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A Small Molecule for Controlled Generation of Reactive Oxygen Species (ROS)

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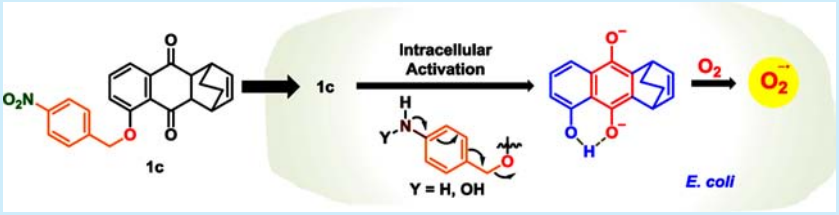
Supporting Information



ABSTRACT: Due to the short half-life of reactive oxygen species (ROS) such as a superoxide radical, controlled and localized generation of ROS is challenging. Here, we report a rationally designed small-molecule **1c** that generates ROS only when triggered by a bacterial enzyme. We provide evidence for **1c** predictably enhancing the intracellular superoxide radical in a model bacterium. Spatiotemporal control over ROS generation offered by **1c** should help better understand stress responses in bacteria to increased ROS.

Nearly all organisms inadvertently produce superoxide $O_2^{\bullet-}$, by 1-electron transfer to oxygen during respiration.[1] $O_2^{\bullet-}$ is subsequently converted to hydrogen peroxide H_2O_2, which through the Fenton reaction generates the highly reactive $^{\bullet}OH$.[1] Together, these reactive oxygen species (ROS) can damage vital cellular components and are hence deployed by the immune system to counter infectious pathogens. Several recent studies have shown that ROS can sensitize infectious bacteria to clinical antibiotics suggesting the possible therapeutic utility for ROS. Due to a weak pipeline of antibiotics in preclinical development and the global emergence of antibiotic resistance, methodologies for selectively enhancing intracellular ROS including $O_2^{\bullet-}$ might help better understand[2] and evaluate the therapeutic potential of ROS.[3]

Due to its short life, $O_2^{\bullet-}$ must be produced *in situ* by reaction with oxygen for use in biochemical studies. Hence, either small organic molecules that spontaneously generate $O_2^{\bullet-}$ or enzymatic methods that process a substrate to generate $O_2^{\bullet-}$ are used.[4] For example, a combination of hypoxanthine and xanthine oxidase (X + XO) where hypoxanthine is metabolized by XO predominantly produces $O_2^{\bullet-}$ in the proximity of cells.[4] Any $O_2^{\bullet-}$ that is produced must diffuse across a lipid bilayer to exert its effects. However, $O_2^{\bullet-}$ is not highly permeable at neutral pH and such a method may not be useful for enhancing intracellular ROS.[5] Small molecules such as paraquat or menadione, which require bioactivation for $O_2^{\bullet-}$ production, have often been used but at elevated concentrations that can potentially complicate mechanistic interpretations (see Supporting Information, Chart S1).[3] Thus, a cell permeable small molecule that can predictably and exclusively increase $O_2^{\bullet-}$ within cells is not available. Herein, we report a small molecule that is activated by a bacterial enzyme to generate $O_2^{\bullet-}$ that is well suited to simulate increased ROS in bacteria.

5-(4-Nitrobenzyloxy)-1,4,4a,9a-tetrahydro-1,4-ethanoanthracene-9,10-dione (**1c**) was considered as a cell-permeable $O_2^{\bullet-}$ generator (Scheme 1).[6] The 4-nitrobenzyl group is a known substrate for *E. coli* nitroreductase (NTR), a commonly expressed oxygen-insensitive bacterial enzyme that reduces a broad range of aromatic nitro compounds to amines.[7] Reduction of the nitro group might lead to a rearrangement leading to the departure of **1a**, which has been previously reported to generate $O_2^{\bullet-}$ in ambient aerobic buffer through a keto−enol tautomerism as the first step (Scheme 1). Enolization of the carbonyls in **1a** is promoted by a proximal H-bonding hydroxyl group, and as a consequence, ROS generation by this compound was enhanced in comparison with an analogous benzylated derivative **1b** (Scheme 1). Hence, similarly in the case of **1c**, where intramolecular H-bonding is not possible, enolization is disfavored and this compound is predicted to be a poor $O_2^{\bullet-}$ generator in buffer.

Compound **1c** was synthesized from **1a** by reaction with 4-nitrobenzylbromide (Supporting Information, Scheme S1). During the incubation of **1c** in ambient aerobic buffer, increased $O_2^{\bullet-}$ was observed only in the presence of NTR as determined by a luminol-based chemiluminescence assay (Figure 1a and 1b).[8] An HPLC-based dihydroethidium (DHE) assay was used to independently assess $O_2^{\bullet-}$ production (Figure 1c). During incubation of **1c** in the presence of NTR, the nearly complete disappearance of DHE with concomitant formation of 2-hydroxyethidium (2-OH-E^+)

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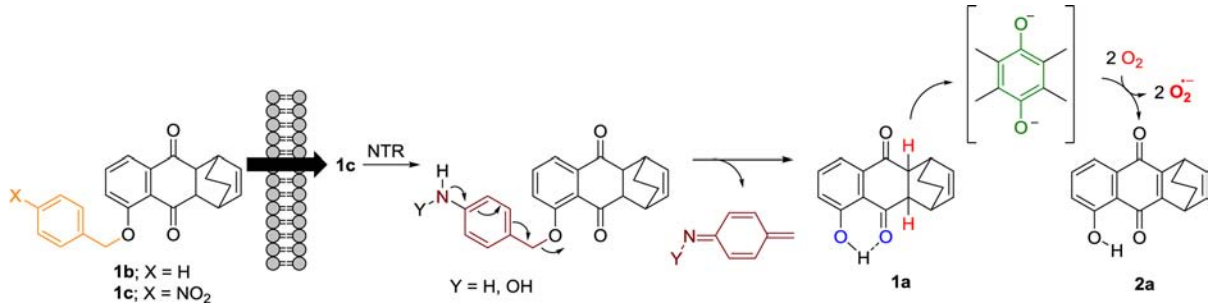
Scheme 1. Design of a Cell Permeable ROS Generator[a]



[a]The 4-nitrobenzyl derivative **1c** is a substrate for nitro reduction, which would convert **1c** to the active ROS producing **1a**. When this transformation is catalyzed by *E. coli* nitroreductase (NTR), exclusive intracellular accumulation of ROS is predicted.

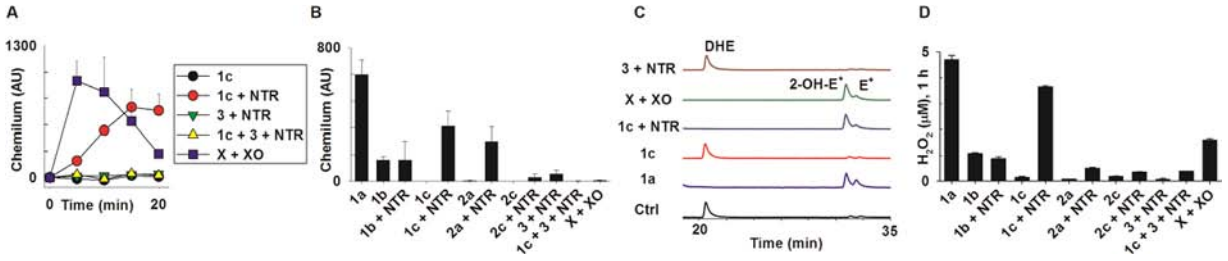


Figure 1. Estimation of ROS in ambient aerobic aqueous buffer. + NTR implies *E. coli* nitroreductase and NADPH; X + XO: hypoxanthine and xanthine oxidase were used. (a) Chemiluminescence measurement upon reaction with luminol as a measure of $O_2^{\bullet-}$ production was carried out with various compounds (10 μM) and combinations in pH 8.0 buffer. (b) Results of the above assay conducted after 30 min; (c) A HPLC-based dihydroethidium (DHE) assay was used to infer generation of $O_2^{\bullet-}$ after incubation of compounds (100 μM) in pH 8.0 buffer for 3 h. 2-Hydroxyethidium (2-OH-E^+), which is exclusively formed by the reaction of $O_2^{\bullet-}$ with DHE, elutes at 30.9 min, and ethidium E^+, which is formed by nonspecific oxidation of DHE, elutes at 31.7 min. (d) The amount of hydrogen peroxide generated during incubation of 10 μM of each compound in pH 7.4 was estimated using an Amplex Red fluorescence assay.

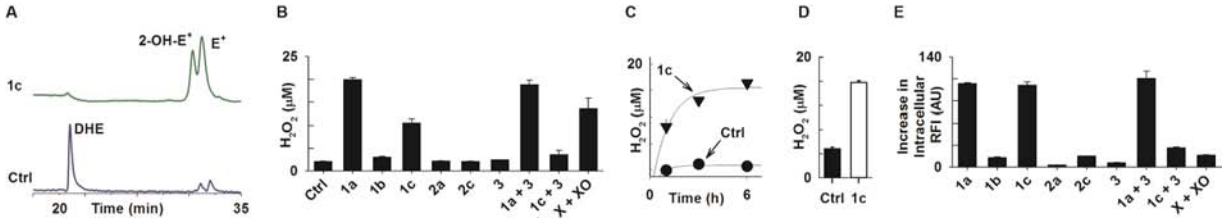


Figure 2. ROS generation during incubation with *E. coli*: (a) HPLC traces of assay for intracellular $O_2^{\bullet-}$ production using a hydroethidine (DHE) assay. Incubation with **1c** (250 μM) was for 30 min; DHE levels indicate unoxidized dye while 2-OH-E^+ formed is an indicator for $O_2^{\bullet-}$ production and E^+ is indicative of an increase in oxidative species. Ctrl: untreated bacteria. (b) Extracellular H_2O_2 generated during incubation of *E. coli* with compounds (100 μM) for 1 h was estimated using an Amplex Red fluorescence assay. Ctrl: untreated bacteria. (c) H_2O_2 generation during incubation of *E. coli* with **1c** (100 μM) was recorded during 6 h as described above. A first-order rate constant for ROS production was calculated as 0.96 h^{-1}. Ctrl: untreated bacteria. (d) *E. coli* treated with **1c** (100 μM) for 1 h; centrifugation and removal of the supernatant followed by resuspension of bacteria in fresh media. H_2O_2 generated after incubation of bacteria for 3 h was recorded as described above. Ctrl: untreated bacteria. (e) A 2,7-dichlorodihydrofluorescein-diacetate ($DCFH_2$-DA)-based fluorescence assay was used to estimate oxidative species generated intracellularly in *E. coli*. Increase in relative fluorescence intensity (RFI) with respect to DMSO (0.5%).

was observed, thus, confirming the intermediacy of $O_2^{\bullet-}$ (Figure 1c).[9] H_2O_2 is produced by $1e^-$ transfer to $O_2^{\bullet-}$, and we estimated H_2O_2 using an Amplex Red fluorescence assay.[10] In the absence of NTR, negligible H_2O_2 was produced during incubation of **1c** (10 μM), but in the presence of NTR, a yield of 3.65 μM H_2O_2 was recorded after 1 h (Figure 1d). 4-Nitrobenzyl benzoate **3** (Chart S1), which is a substrate for NTR but should not produce ROS during its metabolism by this enzyme, was synthesized using a reported procedure.[11] Compound **3** (100 μM) was metabolized by NTR, and as predicted no evidence for $O_2^{\bullet-}$ or H_2O_2 was found (Figure 1) indicating that possible products of metabolism of the 4-nitrobenzyl group were incapable of generating ROS. When **1c** and **3** (10 equiv) were coincubated in the presence of NTR, diminished $O_2^{\bullet-}$ and H_2O_2 were produced possibly due to inhibition of turnover of **1c** to **1a** (Figure 1). Under similar experimental conditions, the amounts of $O_2^{\bullet-}$ and H_2O_2 generated by **1a** and **1b** were consistent with previously reported data (Figure 1b and 1d).[6]

Next, the possibility of the use of **1c** as a tool for predictably enhancing intracellular $O_2^{\bullet-}$ in bacteria was examined. The production of $O_2^{\bullet-}$ in *E. coli* was estimated using a DHE assay that is selective for intracellular $O_2^{\bullet-}$ (Figure 2a).[9] The bacterial control showed unreacted DHE with negligible oxidized DHE (Figure 2a), but in the presence of **1c**, the formation of 2-OH-E^+ was observed with concomitant loss of DHE suggestive of

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$O_2^{\bullet-}$ production intracellularly (Figure 2a). Due to the difficulty in accurately quantifying $O_2^{\bullet-}$ using the DHE assay, measurement of H_2O_2, which provides a quantitative basis for assessing ROS production, was carried out. During incubation of *E. coli* with **1c** (100 μM), a yield of 10.5 μM H_2O_2 after 1 h was recorded (Figure 2b). The formation of H_2O_2 was nearly completely abrogated when **1c** was cotreated with **3** (10 equiv), a substrate for intracellular nitroreductases including NTR (Figure 2b).

Treatment of *E. coli* with **3** did not increase H_2O_2 and this compound did not inhibit ROS generation by **1a** (Figure 2b), suggesting that **3** inhibited the metabolism of **1c** to **1a**. A time course for ROS generation during incubation of *E. coli* with **1c** showed an increase in H_2O_2 levels during 6 h, which is consistent with temporally controlled enhancement in intracellular ROS (Figure 2c).

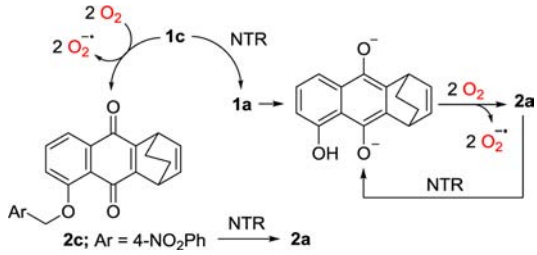
Next, a series of experiments to test if **1c** was metabolized only intracellularly in bacteria to enhance levels of ROS were carried out. *E. coli* was incubated with **1c** for 1 h, which was followed by removal of media, and cells were resuspended in fresh media: under these conditions, any **1c** that does not permeate cells would be removed and any H_2O_2 produced would be due to intracellular activation. The difference in yields of H_2O_2 obtained during this experiment (Figure 2d) and during a bolus treatment of **1c** (Figure 2b) was minimal, which supports intracellular metabolism to produce ROS as the major pathway. Next, *E. coli* was grown overnight and the cell-free media was incubated with **1c**: any reductases secreted might activate **1c** to produce ROS. However, no significant increase in H_2O_2 was found even after 3 h suggesting that extracellular activation of **1c** was not important (see Supporting Information, Figure S3).

Next, the intermediacy of hydroxyl radical $^{\bullet}$OH was assessed by a supercoiled plasmid DNA cleavage assay. When cotreated with Fe(II), the ROS generator **1c** produced nicks only in the presence of NTR, supporting the use of this protocol for enhancement of ROS (Figure S4). For cellular assays, the ability of **1c** to increase intracellular oxidative species including $^{\bullet}$OH was studied.[12] Incubation of *E. coli* with dichlorofluorescein-diacetate, $DCFH_2$-DA, followed by treatment with **1c** resulted in an increased intracellular fluorescence attributable to oxidative species (Figure 2e).[13] In addition, formation of ethidium (E^+) in the DHE assay is suggestive of an increased oxidative intracellular environment caused by **1c** (Figure 2a).

Taken together, our data suggest that the use of **1c** predictably increases ROS in bacteria. Compound **1c** was next compared with the X + XO protocol. The time course of X + XO indicates that a burst of $O_2^{\bullet-}$ is produced while **1c** + NTR showed a gradual increase in ROS during this time period (Figure 1a). Thus, for biochemical studies, the use of **1c** + NTR might simulate gradual ROS production. In cell-based assays, the ability of X + XO to increase intracellular ROS was diminished in comparison with **1c** (see Supporting Information, Figure S1).[14] Thus, **1c** complements the existing repertoire of ROS generators while offering distinct advantages such as cell permeability and temporal control.

Two possible pathways for metabolism of **1c** to generate $O_2^{\bullet-}$ were considered (Scheme 2). First, NTR-induced reduction of the nitroaryl group should produce an amine or hydroxylamine, which might initiate a deprotection cascade to produce **1a** (Scheme 1). Under chemoreductive conditions ($Zn/HCOONH_4$) in pH 7.4 buffer, complete consumption of

Scheme 2. Proposed Mechanisms for ROS Generation during Incubation of 1c in Buffer in the Presence of NTR



1c with concomitant formation of **1a** as the major product was observed (Figure S5).

Mass spectrometric analysis of this reaction mixture (Figure S6) revealed the formation of an amine and hydroxylamine byproducts (Scheme 1), which is consistent with a reduction−rearrangement cascade.[15] When **1c** was treated with NTR and NADPH, mass spectrometric analysis revealed the formation of **2a**, again consistent with the proposed mechanism.

Incubation of **1a** in aerobic buffer generated $O_2^{\bullet-}$ (Figure 1b and 1c) and produced **2a** as the major organic product (Figure S7). The role of intramolecular H-bonding is apparent in the large difference in the rate of conversion of **1c** to **2c** (<20% conversion after 18 h) and the oxidation of **1a** to **2a**, which was nearly complete in the same time period (Figure S7). The quinone **2a** is also a candidate for ROS generation in the presence of bioreductive enzymes including NTR. However, our cellular assays showed that **2a** did not produce ROS (Figure 2b and 2d) perhaps due to the diminished rates of redox cycling of this compound intracellularly.[16] This observation also implies that once **1a** converts to **2a**, no further ROS is produced suggesting that **1c** might produce up to 2 mol of $O_2^{\bullet-}$ per mol of compound (Scheme 2).[17] An alternate ROS producing pathway occurred during conversion of **1c** to **2c**, which in turn can be cleaved in the presence of NTR to produce **2a** (Scheme 2). If this was a major pathway, ROS generation from **1c** neither would depend on NTR nor should be inhibited by **3**. However, ROS generation by **1c** is significant only in the presence of NTR and was nearly completely inhibited by **3** in both test tube and cellular assays (Figures 1 and 2). In addition, the nitrobenzyl derivative **2c** was a poor ROS generator (Figures 1 and 2). Thus, ROS generation by direct conversion of **1c** to **2c** appears to be minor. Although biocatalytic reduction of **1c** by NTR requires NADPH as a cofactor, our observation that treatment of *E. coli* with **3** resulted in no significant increase of H_2O_2 (Figure 2b) suggests that such perturbations of NADPH levels (estimated millimolar concentrations) do not result in ROS generation.[18]

Recently, compounds that potentiate ROS in bacteria were proposed as adjuvants, which are small molecules that may not have significant antimicrobial properties themselves but when cotreated with antibiotics can reduce effective dosages and minimize side effects.[4,19] As few drug candidates with novel mechanisms are in the pipeline, the development of adjuvants assume importance.[18] The ROS generator **1c** did not inhibit *E. coli* growth (Table S5), but this compound significantly sensitized bacteria to aminoglycosides supporting possible applications of ROS as an adjuvant to this class of antibiotics (Table S6).[20]

In conclusion, we report that **1c** is capable of predictably increasing intracellular levels of ROS in a model bacterium. To our knowledge, this is the first report of a rationally designed

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enzyme activated $O_2^{\bullet-}$ generator that is suitable for enhancing ROS in bacteria. It is anticipated that **1c** would facilitate the understanding of bacterial responses to ROS, mechanisms of antibiotic action, and the therapeutic potential of ROS. The use of **1c** to predictably enhance ROS in bacteria other than *E. coli* is expected to depend on the expression levels of NTR and on the uptake of this small molecule. These studies are underway in our laboratory.

■ ASSOCIATED CONTENT

Supporting Information

Synthesis, characterization data, assay protocols, and associated data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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(12) Due to its extremely short half-life and indiscriminate reactivity, *in vivo* detection of the hydroxyl radical is challenging and the specificity of previously used fluorescence assays for $^{\bullet}$OH has been questioned (see ref 1c).

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(14) In cellular assays at comparable concentrations with menadione, despite several attempts, nonspecific decomposition of DHE without formation of 2-OH-E^+ or E^+ was observed (Figure S1).

(15) Attempts to isolate these amine byproducts were unsuccessful and instead produced **1a** or **2a** as the major product.

(16) The precise rationale for this observation is unclear, but steric hindrance might hamper the accessibility of the quinone ring to reductive enzymes.

(17) Intracellular ROS generation yield and rate are dictated by the efficiency and kinetics of metabolism of **1c** by NTR in *E. coli*.

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(20) A Clinical and Laboratory Standards Institute (CLSI) recommended protocol was used to determine the minimum inhibitory concentration (MIC) against *E. coli* (Table S5). No significant inhibition of bacterial growth by **1c** was observed at elevated concentrations (MIC >100 μM) suggesting that perhaps ROS alone is well tolerated by *E. coli*. However, when gentamycin was cotreated with **1c** (50 μM), an 8-fold decrease in MIC for this antibiotic was observed suggesting that a lower antibiotic dosage was required to obtain similar efficacy.