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Graphical Abstract

Syntheses and evaluation of substituted aromatic hydroxamates and hydroxamic acids that target *Mycobacterium tuberculosis.*

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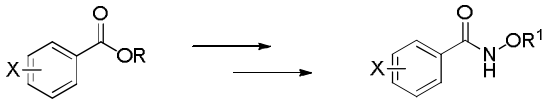

R^1 = H, X = *p*-NO_2
MIC H37RV (GAS) = 0.71 μM
MIC H37RV (7H12) = 7.79 μM

R^1 = CH_2Ph, X = 3-nitro-5-(trifluoromethyl)
MIC H37RV (GAS) = 10.65 μM

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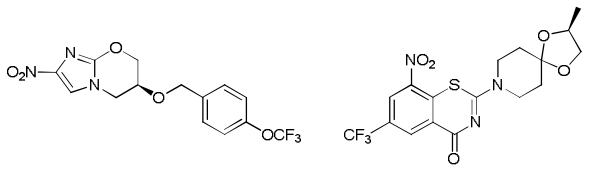
ABSTRACT

Tuberculosis (TB) continues to remain one of the most threatening diseases in the world. With the emergence of multi-drug resistant (MDR) and extensively drug resistant (XDR) strains, the need to develop new therapies is dire. The syntheses of a focused library of hydroxamates and hydroxamic acids is described, as well as anti-TB activity in the microplate alamar blue assay (MABA). A number of compounds exhibited good activity against *Mtb*, with notable compounds exhibiting MIC values in the range of 20-0.71 μM. This work suggests that both hydroxamates and their free acids may be incorporated into more complex scaffolds and serve as potential leads for the development of anti-TB agents.

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*Mtb*), is a harsh disease that infects approximately one-third of the global population. TB is a highly contagious disease, the second major cause of death as the result of a bacterial infection worldwide.[1] TB targets the lungs in most cases and can spread quite rapidly, a result of the ease in which this infection can be transmitted among individuals. On average, there is a new case of TB each second[2] and, although most are latent infections, the actual cases of active TB continue to grow, approaching 10 million as of 2010.[3] TB exhibits a high mortality rate, with up to 2 million deaths associated with this pathogen every year.[4] Over the last 25 years, TB has seen a resurgence in both the number of cases and severity of infection.[5] The emergence of multi-drug resistant (MDR), extensively drug resistant (XDR), and totally drug resistant (TDR) strains has further exacerbated this situation.[6] TB drug discovery is an arduous process primarily because of the obstacles that need to be circumvented such as the thick, lipophilic cell wall characterized by the presence of mycolic acids and arabinogalactan.[7] Prior to December 31st 2012, there was a 40 year gap between an FDA approved anti-TB agent, with bedaquiline recently approved.[8] With this in mind, a current interest in our lab is in the development of new anti-TB agents[9] based on a scaffold simplification strategy.[10] Herein we report on the syntheses of such compounds and their anti-tubercular activities.

Although there are a number of classic treatments for TB (i.e. isoniazid, rifampin), there remain many issues with them, including drug toxicity, long treatment duration, and adverse drug-drug interactions. In cases where the TB infection is treatable, the regimen consists of administration of multiple drugs for at least six months.[11] Fortunately, a number of promising new compounds have been reported that have demonstrated potent activity against TB. Although the majority of these are still in pre-clinical investigations, they may serve as welcome additions in the fight against TB. This list is highlighted by PA-824[12] and BTZ-043[13] (Figure 1). A common feature to both of these classes of anti-TB agent is the presence of an electron deficient aromatic ring. This moiety continues to be reported as crucial for activity, especially for the BTZ class of compounds.[14]

Figure 1. Two Promising Anti-TB Compounds in Development.

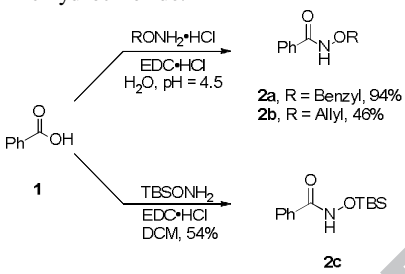


PA-824 BTZ-043

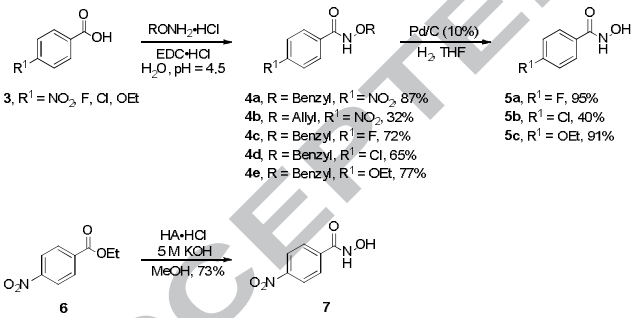
With these findings in mind, we were interested in combining electron deficient systems with more lipophilic hydroxamates and hydroxamic acids and testing them against *Mtb*. Both the hydroxamate and hydroxamic acid functional group have proven to be very important in a multitude of medicinally useful agents.[15] The ability of hydroxamic acids to readily chelate metal ions is well established in the literature,[16] thus making them useful moieties for interactions with metalloproteases. Notably, this family of iron chelators has been incorporated in siderophores and has been found to inhibit lipoxygenase, matrix

metalloproteinases, and histone deacetylases.[17] Beyond just the consideration of metal chelation, we were interested to determine if the combination of the hydroxamate and hydroxamic acid functionalities with aromatic rings of varying electron densities would generate an anti-mycobacterial agent. Generally, the utilization of both hydroxamates and hydroxamic acids in TB therapy is scarce.[18] Here we report on the syntheses of these compounds and evaluation of their anti-TB and anti-microbial activities.

First, simple unsubstituted hydroxamates of interest were generated (Scheme 1). Here, benzoic acid was coupled to both *O*-benzylhydroxylamine hydrochloride and *O*-allylhydroxylamine hydrochloride using *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC•HCl) under aqueous conditions to give hydroxamates **2a-b**. To enhance lipophilicity, silylated hydroxamate **2c** was synthesized in 54% yield from **1** and *tert*-butyldimethylsilyl hydroxylamine and EDC•HCl in DCM. Next, hydroxamates with *para*-substituted aromatic rings were generated (Scheme 2). First, the appropriately substituted benzoic acid, **3**, and substituted hydroxylamine was coupled with EDC•HCl to give hydroxamates **4a-e** in varying yields. Intermediates **4c-e** were then subjected to hydrogenolysis conditions to give hydroxamic acids **5a-c**. Lastly, the *para*-nitrohydroxamic acid **7** was made via the direct reaction of ethyl 4-nitrobenzoate, **6**, with hydroxylamine hydrochloride.

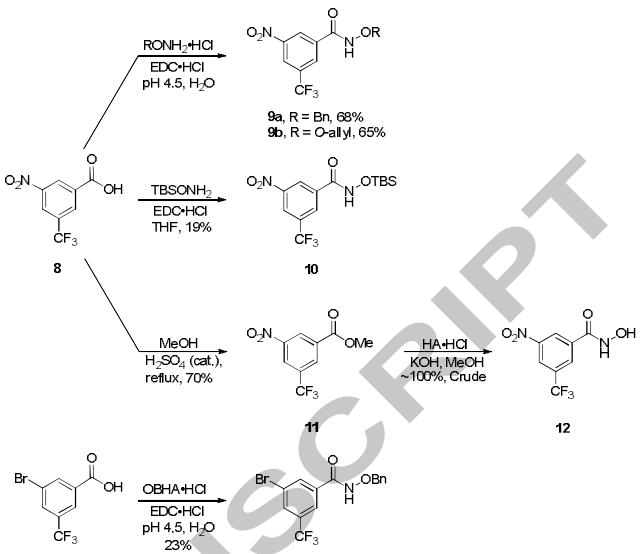


Scheme 1. Syntheses of Hydroxamates **2a-c**.



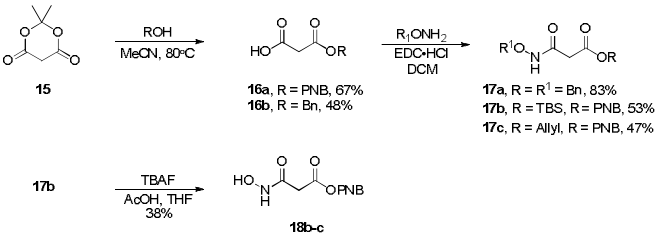
Scheme 2. Syntheses of *para*-Substituted Compounds.

The syntheses of hydroxamates with the 3-nitro-5-(trifluoromethyl) aromatic group are shown in Scheme 3. This is the same moiety that is featured in BTZ-043 and shown to be crucial for activity (Figure 1). Thus, we were curious if combining simple hydroxamates and hydroxamic acids with this key functionality would affect anti-TB activity. EDC coupling of 3-nitro-5-(trifluoromethyl)benzoic acid, **8**, with the appropriate substituted hydroxylamine hydrochloride gave **9a-b** and **10** in varying yields. For the synthesis of **12**, a number of different paths were tested; however, the best involved esterification to give methyl ester **11** followed by treatment with KOH and hydroxylamine hydrochloride (HA•HCl) in MeOH to give **12**. As a result of challenges in the purification of **12**, a crude sample was tested (80-85% pure). We were also interested to see how the absence of the nitro group would affect TB activity, so we coupled 3-bromo-5-(trifluoromethyl)benzoic acid, **13**, to give hydroxamate **14**.



Scheme 3. Syntheses of 3,5-Substituted Compounds.

Lastly, Scheme 4 shows the syntheses of hydroxamates from simple malonate monoacids. As an extension of our structure-activity relationship studies, we were interested in further varying substrate polarity and see if malonate based hydroxamates and their corresponding acids demonstrated anti-TB activity. Here Meldrum's acid, **15**, was opened to give malonates **16a-b** which were then coupled to the appropriate substituted hydroxylamine to give hydroxamates **17a-c**. Finally, silyl deprotection with tetrabutylammonium fluoride (TBAF) afforded **18b-c**.



Scheme 4. Syntheses of Hydroxamate Malonates.

All hydroxamates and hydroxamic acids were then subjected to anti-microbial evaluations. Minimum inhibitory concentration (MIC) data for select compounds against *Mtb* in the microplate alamar blue assay (MABA)[19] are displayed in Table 1. From this data, we observed that some compounds exhibited good media-dependent activity against *Mtb*. Often, differences in MIC values between the two media are attributed to factors such as compound solubility and media age.[20] Among the compounds tested, both the hydroxamate protecting group and electronics of the aromatic system did affect activity. The data showed that the benzyl protected hydroxamates had, in general, better activity than the allyl hydroxamates. This is possibly due to the fact that the allyl group represents a decrease in lipophilicity and, as a result, diminished the compound's ability to pass through the thick cell wall of *Mtb*.

Interesting results were also obtained when the electronics of the aromatic ring were varied. When comparing activities among the *para*-substituted hydroxamates and their corresponding acids,

Table 2. Antibacterial Assay Results

Compound	Agar Diffusion Zones (mm)							MIC (μM)
	B. subtilis	*S. aureus*	*M. luteus*	*M. vaccae*	*P. aeruginosa*		*E. coli*	*M. vaccae*
	ATCC 6633	SG511	ATCC 10240	IMET 10670	K799 /wt	K799/61	DC2	IMET 10670
2c	0	0	0	**26/30P**	14/18P	0	0	**12.5**
4d	19* ppt	19 ppt	19P* ppt	15 ppt	14P ppt	14 ppt	0	nt
5b	0	0	0	15.5V	0	18P	16	nt
5c	20	17	0	0	0	14P	0	nt
7	0	0	0	**30/33P**	13	0	0	**6.25**
9a	16	20	14/20V	**45**	0	0	0	**3.13**
10	20	0	0	14	0	0	17P	nt
14	17.5	20	16	**26/32P**	0	0	10/15P	**12.5**
DMSO/MeOH	0	0	0	0	0	0	0	nt
Ciprofloxicin	1.66 μg/mL 19	18	0	20	23	1.66 μg/mL 19/26P	21	nt

*All solutions were prepared by first making a 20 mM solution in DMSO and diluting 10-fold with MeOH to give 2 mM solutions. Plates were incubated at 37 ºC for 21 h. *M. vaccae* was incubated 37 ºC for 44 h. All zones reported are in mm.

KEY:

P:	Unclear inhibition zone	V:	Very unclear inhibition zone
*:	Misshapen zone of inhibition	ppt:	Compound precipitated in media
nt:	Not tested		

we were surprised to see only small changes in anti-TB activity among compounds as both the number and nature of the substituents were varied. Anticipating that activity may be enhanced as the aromatic ring became increasingly electron deficient, we were intrigued by the small and non-linear changes in TB activity among compounds **4c-e** and **5a-c**. Thus, it was especially interesting that the most polar hydroxamate, free acid **7**, demonstrated the best overall anti-TB activity in our library, while other *para*-substituted hydroxamates, acids, and even benzohydroxamic acid (not shown in Table 1) were substantially less active. Hypothesizing that activity may be further potentiated by making the aromatic ring increasingly electron deficient, we then studied systems featuring 3-nitro-5-(trifluoromethyl) substitution. Here we saw that **9a** was the most active in this series; however, the activity was again inferior to *para*-nitro hydroxamic acid **7**.

Table 1. MIC Determinations (μM) for Select Compounds against *Mycobacterium Tuberculosis* in the Microplate Alamar Blue Assay

Compound	7H12[a]	GAS[b]	Compound	7H12[a]	GAS[b]
2a	>100	48.45	**7**	**7.79**	**0.71**
2b	>100	>100	**9a**	>100	**10.65**
2c	>50	24.29	**9b**	>100	43.17
4a	90.42	**13.60**	**10**	>100	>100
4b	>100	37.61	**11**	>100	29.96
4c	>100	42.26	**12***	19.48	>100
4d	47.31	**15.54**	**14**	>100	80.53
4e	>100	47.31	**17a**	86.59	27.11
5a	>100	>100	**17b**	>100	**3.04**
5b	>100	>100	**17c**	98.65	98.58
5c	48.07	>100	**18**	>100	>100
[a]7H12 = 7H9 medium + casitone, palmitic acid, albumin, and catalase [b]GAS = glycerol-alanine-salts medium * Crude Material Screened			**Rifampin**	**0.03**	**<0.016**

With regards to hydroxamate-malonate compounds, compound **17b** was our most potent agent. This hydroxamate, like compounds **4d** and **9a**, exhibited both a very lipophilic group (TBS) and an electron deficient ring (PNB). Anticipating that activity could be further optimized, compounds **17a**, **17c**, and free acid **18** were made and tested. These compounds were inferior in overall activity. Overall, these results helped further solidify the notion that electron deficiency, specifically the incorporation of nitro functionality, may transform simple scaffolds into anti-TB agents. Further, our data illustrated the point that greater lipophilicity may assist in potentiating activity (e.g. **2c**, **4a**, and **9**); however, is not necessarily a requirement for the anti-TB activity of this library.

Representative antibacterial evaluations performed using the Kirby-Bauer agar diffusion assay,[21] are shown in Table 2. As shown, some compounds that were active against TB were also quite active against *M. vaccae.* Based on the aforementioned anti-TB activity, this result was not completely unexpected since both bacterial strains are from the mycobacteria genus. Additionally, for compounds that showed notable zones of inhibition against *M. vaccae* (**2c**, **7**, **9a**, and **14**) minimum inhibitory concentrations (MICs) were determined. As can be seen **9a** was the most active against *M. vaccae*, with a MIC of 3.13 μM.

In conclusion, a number of compounds synthesized exhibited notable anti-TB activity (MIC values 15.54 to 0.71 μM) and specific antimicrobial activity. Although at this stage of our studies the exact target and/or general mode of action remain unknown, these compounds may serve as potential scaffolds for the generation of new anti-TB agents.

Acknowledgments

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Supplementary Material

Data associated with this article, including experimental procedures and characterization data for all compounds, is available with the online version of this article.

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