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The effect of 5-substitution on the electrochemical behavior and antitubercular activity of PA-824

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ABSTRACT

Nitroimidazole PA-824 is part of an exciting new class of compounds currently undergoing clinical evaluation as novel TB therapeutics. The recently elucidated mechanism of action of PA-824 involves reduction of the nitroimidazole ring and subsequent nitric oxide release. The importance of this compound and its unique activity prompted us to explore how substitution of the nitroimidazole ring would affect electrochemical reduction and antitubercular activity. We prepared analogs of PA-824 with bromo, chloro, cyano, and amino substituents in the 5-position of the aromatic ring. We found that substitution of the imidazole ring greatly influences reduction and the stability of the corresponding nitro radical anion. Further, the antitubercular activities of the bromo and chloro analogs may indicate that an alternate nitroreductase pathway within *Mycobacterium tuberculosis* exists.

Each year, tuberculosis, caused by *Mycobacterium tuberculosis* (Mtb), kills over 2 million people and 8 million are newly infected.[1] While there is a WHO-approved therapy for this disease, drug resistance is widespread and the need for new drugs is pressing.[2] In 2000, the structure and antitubercular activity of a unique nitroimidazole, PA-824 (**1**), was reported.[3] Since then, many research groups have investigated its potential as an addition to antitubercular therapy and it has entered Phase 2 clinical trials.[4–9] Interestingly, the mechanism of action of PA-824 remained elusive for some time and is unique with respect to antibiotic therapies.[7,10] The action of PA-824 relies on intracellular reduction of the nitro-bearing imidazole by an Mtb-specific nitroreductase, Ddn. This $2e^-$ reduction is coupled to the oxidation/reduction of F_{420}, an unusual deazaflavin cofactor that delivers a hydride atom in the reaction.

In previous work we reported the cyclic voltammetric behavior of PA-824 (**1**) with the aim of revealing the formation and stability of the corresponding nitro radical anion.[11] In that work we concluded that an aprotic medium was the best experimental condition to perform this study. Our work showed that PA-824 was electrochemically reduced via formation of a relatively stable nitro radical anion in a manner similar to that of the electrochemical reduction of the well-known bactericidal drug metronidazole. However, the PA-824-derived radical required more energy for formation (about 200 mV) and was approximately 100 times less stable than the corresponding metronidazole radical anion.

In light of the importance of reduction in the bioactivity of PA-824, we asked whether or not this reduction could be modulated by substitution on the imidazole ring and, if so, what the consequences would be on the antitubercular activity of such compounds. Because of its proximity to the nitro group, substituents at the 5-position of the imidazole ring might be expected to have the most influence on the reduction potential of this series. We therefore synthesized PA-824 analogs bearing either electron-withdrawing (cyano, chloro, and bromo) or electron-donating (amino) substituents in the 5-position to explore a breadth of electronic characteristics. We report here both the electrochemistry of the nitro radical anion formation and its stability, as well as the antitubercular activities of each of these compounds.

The synthetic process used to prepare compounds **2**–**5** is shown in the Scheme 1. PA-824 (**1**) was prepared as described.[12,13] Bromination of PA-824 (**1**) under basic conditions proceeded in high yield to give compound **2**.[14] Compound **3** was prepared in moderate yield from compound **2** using potassium cyanide and potassium iodide.[15] Aromatic amination of compound **1** was

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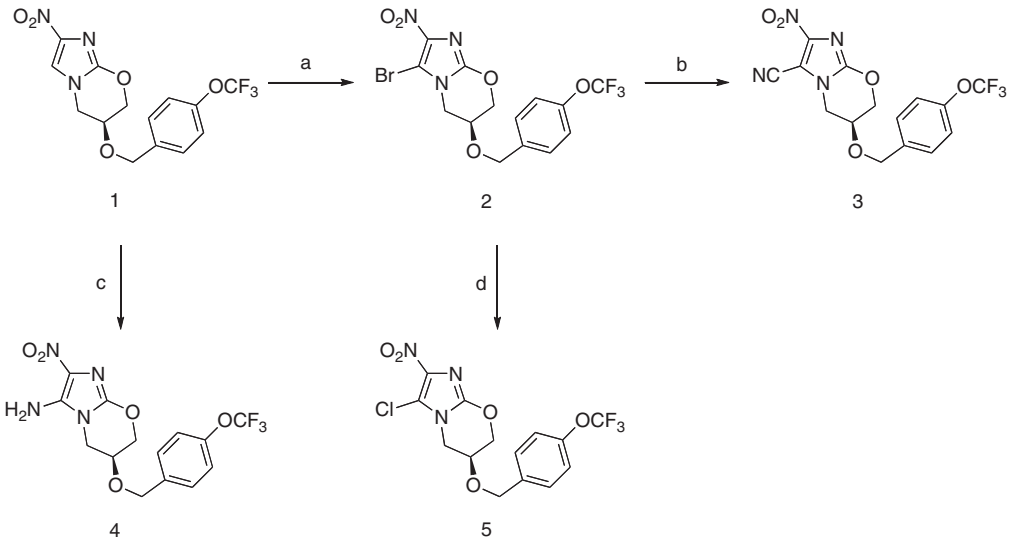


Scheme 1. Synthesis of 5-position Analogs of PA-824. Reagents and conditions: (a) Br_2, $KHCO_3$, DMF, 0 °C, 89%; (b) KI, KCN, DMF, 100 °C, 65%; (c) KOtBu, $(CH_3)_3NNH_2I$, DMSO, rt, 29%; (d) $NiCl_2$, DMF, mw, 100 W, rt to 170 °C, 5 min, 72%.

achieved using 1,1,1-trimethylhydrazinium iodide and potassium *tert*-butoxide in low yield to give compound **4**.[16] Chloro analog **5** was prepared from bromo analog **2** by halogen exchange using nickel(II) chloride in DMF under microwave conditions.[17] This reaction was also successful using standard conditions upon heating at 70 °C (not shown). Attempts to prepare the 5-fluoro compound were unsuccessful.

The effect of different 5-position substituents on reduction of the nitro group was evaluated using different electrochemical techniques and working electrodes.[18] Figure 1 shows differential pulse voltammograms obtained in aprotic medium (100% DMF) for each nitroimidazole. Each voltammogram shows a main peak

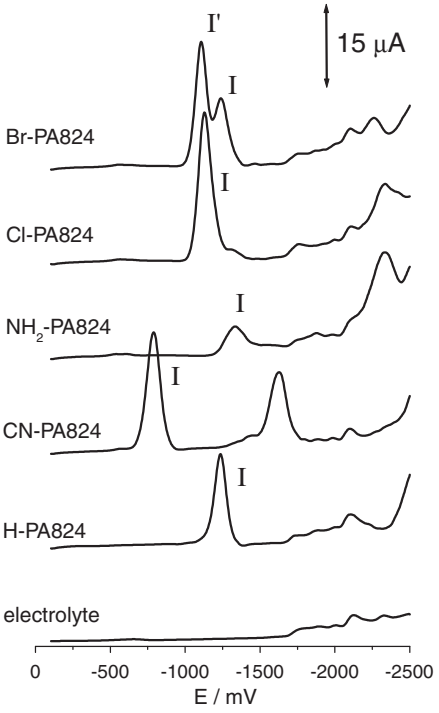


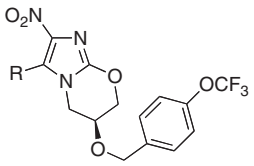
Figure 1. Differential pulse voltammograms obtained for the reduction of 1 mM 5-position substituted PA-824 analogs using a glassy carbon electrode (GCE) in DMF and 0.1 M tetrabutylammonium perchlorate (TBAP).

(I) due to reduction of the nitro group of the corresponding nitroimidazole derivative. This value is the peak potential value (E_P), a parameter that reflects the tendency of the molecule to be reduced. As can be seen, substituents adjacent to the nitro group influence the voltammograms significantly. Thus, compounds **2** (bromo), **3** (cyano), and **5** (chloro), with electron-withdrawing substituents, induce a positive shift in the reduction potential while the amino compound (**4**) produces a negative shift. In Table 1 the peak potential values of each nitro compound are shown. Comparing all of the derivatives, the cyano compound (**3**) shows the greatest shift in peak potential value, suggesting that the nitro radical anion resulting from this compound was the most thermodynamically favored. As expected, the inclusion of an electron-donating amino substituent (**4**) produced a negative shift, hindering the reduction process. This would impede formation of the nitro radical anion in biological systems.

Cyclic voltammetry (CV), unlike differential pulse voltammetry, not only gives information about the energy required for the reduction of the nitro group (E_p values) but also provides information about the kinetics of the nitro group electron transfer reaction (reversibility) and stability of the nitro radical anion formed (k_2 values). The advantage of CV is that information about both the cathodic and anodic electrochemical reactions is uncovered. In this case, the one-electron reduction of the nitro compound forms the corresponding nitro radical anion. This reaction generates a cathodic peak characterized by a cathodic peak potential (E_{pc}) and a cathodic peak current (I_{pc}). When the scan is reversed, the nitro radical anion is oxidized back to the initial nitro derivative, generating an anodic peak characterized by an anodic peak potential (E_{pa}) and an anodic peak current (I_{pa}). The current ratio, I_{pa}/I_{pc}, indicates the stability of the nitro radical. Thus, a current ratio of one indicates a stable nitro radical and the absence of further reaction. The ratio decreases if the radical reacts subsequently.

Using CV, it was possible to evaluate the influence of each substituent on the reduction mechanism of the nitro group. Figure 2 shows the cyclic voltammograms obtained using a mercury electrode. As expected the cyano (**3**), bromo (**2**), and chloro (**5**) derivatives produce a positive shift in the nitro reduction, but the $ArNO_2/ArNO_2^{\cdot-}$ couple is only clearly formed in the cyano derivative. Moreover, the redox mechanism is clearly more complex in the case of the bromo derivative, with two close signals (i.e., *I* and *I'*) that prevent the exact determination of which peak corresponds to the nitro radical

Table 1
Peak potentials (E_{pc}) for reduction of 5-substituted PA-824 analogs



Compound	R	DPV–GCE[a]	CV–Hg[b]		
		E_{pc} (mV)	E_{pc} (mV)	ΔE_p (mV)	$k_{2,dim}$ (M^{-1} s^{-1})
1	H	−1236	−1312	72	1.9×10^5
2	Br	−1107/−1236	−1084/−1281	—	—
3	CN	−784/−1635	−852/−1808	71	6.7×10^2
4	NH_2	−1331	−1528	—	—
5	Cl	−1126	−1217	276	—

[a] Values obtained by differential pulse voltammetry (DPV).
[b] Values obtained from cyclic voltammetric (CV) experiments.

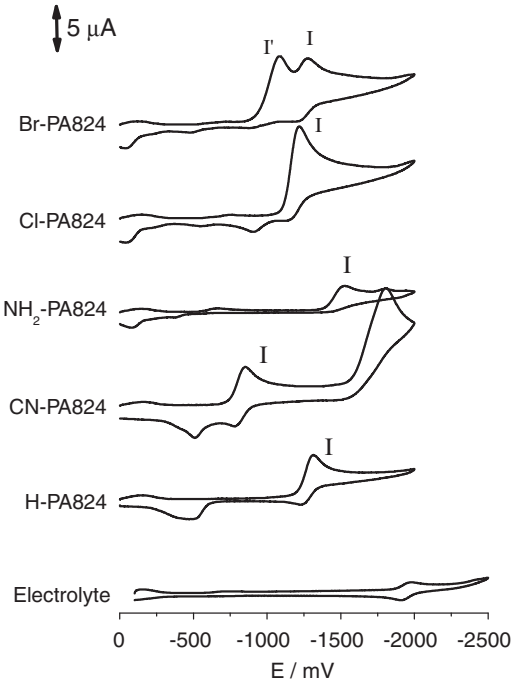


Figure 2. Cyclic voltammograms obtained for the reduction of 1 mM 5-position substituted PA-824 analogs using a drop mercury electrode (DME) in DMF and 0.1 M TBAP. Sweep rate = 1 V/s.

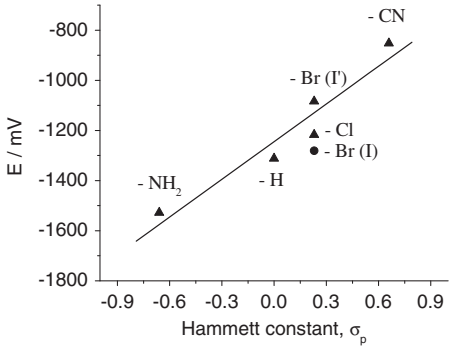


Figure 3. Dependence of the peak potential (E) for the reductions of 5-position substituted PA-824 analogs on the Hammett substituent constants (σ_p).[27] Peak potentials were obtained from cyclic voltammetric measurements at 1 V/s. The two points for bromine are from the two peaks found in the voltammogram.

anion. In fact the voltammetric behavior for the bromo derivative is indicative of a strong adsorption of the reaction product on the electrode surface as shown below.

The peak potential values obtained for each derivative using a mercury electrode are presented in Table 1. As can be seen, the order of reduction is similar to that obtained using a glassy carbon electrode (GCE). Plotting reduction potential versus Hammett substituent constants often leads to a linear correlation.[19] Figure 3 clearly shows a good correlation between the Hammett constant and the peak potential value (correlation coefficient = 0.96).

The nitro radical anion stability was evaluated by CV measurements varying the sweep rate during the experiment (Fig. 4). Variation of the sweep rate in CV is equivalent to changing the time schedule of the experiment thus permitting us to visualize free radical species like the nitro radical anion. Under these conditions, well-resolved, reversible cyclic voltammograms without interfering signals were obtained only for the parent PA-824 (**1**) and the cyano (**3**) derivative. For the chloro (**5**) derivative, an oxidation signal was observed but with a difference between cathodic and anodic peak potentials, ΔE_p, that far exceeds the value expected for a reversible system suggesting a quasi-reversible behavior. Finally, the bromo (**2**) and amino (**4**) derivatives did not show any oxidation signal, meaning that the nitro reduction was completely irreversible. The calculated ΔE_p values are shown in Table 1. Furthermore, according to the different evolution with the sweep rate of both peaks in the bromo derivative (I and I', Fig. 4) and knowing that adsorption peaks depend on sweep rate and diffusion peaks depend on square root of the sweep rate, we conclude that the product of the reduction is strongly adsorbed on the electrode producing an adsorptive pre-peak I' and a diffusion peak I.[20]

Kinetic analyses were possible only for the derivatives that showed a reversible mechanism on the mercury electrode.[21] The dependence of ΔE_p and the current ratio, I_{pa}/I_{pc}, on the sweep rate and the ω versus τ plot are shown in Figure 5. From the ΔE_p behavior, we can conclude that PA-824 (**1**) in DMF follows a quasi-reversible mechanism at low scan rates, but over 1 V/s the value is ~70 mV indicating that the transference is reversible. For the cyano derivative (**3**), the ΔE_p value was about 60–80 mV over all sweep rates studied. On the other hand, the I_{pa}/I_{pc} values of both compounds increase with increasing sweep rate up to 1, showing the typical variation of an ECi mechanism,[22] that is, with values lower than 1 at low sweep rates and values ~1 at higher sweep rates. According to the literature,[23] an electrochemically formed nitro radical anion in this medium can undergo dimerization. Olmstead and Nicholson developed theoretical approaches to evaluate

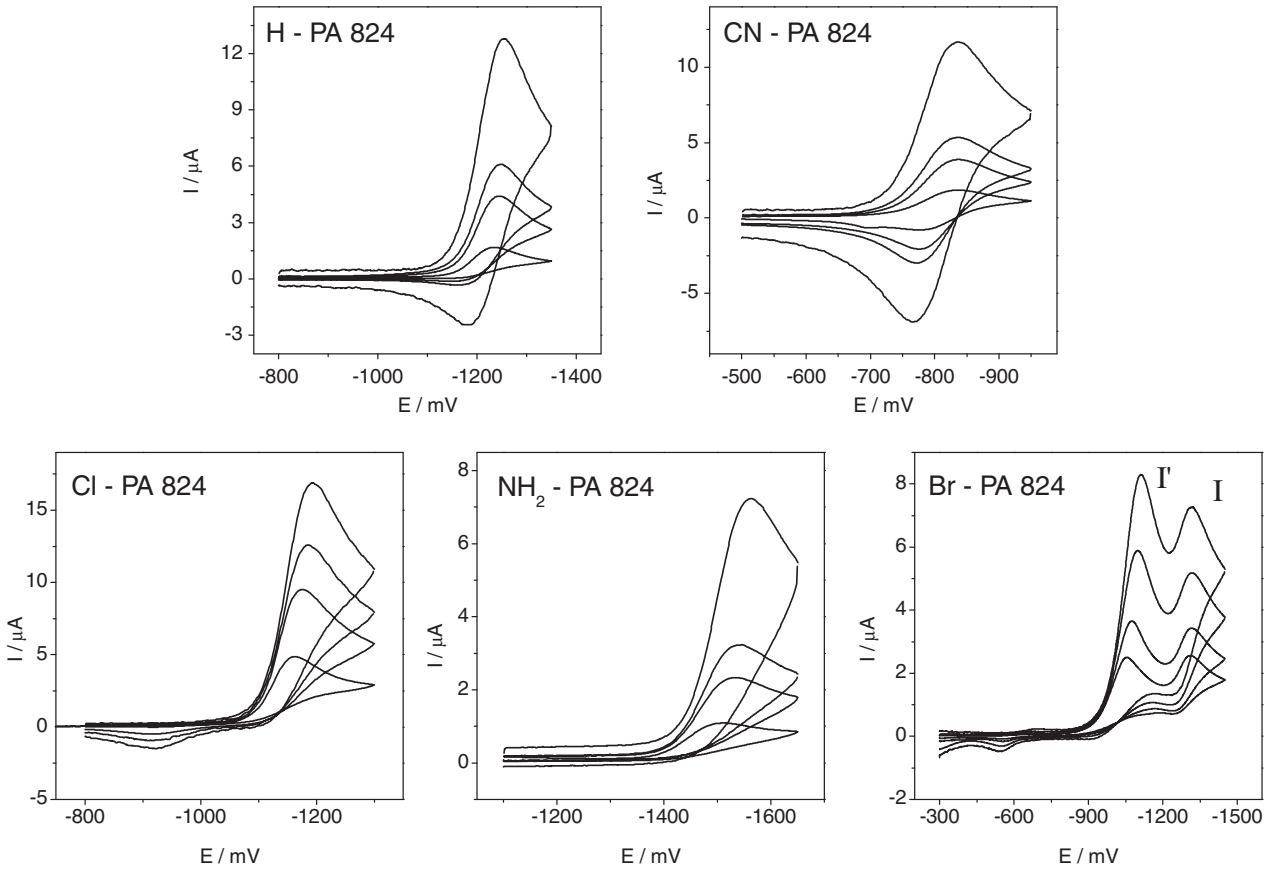


Figure 4. Cyclic voltammograms of the isolated couple nitro/nitroradical anion from 1 mM PA-824 and its analogs in DMF and 0.1 M TBAP at different sweep rates.

the corresponding decay constants, $k_{2,dim}$.[24,25] Applying these theoretical curves to our experimental data, we found a straight line in the ω versus τ plot (Fig. 5). From this, we were able to determine the decay constant values (Table 1). We conclude that the nitro radical anion formed from the cyano derivative (**3**) is almost 300-times more stable than the similar species formed from the parent PA-824 (**1**).

The biological activity of compounds **2–5** is shown in Table 2.[26] The data for PA-824 is given as a reference and has been reported previously.[5] Against wild-type Mtb, the bromo (**2**) and chloro (**5**) derivatives retained antitubercular activity, albeit 30-fold lower compared to the parent PA-824 (**1**). The cyano (**3**) and amino (**4**) derivatives were inactive. Superficially, this data suggests that while substitution at the 5-position is tolerated, strongly electron-withdrawing or electron-donating substituents in this position are not. Looking further, we see that compounds **2** and **5** are also active against Class B1 and C mutant Mtb strains where PA-824 is inactive (B1 mutants are defective in production of coenzyme F_{420} while C mutants are defective in Ddn, the enzyme which catalyzes reduction of this class of compounds using F_{420}).[7] These data suggest that the antitubercular activity of compounds **2** and **5** is not reliant on the biosynthesis of cofactor F_{420} in the same way that PA-824 is. The most likely explanation of these results is that reduction of these compounds proceeds by a different pathway than PA-824. Since the mechanism of action of PA-824 involves reduction not of the aromatic nitro group per se but rather hydride transfer to C-4 of the imidazole ring, one possible explanation is that the 5-substituted compounds are instead reduced at the nitro substituent. Due to the modest activity of these compounds, we cannot rule out a non-specific mechanism within the cell or cell membrane. It is interesting to note that the bromo derivative **2** is active against anaerobic (MAC) Mtb cultures at 2–4-fold higher concentrations compared with PA-824.

The cyclic voltammetric results show that substitution in the 5-position of the nitroimidazole greatly influences the electroreduction of the adjacent nitro group. Thus, the bromo and chloro derivatives produced a decrease in the redox potential but the resulting nitro radical anion is not a stable species. On the other hand, the amino derivative hindered the electroreduction of the nitro group at about 200 mV (mercury electrode) and study of the radical was not possible because it was not sufficiently stable in the timescale of the cyclic voltammetric experiment. Finally, incorporation of a cyano group in the 5-position produced a dramatic decrease in the energy required for nitro reduction (~460 mV) and its nitro radical anion was shown to be ~300 times more stable than the parent PA-824. Interestingly, the bromo and chloro derivatives were active against wild-type and F_{420} mutant strains of Mtb, while the amino and cyano derivatives were devoid of antitubercular activity.

Taken together, our results indicate that: (1) reduction of the bromo and chloro derivatives may occur via an Mtb nitroreductase that is distinct from Ddn and which may follow a different mechanism compared with PA-824; (2) electron-rich nitroimidazoles may not be reducible within the Mtb cell; and (3) electron-deficient nitroimidazoles are reduced slowly and result in a more stable nitro radical anion. One might expect these latter compounds to have greater antitubercular activity due to an increased production of NO. They did not display higher activity against wild-type H37Rv. One reason for this may be that electrochemical reduction is not perfectly mimicked within the cell, and steric constraints of the relevant nitroreductase prevented facile nitroreduction. Additionally, NO production could be measured in a manner similar

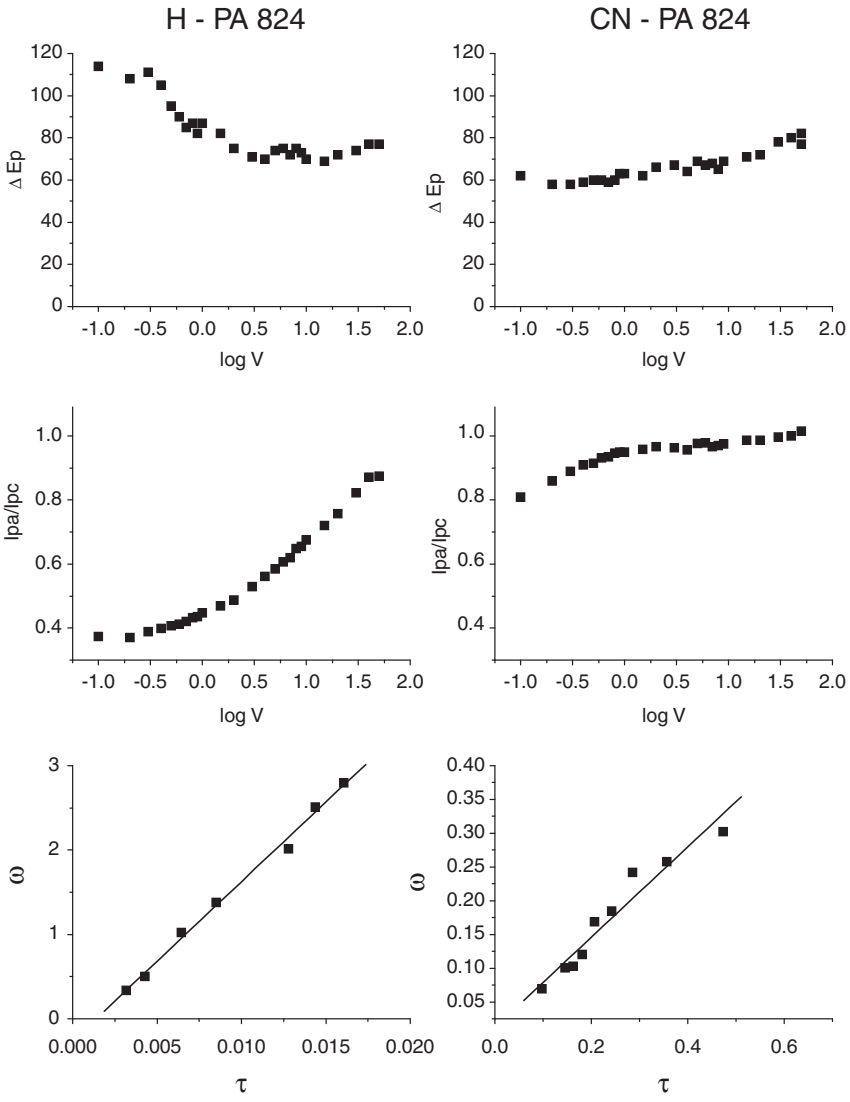
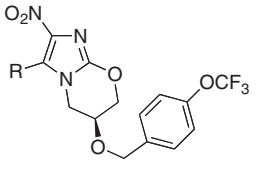


Figure 5. ΔE_p and I_{pa}/I_{pc} vs. log sweep rate plots and plot of the kinetic parameter, ω, with the time constant, τ, for PA-824 and its cyano analog according to the theoretical approach of Nicholson and Shain for dimerization.[21]

Table 2
Biological activities of 5-substituted PA-824 analogs



Compound	R	wtH37Rv MIC (μM)	wtH37Rv MAC (μM)	Class B1[a] MIC (μM)	Class C[b] MIC (μM)
1	H	0.8	8–16	>100	>100
2	Br	25	32.5	25	25
3	CN	>100	nd	>100	>100
4	NH_2	>100	>500	>100	>100
5	Cl	25	nd	25	50

nd = not done.
[a] B1 mutant is F_{420}^-.
[b] C mutant is Ddn^-.

to that for PA-824[10] to show that a nitroreductive mechanism remains at work. These experiments are beyond the scope of this work.

The most intriguing result to come from this data is the possibility of an alternate nitroreductase that reduces the bromo/chloro derivatives. This possibility was raised in previous work by Hurdle

et al.[28] If such a protein exists, pursuing it and its substrate toward a second-generation nitroimidazole series would seem worthwhile. These compounds would theoretically overcome resistance to PA-824 and related clinical candidates.

Acknowledgements

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14. Preparation of **2**. 3-Bromo-2-nitro-6-(4-trifluoromethoxy-benzyloxy)-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazine. Bromine (62 μL, 0.50 mmol) was added to a stirred suspension of PA-824 (**1**, 0.15 g, 0.42 mmol) and $KHCO_3$ (0.063 g, 0.63 mmol) in anhydrous DMF (5 mL) at 0 °C. The resulting mixture was stirred at room temperature for 2 h. The solvent was removed under reduced pressure, and water (10 mL) was added to the residue, and the mixture was extracted with ethyl acetate (10 mL × 2). The organic layer was washed with brine (10 mL), dried ($MgSO_4$) and evaporated. The crude residue was purified by column chromatography (methylene chloride/methanol = 30:1 ratio) to give **2** (pale yellow solid, 0.16 g, 89%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.98 (dd, J = 3.9, 13.2 Hz, 1H), 4.08–4.14 (m, 1H), 4.18–4.19 (m, 1H), 4.33–4.37 (m, 1H), 4.61–4.68 (m, 2H), 4.71 (d, J = 12.0 Hz, 1H), 7.21–7.23 (m, 2H), 7.34–7.36 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 47.2, 66.5, 67.7, 70.5, 99.9, 118.9, 121.4, 122.3, 129.3, 135.3, 147.3, 149.4; MS(ESI) m/e 438, 440 $[M+H]^+$ (1:1 ratio of Br isotope pattern); mp 124.8–126.1 °C; HRMS m/e calcd for $C_{14}H_{12}N_3O_5BrF_3$ 437.9912, found 437.9911.
15. Preparation of **3**. 2-Nitro-6-(4-trifluoromethoxy-benzyloxy)-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazine-3-carbonitrile. To a solution of **2** (0.20 g, 0.46 mmol) in DMF (10 mL) was added KI (0.015 g, 0.091 mmol) and KCN (0.059 g, 0.91 mmol). The reaction mixture was heated to 100 °C and stirred overnight. The solvent was removed and water (40 mL) was added. The resulting mixture was extracted with methylene chloride (40 mL × 2). The organic layer was washed with brine (40 mL), dried ($MgSO_4$) and evaporated. The crude residue was purified by column chromatography (hexanes/ethyl acetate = 1:3 ratio) to give **3** (white solid, 0.11 g, 65%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.19–4.32 (m, 3H), 4.39–4.44 (m, 1H), 4.63 (d, J = 12.0 Hz, 1H), 4.71–4.77 (m, 2H), 7.22–7.26 (m, 2H), 7.34–7.37 (m, 2H); MS(ESI) m/e 385 $[M+H]^+$; mp 158–159 °C; HRMS m/e calcd for $C_{15}H_{12}N_4O_5F_3$ 385.0760, found 385.0757; $[\alpha]_D^{20}$ −34.1 (c, 0.51, $CHCl_3$).
16. Preparation of **4**. 2-Nitro-6-(4-trifluoromethoxy-benzyloxy)-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazin-3-ylamine. KOtBu (0.44 g, 3.90 mmol) was added to a stirred solution of **1** (0.70 g, 1.95 mmol) and 1,1,1-trimethylhydrazinium iodide (0.43 g, 2.15 mmol) in DMSO (10 mL) at rt. The reaction mixture was stirred further under an inert atmosphere overnight. Water (200 mL) was added to the mixture, and the precipitate was filtered and washed with water to give **4** (yellow solid, 0.21 g, 29%): ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.89–4.05 (m, 2H), 4.20–4.24 (m, 1H), 4.36 (d, J = 11.7 Hz, 1H), 4.59–4.71 (m, 3H), 7.33–7.35 (m, 1H), 7.43–7.46 (m, 2H), 7.83 (br s, 2H); MS(ESI) m/e 375 $[M+H]^+$; mp 235 °C (dec); HRMS m/e calcd for $C_{14}H_{14}N_4O_5F_3$ 375.0916, found 375.0918.
17. Preparation of **5**. 3-Chloro-2-nitro-6-(4-trifluoromethoxy-benzyloxy)-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazine. Compound **2** (0.020 g, 0.045 mmol) was placed in a glass tube with nickel(II) chloride (5.9 mg, 0.045 mmol), DMF (0.5 mL) and stirring bar. The vessel was sealed with a septum and placed into the microwave cavity. Microwave irradiation of 100 W was used, the temperature being ramped from rt to 170 °C. Once this temperature was reached, the reaction mixture was held at this temperature for 5 min. After the reaction, the solvent was removed under reduced pressure. Water (5 mL) was added to the residue and the mixture was extracted with ethyl acetate (5 mL × 2). The organic layer was washed with brine (5 mL), dried ($MgSO_4$) and evaporated. The crude residue was purified by recrystallization (mixture of ethyl acetate and hexanes) to give **5** (white solid, 0.013 g, 72%): ^{1}H NMR (300 MHz, $CDCl_3$ + CD_3OD) δ 4.02–4.17 (m, 2H), 4.22–4.25 (m, 1H), 4.36 (dd, J = 12.0 Hz, 1.5 Hz, 1H), 4.62 (d, J = 12.3 Hz, 1H), 4.66–4.71 (m, 1H), 4.72 (d, J = 12.3 Hz, 1H), 7.21–7.23 (m, 2H), 7.35–7.39 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 46.1, 66.3, 67.6, 70.4, 114.1, 118.9, 121.4, 122.3, 129.3, 135.4, 138.4, 145.4, 149.3; MS(ESI) m/e 394, 396 $[M+H]^+$ (3:1 ratio of Cl isotope pattern); mp 141–142 °C; HRMS m/e calcd for $C_{14}H_{12}N_3O_5Cl_1F_3$ 394.0418, found 394.0413; $[\alpha]_D^{20}$ −34.09 (c, 0.87, $CHCl_3$).
18. Electrochemical experiments were performed using a totally automated BAS CV-100 voltammetric analyzer. All experiments were carried out at a constant temperature of 25 ± 0.1 °C using a 10 mL thermostatic cell. A static mercury drop electrode (SMDE) mode in a Controlling Growth Mercury Electrode stand from BAS, with a drop area of 0.42 mm^2, and a 3 mm diameter Glassy Carbon Electrode (GCE) as working electrodes and a platinum wire as a counter electrode were used. All potentials were measured against 3 M Ag/AgCl.
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21. For voltammetric studies, 1 mM of each drug in anhydrous dimethylformamide (DMF) and 0.1 M of tetrabutylammonium perchlorate (TBAP) was used.[18] In the kinetic analysis carried out by cyclic voltammetry, the return-to-forward peak current ratio I_{pa}/I_{pc} for the reversible first-electron transfer (the $ArNO_2/ArNO_2^{\cdot-}$ couple) was measured from each cyclic voltammogram, varying the scan rate from 0.1 to 10 V/s according to the procedure described by Nicholson.[21] Using the theoretical approach of Olmstead et al.,[23,24] the I_{pa}/I_{pc} values measured experimentally at each scan rate were inserted into a working curve to determine the parameter ω, which incorporates the effects of rate constant, drug concentration and scan rate. A plot of ω versus τ resulted in a linear relationship described by the equation

$$\omega = k_2 \times C_o \times \tau$$

where k_2 is the second-order rate constant for the chemical reaction of $ArNO_2^{\cdot-}$, C_o is the compound concentration and $\tau = (E_\lambda - E_{1/2})/v$. We obtained the second-order rate constant for the decomposition of the nitro radical anion from the slope of the straight line ω versus τ. The assumption that the decomposition of $ArNO_2^{\cdot-}$ follows second-order kinetics is supported by the linear relation between the kinetic parameter ω and the time constant τ.
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26. The antitubercular activities of all compounds were measured as has been previously described using the H37Rv laboratory strain of *Mycobacterium tuberculosis*.[5] Mutant strains B1 and C have been described previously.[7]
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