Ferguson, B.A. Granger, L. McWilliams, C. Stacey, L.J. Leach, S. Narita, H. Tokuda, A.A. Miller, D.G. Brown, S.M. McLeod, Novel antibacterial targets and compounds revealed by a high-throughput cell wall reporter assay, J. Bacteriol. 197 (2015) 1726–1734.

[33] P. Chaudhary, R. Kumar, A.K. Verma, D. Singh, V. Yadav, A.K. Chhillar, G.L. Sharma, R. Chandra, Synthesis and antimicrobial activity of N-Alkyl and N-aryl piperazine derivatives, Bioorg. Med. Chem. 14 (2006) 1819–1826.

[34] J.E. Saavedra, M.N. Booth, J.A. Hrabie, K.M. Davies, L.K. Keefer, Piperazine as a linker for incorporating the nitric oxide-releasing diazeniumdiolate group into other biomedically relevant functional molecules, J. Org. Chem. 64 (1999) 5124–5131.

[35] K.M. Davies, D.A. Wink, J.E. Saavedra, L.K. Keefer, Chemistry of the diazeniumdiolates. 2. Kinetics and mechanism of dissociation to nitric oxide in aqueous solution, J. Am. Chem. Soc. 123 (2001) 5473–5481.

[36] J. De Ruyck, M. Famerée, J. Wouters, E.A. Perpète, J. Preat, D. Jacquemin, Towards the understanding of the absorption spectra of NAD(P)H/NAD(P)+ as a common indicator of dehydrogenase enzymatic activity, Chem. Phys. Lett. 450 (2007) 119–122.

[37] H. Guillén, J.A. Curiel, J.M. Landete, R. Muñoz, T. Herraiz, Characterization of a nitroreductase with selective nitroreduction properties in the food and intestinal lactic acid bacterium *Lactobacillus plantarum* WCFS1, J. Agric. Food Chem. 57 (2009) 10457–10465.

[38] P.H. Ruane, K.M. Bushan, C.M. Pavlos, R.A. D'S, J.P. Toscano, Controlled photochemical release of nitric oxide from O^2-benzyl-substituted diazeniumdiolates, J. Am. Chem. Soc. 124 (2002) 9806–9811.

[39] C.M. Maragos, D. Morley, D.A. Wink, T.M. Dunams, J.E. Saavedra, A. Hoffman, A.A. Bove, L. Isaac, J.A. Hrabie, L.K. Keefer, Complexes of ˙NO with nucleophiles as agents for the controlled biological release of nitric oxide. Vasorelaxant effects, J. Med. Chem. 34 (1991) 3242–3247.

[40] M.M. Reynolds, Z. Zhou, B.K. Oh, M.E. Meyerhoff, Bis-diazeniumdiolates of dialkyldiamines: enhanced nitric oxide loading of parent diamines, Org. Lett. 7 (2005) 2813–2816.

[41] M.U. Rasheed, N. Thajuddin, P. Ahamed, Z. Teklemariam, K. Jamil, Antimicrobial drug resistance in strains of *Escherichia coli* isolated from food sources, Rev. Inst. Med. Trop. Sao Paulo 56 (2014) 341–346.

[42] H.J. Morrill, J.B. Morton, A.R. Caffrey, L. Jiang, D. Dosa, L.A. Mermel, K.L. LaPlante, Antimicrobial resistance of *Escherichia coli* urinary isolates in the veterans affairs health care system, Antimicrob. Agents Chemother. 61 (2017).

[43] D.A. Wink, J.B. Mitchell, Chemical biology of nitric oxide: insights into regulatory, cytotoxic, and cytoprotective mechanisms of nitric oxide, Free Radic. Biol. Med. 25 (1998) 434–456.

[44] L.K. Green, S.P. Syddall, K.M. Carlin, G.D. Bell, C.P. Guise, A.M. Mowday, M.P. Hay, J.B. Smaill, A.V. Patterson, D.F. Ackerley, *Pseudomonas aeruginosa* NfsB and Nitro-CBI-DEI – a promising enzyme/prodrug combination for gene directed enzyme prodrug therapy, Mol. Cancer 12 (2013) 58.

[45] P.D. Lister, D.J. Wolter, N.D. Hanson, Antibacterial-resistant *Pseudomonas aeruginosa*: clinical impact and complex regulation of chromosomally encoded resistance mechanisms, Clin. Microbiol. Rev. 22 (2009) 582–610.