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PA-824 Kills Nonreplicating *Mycobacterium tuberculosis* by Intracellular NO Release

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Abstract

Bicyclic nitroimidazoles, including PA-824, are exciting candidates for the treatment of tuberculosis. These prodrugs require intracellular activation for their biological function. We found that Rv3547 is a deazaflavin-dependent nitroreductase (Ddn) that converts PA-824 into three primary metabolites; the major one is the corresponding des-nitroimidazole (des-nitro). When derivatives of PA-824 were used, the amount of des-nitro metabolite formed was highly correlated with anaerobic killing of *Mycobacterium tuberculosis* (Mtb). Des-nitro metabolite formation generated reactive nitrogen species, including nitric oxide (NO), which are the major effectors of the anaerobic activity of these compounds. Furthermore, NO scavengers protected the bacilli from the lethal effects of the drug. Thus, these compounds may act as intracellular NO donors and could augment a killing mechanism intrinsic to the innate immune system.

Drug-resistant tuberculosis (TB) has emerged as a major threat to global health (1). Promising new candidate drugs are the bicyclic nitroimidazoles, including PA-824 and OPC-67683, which are currently in human clinical trials (2,3). These molecules are active not only against actively replicating bacteria, but also against bacteria that are nonreplicating by virtue of hypoxia (4). These nonreplicating cells are thought to be particularly difficult to eradicate and may be a major determinant of the extended treatment periods (6 to 8 months) necessary to cure a patient and to avoid disease relapse (5). In addition, such nonreplicating bacteria have been proposed to be responsible for latent tuberculosis, a condition that affects one-third of the entire human population (6).

The mechanism of cell killing by these prodrugs is complex. Treatment with PA-824 or OPC-67683 disrupts the formation of mycolic acids, major constituents of the cell envelope of Mtb (4,7). However, this effect seemed unlikely to be responsible for cell killing under nonreplicating conditions, because the bacilli do not extensively remodel mycolic acids under anaerobic conditions (8). The deazaflavin cofactor F_{420} and the nonessential F_{420}-dependent glucose-6-phosphate dehydrogenase Fgd1 are both essential for the activation of bicyclic nitroimidazoles, and loss of either confers high-level resistance to PA-824 (4,9,10). We recently identified a rare third class of mutants that had lesions in Rv3547, a member of a large

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family of proteins in Actinobacteria of unknown function (fig. S1) (11). We speculated that Rv3547 was a deazaflavin (F_{420})-dependent nitroreductase and renamed it Ddn (12).

To test this, purified F_{420} was reduced by the use of recombinant Mtb Fgd1 and glucose-6-phosphate (12). Recombinant Ddn was capable of reoxidizing this cofactor in a time- and enzyme-dependent fashion in the presence of PA-824 (Fig. 1, A and B). To test the specificity of this reaction, the opposite enantiomer of PA-824 (which has the *R* configuration at the 6-position and is about 1/100th as active against whole cells of Mtb) was tested and found to be a poor substrate for Ddn (Fig. 1B).

We next studied the chemical structures of the products of PA-824 reduction by Ddn. By titrating the enzyme with $F_{420}H_2$ and analyzing an aliquot by liquid chromatography—mass spectrometry (LC-MS), we characterized three reduction products (Fig. 1C). The major product had a molecular mass of 315 and was labeled metabolite 1. The formation of this metabolite was mostly complete after addition of a single equivalent of $F_{420}H_2$, as was formation of a minor peak showing a mass/charge ratio (*m/z*) of 331 (metabolite 2). Additional $F_{420}H_2$ equivalents increased mainly metabolite 3 (mass 291), and an unstable intermediate with a molecular mass of 346 (triangles in Fig. 1C). This intermediate (compound C in Figs. 1C and 2) was not stable enough to isolate, and it decomposed quickly and cleanly to metabolite 3. Comparing the metabolites produced from radioactive PA-824 in vitro by using purified Ddn with those produced in whole cells of Mtb revealed only slight differences in the proportion of products formed (Fig. 1D).

On the basis of these results and the previous report that the major metabolite formed from OPC-67683 by whole cells of Mtb was the des-nitro form of this compound (7), we proposed the structures for these metabolites shown in Fig. 2. The structures and retention times on high-performance liquid chromatography (HPLC) of all three compounds were confirmed by chemical synthesis and complete analytical characterization (fig. S2). Using synthetic material, we showed that neither the des-nitro nor either of the other two stable metabolites formed by Ddn-mediated reduction of PA-824 had any detectable activity against Mtb, which suggested that the bactericidal event occurred during the process of reduction.

Unlike flavoproteins that perform nitroreduction by single-electron chemistry, F_{420} is an obligate two-electron, or hydride, donor resembling the nicotinamide cofactors (13). To explore the chemical mechanism for this unusual reduction, we performed the analogous chemical reduction of PA-824 by sodium borohydride (fig. S2). Desnitro PA-824, although not the major product, was also produced by borohydride-mediated reduction of PA-824. We unambiguously assigned H-2 (d6.68 ppm) and H-3 (d6.48 ppm) of desnitro PA-824 by observing a nuclear Overhauser effect (NOE) between the two protons at C-5 on the oxazine ring (δ4.01 ppm) and the imidazole proton at C-3 (δ6.48 ppm) (fig. S3). By performing the reduction with sodium borohydride in deuterated methanol and sodium borodeuteride in normal methanol, the proton at the C-3 position of des-nitro PA-824 was shown to be derived from reductant, whereas the proton at the C-2 position was derived from solvent (fig. S4).

Thus, we hypothesized that des-nitro PA-824 was formed by hydride transfer from $F_{420}H_2$ to C-3 of the imidazole ring followed by protonation of the resulting nitronic acid to give **A**, which can eliminate nitrous acid (Fig. 2). This is also observed in bacterial enzymes involved in bioremediation of 2,4,6-trinitrotoluene (TNT), at least one of which has been shown to be F_{420}-dependent (14,15). An electrochemical study of the sequential single-electron reduction of PA-824 by a radical mechanism supports the novel reduction of the imidazole ring preferentially to the nitro group (16). The nitrous acid released would be unstable and quickly disproportionate into nitric oxide (NO) and other reactive nitrogen intermediates (17). This same nitronic acid intermediate (or its tautomer) could instead be quenched by water giving

B, followed by elimination of hyponitrous acid (HNO) to give product **2**, the biochemical equivalent of the Nef reaction that typically requires strongly acidic conditions (18). Biological Nef reactions have previously been limited to nitroalkane dioxygenases that utilize flavins and molecular oxygen to convert an alkyl nitro group to a carbonyl (19). Although this is a minor product with PA-824, certain bicyclic nitroimidazoles (e.g., compound **7** in Table 1) show this as the major product. Further reduction of the nitronic acid intermediate produces the aromatic hydroxylamine metabolite **C**, which, upon further reduction and fragmentation, produces **3** (fig. S5) (20).

To test the hypothesis that reduction of PA-824 by Ddn resulted in the release of reactive nitrogen species, we used the Griess reagent to directly detect nitrous acid. A PA-824—dependent production of HNO_2 by Ddn was consistently observed in the presence of $F_{420}H_2$ (Fig. 3A). NO could also be detected in PA-824—treated whole cells, under both aerobic (Fig. 3B) and hypoxic nonreplicating conditions (fig. S6), by preloading cells with diaminofluorescein (DAF-FM) diacetate, a probe that fluoresces specifically in the presence of NO (21,22).

To correlate NO release with anaerobic killing, we selected a series of eight derivatives that had a broad range (from 2 to 125 μM, Table 1) of minimum anaerobicidal concentration (MAC) values (the aerobic potency of these compounds ranges from 0.04 to 6 μM). These derivatives revealed a strong correlation between the amount of the corresponding des-nitro product formed and anaerobic killing activity (Fig. 3C). Des-nitro formation did not correlate with aerobic activity, nor did any other metabolite correlate with either aerobic or anaerobic activity (fig. S7). The rate of NO release [an apparent V_{max}/K_m as RFU per hour divided by substrate affinity (the Michaelis constant) measured intracellularly] also correlated with anaerobic killing activity (Table 1). Finally, the effect of PA-824 on cells could be abrogated by a scavenger of NO, C-PTIO, which showed a dose-dependent decrease in NO production (Fig. 3D) and partially rescued hypoxic cells from killing by PA-824 (Fig. 3E). This effect was muted by both toxicity and poor cell penetrating ability of C-PTIO. Mtb mutants hypersensitive to NO, such as proteasome mutants, also display increased anaerobic sensitivity to PA-824 (fig. S8). The simplest interpretation of these data is that reactive nitrogen species are dominant in anaerobic activity but under aerobic conditions this is augmented by other effects.

Reactive nitrogen species play a major role in mammalian defense against mycobacterial infections, and mice deficient in the ability to produce NO are hypersusceptible to infection with Mtb (23). PA-824—resistant mutants in F_{420} biosynthesis have also been shown to be hypersensitive to killing by NO (24). Nitrite and NO both react with cytochromes and cytochrome c oxidase to interfere with electron flow and to cripple the coupling of respiration to reduction of oxygen. Thus, bicyclic nitroimidazoles may function as highly specific NO donors requiring activation by a powerful deazaflavin cofactor (F_{420}) that is unique to a small group of microorganisms and extremophiles, which makes cross-activation by mammalian enzymes highly unlikely. This unusual mechanism for nitroimidazole reduction avoids the single-electron—reduced nitrogen species typical of oxygen-sensitive, flavin-dependent enzymes and defines the chemistry of a class of F_{420}-dependent enzymatic nitroreductases.

NO-donating drugs have been shown to have potential for a wide variety of human diseases including cardiovascular disease, asthma, hypoxicischemic brain injury, glaucoma, and Alzheimer's disease (25). Indeed, it may be possible to more generally co-opt bacterial nitroreductases to reduce other nitro-containing heterocycles as an avenue for developing anti-infectives. It is noteworthy that the two candidate molecules in phase 2 trials (PA-824 and OPC-67683) were both optimized only for whole-cell aerobic activity and, as a result, have fairly modest anaerobic activity. Thus, these results have the potential to enable structure-based design of a drug specific for the treatment of latent tuberculosis.

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References and Notes

1. Matteelli A, et al. Expert Rev. Anti Infect. Ther 2007;5:857. [PubMed: 17914919]
2. Barry CE 3rd, Boshoff HI, Dowd CS. Curr. Pharm. Des 2004;10:3239. [PubMed: 15544513]
3. StopTB Working Group. New Drugs. 2008. www.stoptb.org/wg/ new_drugs/assets/documents/2007GlobalPipeline.pdf
4. Stover CK, et al. Nature 2000;405:962. [PubMed: 10879539]
5. Boshoff HI, Barry CE 3rd. Nat. Rev. Microbiol 2005;3:70. [PubMed: 15608701]
6. Gomez JE, McKinney JD. Tuberculosis (Edinburgh) 2004;84:29.
7. Matsumoto M, et al. PLoS Med 2006;3:e466. [PubMed: 17132069]
8. Boshoff HI, Barry CE 3rd. Drug Discov. Today Dis. Mech 2006;3:237.
9. Choi KP, Bair TB, Bae YM, Daniels L. J. Bacteriol 2001;183:7058. [PubMed: 11717263]
10. Choi KP, Kendrick N, Daniels L. J. Bacteriol 2002;184:2420. [PubMed: 11948155]
11. Materials and methods are available as supporting material on *Science* Online
12. Manjunatha UH, et al. Proc. Natl. Acad. Sci. U.S.A 2006;103:431. [PubMed: 16387854]
13. Walsh C. Acc. Chem. Res 1986;19:216.
14. Smets BF, Yin H, Esteve-Nunez A. Appl. Microbiol. Biotechnol 2007;76:267. [PubMed: 17534614]
15. Hofmann KW, Knackmuss HJ, Heiss G. Appl. Environ. Microbiol 2004;70:2854. [PubMed: 15128543]
16. Anderson RF, et al. Org. Biomol. Chem 2008;6:1973. [PubMed: 18480913]
17. Lundberg JO, Weitzberg E, Gladwin MT. Nat. Rev. Drug Discov 2008;7:156. [PubMed: 18167491]
18. Ballini R, Petrini M. Tetrahedron 2004;60:1017.
19. Fitzpatrick PF, Orville AM, Nagpal A, Valley MP. Arch. Biochem. Biophys 2005;433:157. [PubMed: 15581574]
20. Beaulieu BB, Mclafferty MA, Koch RL, Goldman P. Antimicrob. Agents Chemother 1981;20:410. [PubMed: 6272633]
21. Kojima H, et al. Anal. Chem 1998;70:2446. [PubMed: 9666719]
22. Boshoff HI, et al. J. Biol. Chem 2004;279:40174. [PubMed: 15247240]
23. MacMicking JD, et al. Proc. Natl. Acad. Sci. U.S.A 1997;94:5243. [PubMed: 9144222]
24. Darwin KH, Ehrt S, Gutierrez-Ramos JC, Weich N, Nathan CF. Science 2003;302:1963. [PubMed: 14671303]
25. Rigas B, Williams JL. Nitric Oxide 2008;19:199. [PubMed: 18486630]

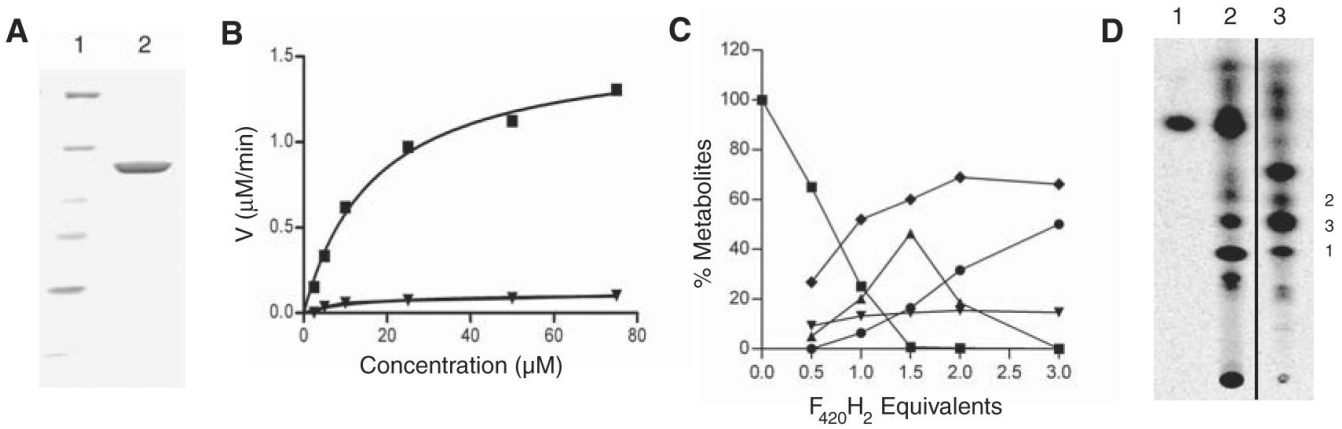


Fig. 1.

Ddn-mediated activation of PA-824. (**A**) Ddn was expressed as a maltose-binding protein fusion in *E. coli* and purified on an amylose resin column. (**B**) Ddn-catalyzed oxidation of $F_{420}H_2$ using PA-824 as substrate (squares, the maximum number of substrate molecules an enzyme can turn over per unit of time, $k_{cat})/K_m = 0.145\ min^{-1}\ \mu M^{-1}$,orthe enantiomer of PA-824 (inverted triangles $k_{cat}/K_m = 0.016\ min^{-1}\ \mu M^{-1}$). (**C**) Reaction was initiated by the addition of the indicated amount of $F_{420}H_2$, followed by incubation and analysis of aliquots by LC-MS. Squares, PA-824 (*m/z* 360); diamonds, des-nitro PA-824 (**1**, *m/z* 315); circles, metabolite **3** (*m/z* 291); inverted triangles, metabolite **2** (*m/z* 331); triangles, *m/z* 346, an unstable intermediate (**C**) quickly convertedto **3**. (**D**) Thin-layer chromatography (TLC) analysis of the conversion of [^{14}C]PA-824 (lane 1) by whole cells of Mtb (lane 2) or by purified Ddn using 25 μM $F_{420}H_2$ (lane 3). The numbered spots in (D) were shown to comigrate with the numbered metabolites from (C) by collecting HPLC peaks from (C) and analyzing by TLC.

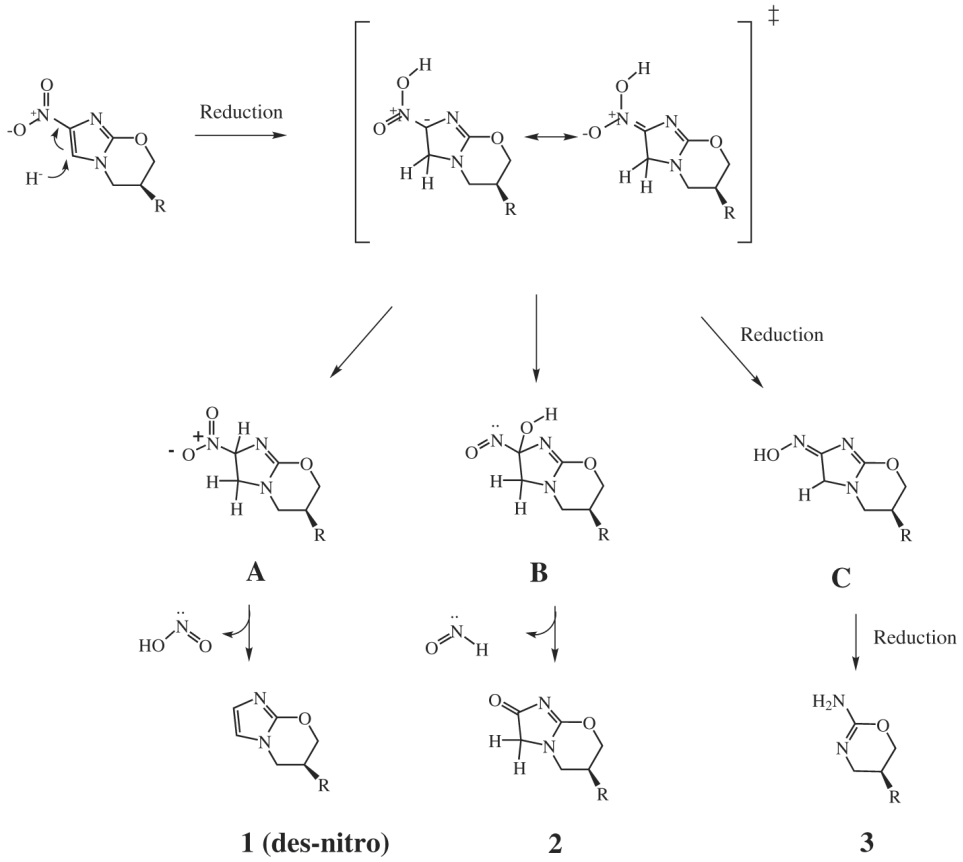


Fig. 2.

Mechanism of nitroimidazole reduction by Ddn. Initial hydride addition to C-3 of the bicyclic nitroimidazole results in formation of an intermediate that gives rise to all three observed stable products through the two resonance forms shown. Protonation of the C-2 position by solvent to give (**A**) followed by the elimination of nitrous acid to give **1**. Hydrolysis gives (**B**) which leads to another loss of a reactive nitrogen species in an “enzymatic Nef reaction” to produce **2**. Further reduction of the intermediate produces the unstable hydroxylamine (**C**) which decomposes to **3**.

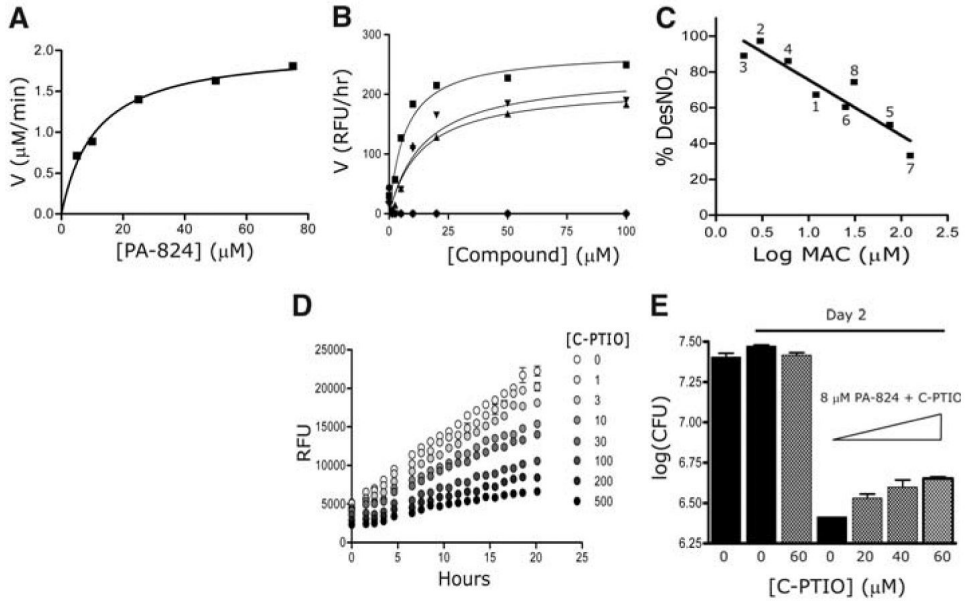


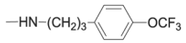
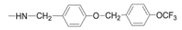
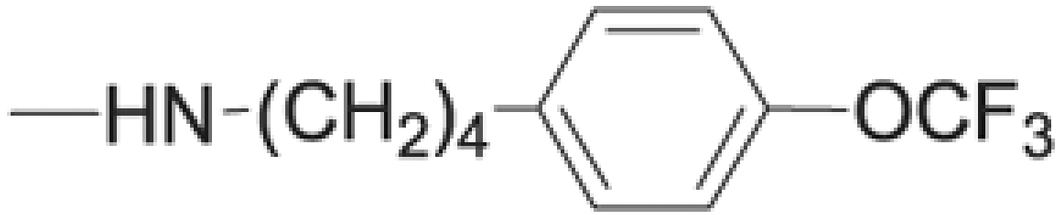
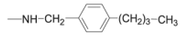
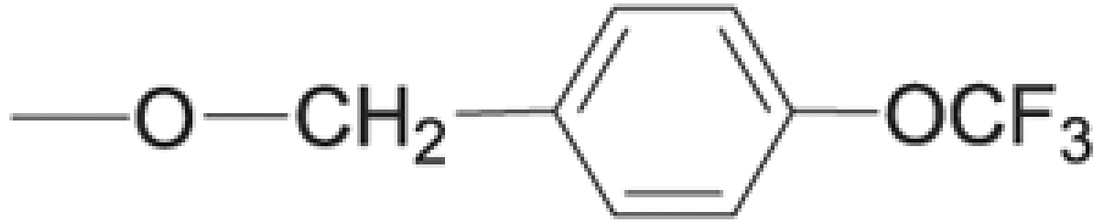
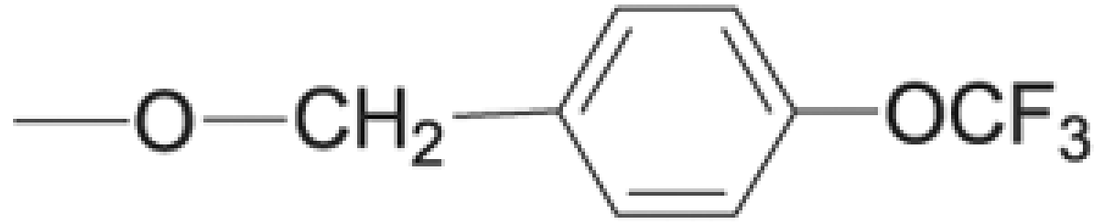
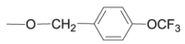
Fig.3.

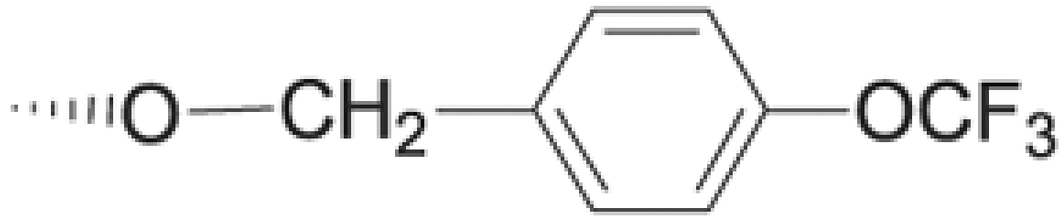
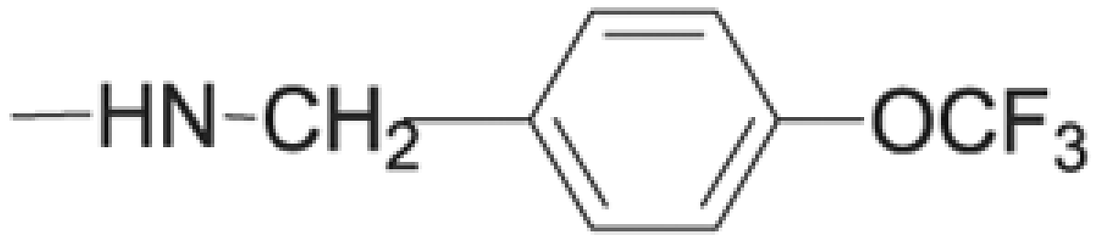
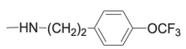
Formation of NO and correlation with anaerobic activity. **(A)** Monitoring of the in vitro reduction of PA-824 by Ddn using the Greiss reagent after incubation of purified Ddn with PA-824 and $F_{420}H_2$. **(B)**Kinetics of in vivo [NO] release in Bacille Calmette-Guérin (BCG)-sensitized cells with PA-824 (triangle), compound 10 (inverted triangle), compound 11 (square), isoniazid (diamond), and metronidazole (circle) by using DAF-FM diacetate. RFU, relative fluorescence units. **(C)** Correlation between anaerobic killing and the percentage of des-nitro metabolite formed by 8 bicyclic nitroimidazoles (Table 1). **(D)** In vivo [NO] release in BCG cells with 20 μM PA-824 in the presence of increasing CPTIO [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide] concentration. **(E)** Protection of PA-824-mediated killing of nonreplicating hypoxic cells by C-PTIO. Error bars are SD of three independent replicates.

Table 1

Structures of bicyclic nitroimidazoles and their aerobic MIC (minimum inhibitory concentration), anaerobic MAC (minimum anaerobicidal concentration), and the apparent V_{max}/K_m for in vivo NO release. MIC values represent the micromolar concentration that will inhibit 99% of the growth of an inoculum of Mtb. MAC values represent the micromolar concentration that will cause a 1 log reduction in bacterial numbers after a 1-week exposure to a compound of hypoxia-adapted Mtb. V_{max}/K_m ratio (RFU hr^{-1} μM^{-1}) values represent the in vivo NO release in *M. bovis* BCG cells. NS, no significant NO release observed; ND, not determined.

R	Z	MIC	MAC	V_{max}/K_m

	R	Z	MIC	MAC	V_{max}/K_m
2		0	0.08	3.1	60.1
3		0	0.16	2.1	76.9
4		0	0.04	8.1	71.6
5		0	0.78	75	4.7
6		S	1.2	25	NS
7		NH	0.80	130	4.8
8		NCHO	6.3	31	ND

	R	Z	MIC	MAC	V_{max}/K_m
9		0	110	250	NS
10		0	0.40	8.0	10.1
11		0	0.04	2.0	28.7

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