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Antitubercular Nitroimidazoles Revisited: Synthesis and Activity of the Authentic 3-Nitro Isomer of Pretomanid

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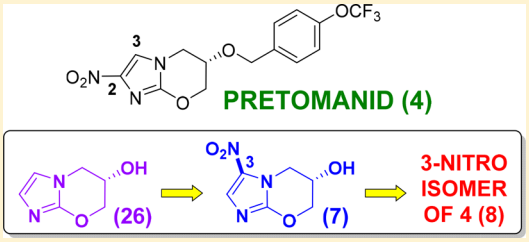
S *Supporting Information*

ABSTRACT: A published study of structural features associated with the aerobic and anaerobic activities of 4- and 5-nitroimidazoles had found that the 3-nitro isomer of pretomanid, **8**, displayed interesting potencies, including against nitroreductase mutant *Mycobacterium tuberculosis*. However, recent nuclear magnetic resonance analyses of two trace byproducts, isolated from early process optimization studies toward a large-scale synthesis of pretomanid, raised structural assignment queries, particularly for **8**, stimulating further investigation. Following our discovery that the reported compound was a 6-nitroimidazooxazole derivative, we developed a *de novo* synthesis of authentic **8** via nitration of the chiral des-nitro imidazooxazine alcohol **26** in trifluoroacetic or acetic anhydride, and verified its identity through an X-ray crystal structure. Unfortunately, **8** displayed no antitubercular activity (MICs > 128 μM), whereas the second byproduct (3′-methyl pretomanid) was eight-fold more potent than pretomanid in the aerobic assay. These findings further clarify target specificities for bicyclic nitroimidazoles, which may become important in the event of any future clinical resistance.



KEYWORDS: *Tuberculosis, pretomanid, drug resistance, nitroimidazole, nitration, silyl migration*

Tuberculosis (TB) currently ranks alongside HIV/AIDS as one of the leading causes of global mortality, claiming about 1.4 million lives every year.[1] The spread of multi- and extensively drug-resistant (MDR/XDR) TB is widely regarded as a burgeoning health crisis, placing great strain on limited healthcare resources for only modest treatment outcomes (cure rates of 20−50% following complex therapy for up to 30 months with several more costly and toxic agents).[1−3] The recent emergence of programmatically incurable tuberculosis has led to many patients being discharged back into the community, further threatening control efforts.[4,5] In this context, the conditional approval of two new MDR-TB drugs, delamanid (OPC-67683, **1**; see Figure 1) and bedaquiline (TMC-207, **2**), is a tremendous advance, although access to these and repurposed agents such as linezolid (**3**) remains very limited in many countries.[4,6] To further address this urgent need, the TB Alliance has been developing shorter acting novel regimens involving the nitroimidazooxazine pretomanid (PA-824, **4**).[7,8] Initial phase III clinical results for the combination of **2**, **3**, and **4** (Nix-TB) in XDR-TB patients look highly encouraging, with 29 of 31 patients who completed the treatment and 6 month follow-up being cured.[9] Another regimen that combines **2** and **4** with pyrazinamide (**5**) and moxifloxacin (**6**) is also demonstrating excellent bactericidal efficacy against both drug-sensitive- and MDR-TB.[10]

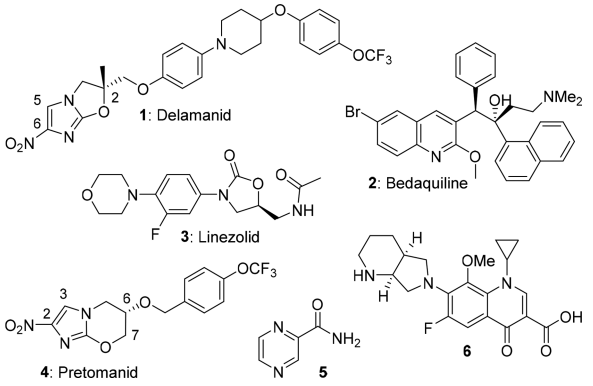


Figure 1. Drugs included in novel regimens for MDR/XDR-TB.

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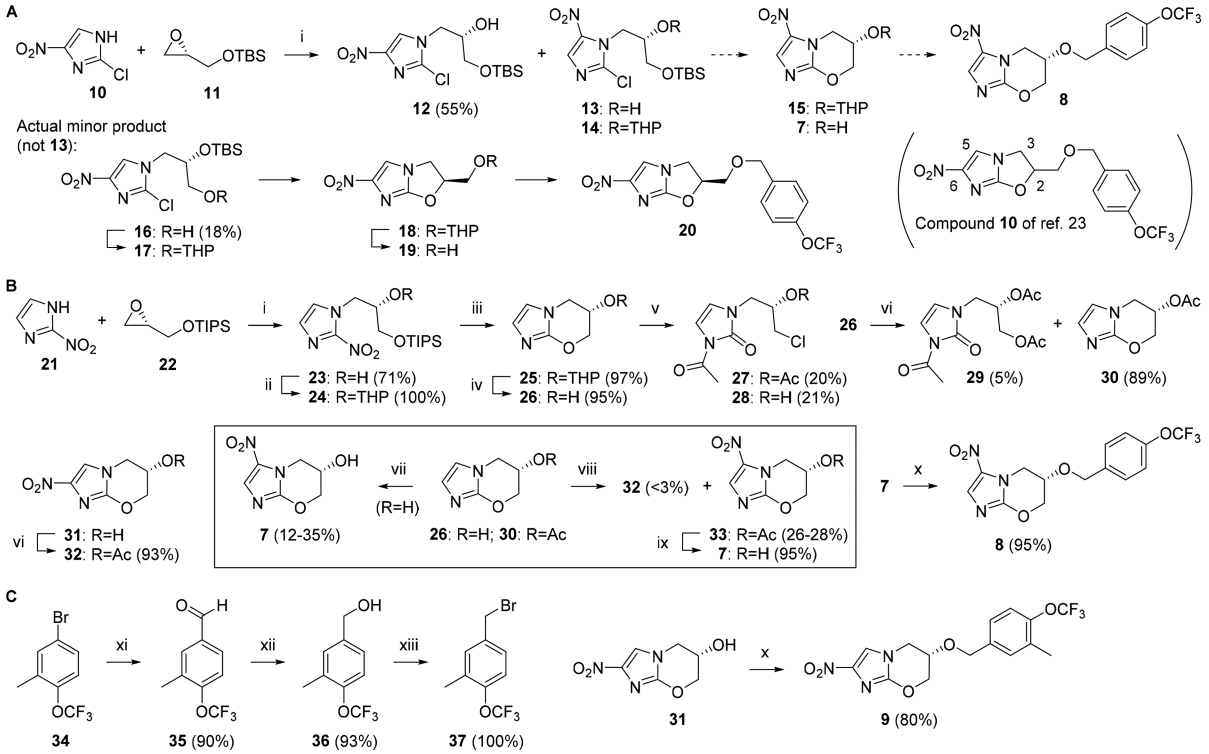
Scheme 1. Synthesis of the Target Compounds 7−9, Highlighting a Key Nitration Step[a]



[a]Reagents and conditions: (i) K_2CO_3, EtOH, 70 °C, 20 h (for **12**/**16**), or 75−82 °C, 23 h (for **23**); (ii) 3,4-dihydro-2*H*-pyran, PPTS, toluene, 20 °C, 5 d; (iii) TBAF, THF, 124 °C, 24 h (sealed tube); (iv) conc. HCl (1.1 equiv), MeOH, 20 °C, 15 h; (v) AcCl, pyridine, 0−20 °C, 4 h; (vi) Ac_2O, pyridine, 0−20 °C, 7−19 h; (vii) TFAA, −5 to 0 °C, 15 min, then conc. HNO_3, −50 to 20 °C, 3 h, then ice, $NaHCO_3$; (viii) Ac_2O, 0−20 °C, 5−80 min, then conc. HNO_3, conc. H_2SO_4, −50 to 0 °C, 1.5−2.5 h, then ice, $NaHCO_3$; (ix) $NaHCO_3$, aq. MeOH, 20 °C, 5 h; (x) 4-OCF_3BnBr or **37**, NaH, DMF, 0−20 °C, 2.5−3.3 h; (xi) *n*BuLi, THF, −78 °C, 1 h, then DMF, −78 to 20 °C, 1.5 h, and then aq. citric acid; (xii) $NaBH_4$, MeOH, 0−20 °C, 1.5 h; (xiii) HBr, AcOH, 20 °C, 13 h.

The mechanism of action of **4** involves bioreductive activation by a deazaflavin (F420)-dependent nitroreductase (Ddn), leading to several metabolites arising from reduction of the imidazole ring at C-3, including a des-nitro derivative.[11] Formation of the latter correlates with the release of nitric oxide, a crucial element in the *anaerobic* bactericidal activity of **4**, which is considered important for treatment shortening.[12] In contrast, the aerobic killing effects of **4** are attributed to the inhibition of cell wall mycolic acid biosynthesis.[13] The 5-nitroimidazole metronidazole also has some antitubercular activity under hypoxic conditions (*vide infra*), but a clinical study in MDR-TB patients showed that it was too neurotoxic for long-term use.[14]

In the past decade, several structure−activity relationship (SAR) studies of the nitroimidazooxazine class have been reported by ourselves and others,[15,16] seeking more effective second-generation analogues of **4**. While the predominant focus has centered on optimizing the aryl side chain (e.g., clinical candidate TBA-354[17]), a few studies examined alternative linkages at C-6[18] or more fundamental modifications to the nitroheterobicyclic "warhead".[19,20] Here, replacement of the nitroimidazole portion by nitrotriazole or nitropyrazole abolished activity, whereas the 8-oxygen could be exchanged for sulfur or nitrogen, and substitution at C-7 was tolerated.[21] Notably, Kim et al.[22] also described the synthesis and biological evaluation of the 3-nitro isomer of **4**, which was recorded as being "only slightly less active" (5- to 10-fold) than **4** itself, whereas its precursor alcohol derivative was 16- to 31-fold less active than **4**. Furthermore, the anaerobic killing of mutant *Mycobacterium tuberculosis* (*M. tb*) by this isomeric compound was suggested to imply a different biological target. This result could be of particular significance in the event of future clinical resistance to **4**. Nevertheless, in the related nitroimidazooxazole class (cf. **1**), we discovered that relocating the nitro group from C6 to C5 reduced the aerobic MIC against *M. tb* by at least 2−3 orders of magnitude.[23]

During initial attempts to optimize a large-scale synthesis of **4**, two trace byproducts were isolated (<0.5%), and one of these was postulated to be the 3-nitro isomer, although its nuclear magnetic resonance (NMR) data did not match that provided by Kim et al.[22] To establish its identity, we sought to make this 3-nitroimidazooxazine analogue of **4** via an unambiguous chiral synthesis. Concurrently, we also targeted the second compound, thought to be a 3′-methyl derivative of **4**. We now report the intriguing findings from this study, including some preliminary biological assessments.

To prepare the 3-nitro isomer of **4**, we first investigated the method of Kim[22] (Scheme 1A). Reaction of 2-chloro-4-nitroimidazole (**10**) with the TBS ether derivative of *R*-glycidol (**11**) gave two products in a ratio of ∼3:1, with the major one being the expected 4-nitroimidazole derivative **12**, as described. However, after low-temperature crystallization of the more polar oily minor product (reportedly the 5-nitroimidazole isomer of **12**, i.e., **13**), we found that its ^{1}H NMR spectrum in $CDCl_3$ contained a D_2O-exchangeable hydroxyl proton resonance at δ_H 1.83 ppm, which appeared as a sharp "dd" (J = 6.7, 5.1 Hz), and coupled to the two proton resonance of a methylene group in a COSY experiment. Moreover, a NOESY

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experiment revealed a strong NOE effect between the imidazole proton resonance and the proton resonances from the directly attached *N*-methylene. HMBC correlations between H-5 and this NCH_2 carbon and between the NCH_2 protons and both C-2 and C-5 (Figure 2) confirmed that the correct structure of

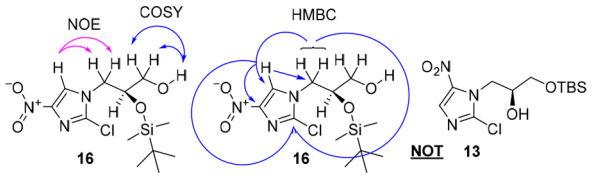

Figure 2. Two-dimensional NMR evidence for structure **16** over the reported **13**.

this minor product was the 4-nitroimidazole **16**. This result is in good accordance with the known tendency of a TBS group to migrate under basic conditions, as employed here.[24]

A critical consequence of this structural assignment error is that subsequent THP protection of the primary hydroxyl of **16** and treatment with TBAF (as reported) would induce cyclization to a 6-nitroimidazooxazole (**18**), rather than the 3-nitroimidazooxazine **15** (Scheme 1A). Therefore, following THP deprotection and installation of the 4-(trifluoromethoxy)-benzyl ether, Kim et al. finally obtained **20** (instead of **8**), equivalent to the 2*S* enantiomer of compound **10** in our nitroimidazooxazole paper,[23] as verified by their identical ^1H and ^{13}C NMR data. We note that while our compound had been prepared by an independent and unequivocal method, we have rigorously confirmed its structure by 2D NMR, including a NOESY experiment, where an NOE effect was observed between the imidazole proton resonance (H-5) and resonances from the adjacent methylene protons at the 3-position in the oxazole ring. This revised structure (**20**) for the compound claimed by Kim et al. as **8** is also consistent with the much smaller than expected optical rotation value obtained ($[\alpha]_D^{20}$ +7.4; cf. −44.7 for **4** in MeOH[25]) as well as the moderate antitubercular potency recorded (*vide infra*), as *S* enantiomers are known to be an order of magnitude less effective than *R* forms in the 2*H*-nitroimidazooxazole class.[23]

In an alternative approach to the 3-nitro isomer of **4**, we explored the novel nitration of known[22] imidazooxazine alcohol **26** (Scheme 1B). This strategy was based on previous success in our laboratory with the nitration of a related 5,6,7,8-tetrahydroimidazo[1,2-*a*]pyridin-6-ol in acetic anhydride,[19] as well as literature precedence for the regioselective C-5 nitration of both 1-methyl-2-(methylsulfanyl)-1*H*-imidazole[26] and 2,3-dihydroimidazo[2,1-*b*][1,3]thiazole.[27] During our initial synthesis of **26**, we again encountered a problem with migration of the TBS group when glycidyl ether **11** was reacted with 2-nitroimidazole (**21**).[22] Fortunately, this was easily overcome by switching the protecting group to the more stable TIPS (**22**).[24,25] We also made improvements to the subsequent THP protection and deprotection steps by adopting methods from Orita et al.,[25] enabling the synthesis of alcohol **26** in 65% yield over four steps. Surprisingly, attempted preparation of its acetate ester (**30**) using acetyl chloride in pyridine gave only ring opened *N*-acetyl imidazol-2-one products, **27** and **28** (having a distinctive ^1H NMR methyl resonance[28] at δ_H 2.64 ppm); this was largely circumvented by employing acetic anhydride.

Several reagent systems were then explored for the nitration of **26** or **30** (Table 1). Neither heating in aqueous nitric acid nor treatment with acetyl nitrate in acetic anhydride gave the desired product, while a reaction of **30** with nitronium tetrafluoroborate was low-yielding and accompanied by minor side products. Overall, the best conditions were 70% nitric acid in trifluoroacetic anhydride[29] or a mixture of concentrated nitric and sulfuric acids in acetic anhydride, which enabled yields of ∼25−30% of the 3-nitro derivatives, **7** and **33**, respectively. Intriguingly, only one nitro isomer was formed in trifluoroacetic anhydride, but this reaction proved to be more capricious than the acetic anhydride alternative, where the isomer ratio was ∼9:1 in favor of nitration at C-3 over C-2 (for confirmation, known[25] 2-nitro acetate ester **32** was prepared from alcohol **31**[30]). Isomers **32** and **33** were separable by careful column chromatography, and cleavage of acetate **33** to alcohol **7** was cleanly achieved through the use of a mild base ($NaHCO_3$).

Importantly, all of the successful nitration methods gave a complete retention of stereochemistry (100% ee by chiral HPLC analysis, using the analogously synthesized racemic form of **7** as a reference standard). This indicated that the in situ formed trifluoroacetate or acetate esters were sufficiently stable to protect the chiral alcohol from potential racemization, e.g., through the formation of a chiral nitrate ester and subsequent hydrolysis, as this can induce inversion of configuration.[31] Alkylation of pure **7** with 4-(trifluoromethoxy)benzyl bromide (NaH/DMF) then gave the desired target **8** in excellent yield (95%), and gratifyingly, this proved to be identical (by NMR, mp, HPLC, and optical rotation) to the first byproduct derived from optimization studies for a large-scale process route to **4**. As expected from findings in the nitroimidazooxazole class, the rotation value for **8** {$[\alpha]_D^{24}$ −150.7 (*c* 1.002, $CHCl_3$)} was indeed much larger than the one recorded for **4**.[25] Conclusive structural proof was gained through a single-crystal X-ray

Table 1. Summary of Nitration Methods Explored for the Synthesis of Alcohol 7 or Acetate 33

compd	solvent	reagents (equiv)	temp range (°C)	time/temp	products (% yield)
26	H_2O	3 M HNO_3 (9)	80 to 97	3 h/97 °C	
26	H_2O	50% HNO_3 (>100)	90	2 h/90 °C	
26[a]	Ac_2O	70% HNO_3 (3.1)[b]	−15 to 20	16 h/20 °C	
26[a]	Ac_2O	96% H_2SO_4 (1.7), 100% HNO_3 (3.0)	−55 to 0	60 min/0 °C	**32** (<3), **33** (20)[c]
26[a]	Ac_2O	96% H_2SO_4 (1.7), 70% HNO_3 (3.0)	−50 to 0	75 min/0 °C	**32** (<3), **33** (26)[c]
26[a]	Ac_2O	96% H_2SO_4 (2.5), KNO_3 (1.7)	−35 to −30	20 min/−30 °C	**32** (<2), **33** (14)[c]
26[a]	TFAA	70% HNO_3 (2.5−2.8)	−50 to 20	2−3 h/20 °C	**7** (12−35)
30	CH_3CN	NO_2BF_4 (1.5)	−48 to 20	90 min/20 °C	**30** (15), **32** (1), **33** (14)[c]
30	Ac_2O	96% H_2SO_4 (1.9), 70% HNO_3 (3.2)	−50 to 0	30 min/0 °C	**32** (<3), **33** (28)[c]

[a]Acetylated or trifluoroacetylated in situ, prior to nitration. [b]Preformed acetyl nitrate. [c]Yields after chromatography and crystallization.

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structure (Figure 3); of note, the benzyloxy side chain adopted a pronounced pseudoaxial conformation at C6, the same as that observed in the previously reported crystal structure of **4**.[21]

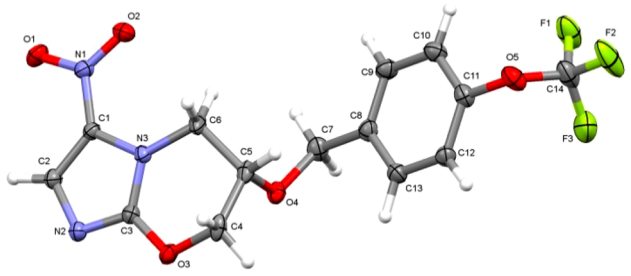

Figure 3. X-ray crystal structure of compound **8**.

Preparation of the second byproduct from the synthesis of **4** was straightforward (Scheme 1C). Lithiation of bromotoluene **34** and quenching with DMF gave the required aldehyde **35**, which was easily reduced ($NaBH_4$) and brominated to give **37**. Alkylation of chiral alcohol **31**[30] with bromide **37** then provided the expected compound **9**, which was also identical to that derived from the nonoptimized process chemistry route for **4**. This byproduct (<0.2%) was postulated to arise from traces of **37** in the bulk commercial 4-(trifluoromethoxy)benzyl bromide. Full experimental procedures and characterization data for all compounds have been provided in the Supporting Information.

The comparative effects of **7**–**9** and several reference drugs against *M. tb* (strain H37Rv) were assessed in two *in vitro* assays, MABA[32] and LORA,[33] conducted under aerobic and hypoxic conditions, respectively. The latter assay employed bacteria preadapted to low oxygen conditions and represented a targeted screen for the identification of agents with better sterilizing ability against nonreplicating persistent bacteria. Recorded minimum inhibitory concentrations (MICs) corresponding to growth inhibitions of ≥90% (Table 2) were mean values obtained from replicate measurements (± standard deviation). Compounds **7**–**9** were also evaluated for cytotoxicity against mammalian cells (VERO) in a 72 h assay[32] and found to be nontoxic (IC_{50} > 128 μM).

Table 2. *In Vitro* Activities of 7–9 versus Other TB Drugs

	MIC[a] (μM)		
compd	MABA	LORA	VERO IC_{50}[b] (μM)
2	0.070 ± 0.018	0.11 ± 0.03	>10
3	2.9 ± 1.2	2.8 ± 0.3	
4[c]	0.50 ± 0.30	2.6 ± 1.4	>128
6	0.42 ± 0.13	>128	
7	>128	>128	>128
8	>128	>128	>128
9	0.063 ± 0.003	1.0 ± 0.1	>128
MET	>512	79 ± 40	
RMP	0.049 ± 0.027	0.64 ± 0.35	>100
INH	0.34 ± 0.18	>128	

[a]Minimum inhibitory concentration against *M. tb*, determined under aerobic (MABA)[32] or hypoxic (LORA)[33] conditions. Each value is the mean of ≥2 independent determinations (**7**–**9** were tested three times). The controls were metronidazole (MET), rifampicin (RMP), and isoniazid (INH). [b]IC_{50} values for cytotoxicity toward VERO cells. [c]MIC data from ref 15.

As expected from our studies in the nitroimidazooxazole class,[23] 3-nitro compounds **7** and **8** were completely inactive in both *M. tb* assays (MICs > 128 μM; Table 2). For **8**, this indicates a >256-fold loss in activity in comparison to its 2-nitro isomer, **4**. Conversely, the 3′-methyl derivative of **4** (**9**) was seven- to eight-fold more active than both **4** and moxifloxacin (**6**) in MABA (similar to rifampicin and bedaquiline **2**) and about three-fold better than both **4** and linezolid (**3**) in LORA (comparable to rifampicin). These results for **9** were in accordance with the findings reported by Cherian et al.[16] for the 3′-methoxy derivative of **4**, which was five-fold superior to **4** in the aerobic assay and two-fold more effective than **4** under hypoxic conditions.

In their original investigation, Kim et al.[22] reported an aerobic MIC_{99} value of 4–8 μM and weak anaerobic activity (MIC_{90} 31 μM) for the compound they believed to be **8** (shown here to be the 2*H*-nitroimidazooxazole **20**). Intriguingly, the same compound also yielded an anaerobic MIC_{90} value of 62.5–125 μM against *M. tb* having mutations in the nitroreductase Ddn, suggesting the participation of a different biological target. While delamanid (**1**) was shown to be primarily triggered by Ddn, several Ddn homologues have been implicated in the cellular activation of simple 2*H*-nitroimidazooxazoles (e.g., 2-Et, 2-Ph).[34] However, in the case of **20**, the lack of significant potency against this Ddn mutant under aerobic conditions (MIC_{99} > 100 μM) renders this explanation unsatisfactory. Overall, the results of the current investigation support findings from previous studies of resistance to **4**, that the activation of nitroimidazooxazines relies exclusively on Ddn.[35] In this class, relocation of the nitro group from C-2 to C-3 destroys all antitubercular activity, implying that, like the *R*-enantiomer of **4**,[34] **8** is not a substrate for Ddn or its homologues and does not release nitric oxide.

In summary, we set out to establish the identities of two novel byproducts from optimization studies around a manufacturing route to **4** through a combination of 2D NMR analysis and *de novo* chiral synthesis. In the case of 3-nitro isomer **8**, this entailed the development of an innovative nitration route, following our discovery of a critical structural assignment error in the published method, and we obtained an X-ray crystal structure of this compound for final confirmation. Preliminary *in vitro* assessments indicated that, whereas the 3′-methyl derivative of **4** (**9**) was markedly more effective than **4**, both **8** and its alcohol precursor **7** were completely inactive, overturning previous misconceptions regarding their aerobic and anaerobic activities and the suggested involvement of another target facilitating their activity against Ddn mutant *M. tb*. These results provide further clarity of fundamental SARs for pretomanid and of the structural features of antitubercular nitroimidazoles that are more likely to overcome any future clinical resistance to **4**.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.7b00356.

Further background, scheme for preparation of racemic **7**, experimental procedures and characterizations for compounds, combustion analytical data, packing diagram for X-ray structure of **8**, crystallographic data, NMR

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spectra for key compounds, chiral HPLC trace for 7 (PDF)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

TB, tuberculosis; *M. tb*, *Mycobacterium tuberculosis*; MDR, multidrug-resistant; XDR, extensively drug-resistant; Ddn, deazaflavin-dependent nitroreductase

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