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Design, synthesis and antibacterial activity against pathogenic mycobacteria of conjugated hydroxamic acids, hydrazides and *O*-alkyl/*O*-acyl protected hydroxamic derivatives

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ABSTRACT

With the aim to discover new antituberculous molecules, three novel series of 23 hydroxamic acids, 13 hydrazides, and 9 *O*-alkyl/*O*-acyl protected hydroxamic acid derivatives have been synthesized, and fully characterized by spectral (¹H NMR, ¹³C NMR, HRMS) analysis. These compounds were further biologically screened for their in vitro antibacterial activities against three pathogenic mycobacteria - *M. abscessus* S and R, *M. marinum*, and *M. tuberculosis* – as well as for their toxicity towards murine macrophages by the resazurin microtiter assay (REMA). Among the 45 derivatives, 17 compounds (3 hydroxamic acids, 9 hydrazides, and 5 *O*-alkyl/*O*-acyl protected hydroxamic acids) were nontoxic against murine macrophages. When tested for their antibacterial activity, hydroxamic acid **9h** was found to be the most potent inhibitor against *M. abscessus* S and R only. Regarding hydrazide series, only **7h** was active against *M. abscessus* R, *M. marinum* and *M. tuberculosis*; while the *O*-acyl protected hydroxamic acid derivatives **14d** and **15d** displayed promising antibacterial activity against both *M. marinum* and *M. tuberculosis*. Since such hydroxamic- and hydrazide-chelating groups have been reported to impair the activity of the peptide deformylase, *in silico* molecular docking studies in *M. tuberculosis* peptide deformylase enzyme active site were further performed with **7h** in order to predict the possible interaction mode and binding energy of this molecule at the molecular level.

1. Introduction

Mycobacterial infections represent an important threat for public health. Tuberculosis (TB), a respiratory disease caused by *Mycobacterium tuberculosis* (*M. tb*) whose resistance to traditional treatment has been growing in recent years,¹ still remains one of the leading cause of death by a single infectious agent according to the World Health Organization.² Moreover, the increased lifespan of immunocompromised patients has led to the emergence of new pathogens, which are now clinically relevant. Among these new pathogens, nontuberculous mycobacteria (NTM) are such opportunistic bacteria, of which the *Mycobacterium abscessus* (*M. abscessus*) complex is the most significant example.³ *M. abscessus* is indeed responsible for cutaneous and pulmonary infections, representing up to 95% of NTM infections in cystic fibrosis (CF) populations, and is considered one of the most drug-resistant mycobacteria for which efficient chemotherapeutics are urgently needed.^{4,6} *Mycobacterium marinum* (*M. marinum*) is another opportunistic non-tuberculous pathogenic mycobacterium typically found in aquatic environments where it causes a systemic tuberculosis-like

illness in fish or frogs.^{7, 8} In humans, this infection is typically limited to skin lesions and soft-tissue extremities, but dissemination of the infection can occur in immunocompromised hosts,^{9, 10} which established *M. marinum* as a clinically relevant human pathogen.

In this context, there is a clear and urgent need to identify new potential targets and therefore to develop new antimicrobial agents for treating such mycobacterial-related diseases.

Among interesting targets, the peptide deformylase (PDF) is a bacterial metalloenzyme responsible for removal of the *N*-formyl group from terminal methionine residue of nascent bacterial polypeptides, a key step in bacterial protein maturation.¹¹ PDF, is not only a highly conserved metalloprotease across bacterial species, but above all, it has no mammalian counterpart.¹² Moreover, the gene (*def*) encoding PDF has been shown as essential for bacterial growth and survival.^{11, 12} Accordingly, the *rv0429c* gene encoding *M. tb* PDF was suggested to be essential through transposon site hybridization;¹³ and its essentiality has been demonstrated through genetic knockout experiments with *M.*

bovis BCG.¹⁴ Although not many studies have been conducted in the field of TB, all these findings make PDF an attractive candidate for the development of novel antibacterial agents. More interestingly, compounds bearing a hydroxamic acid-chelating or *N*-formyl hydroxylamine-chelating groups were also found to display potent PDF inhibition and good antibacterial activity against several bacteria,¹⁵⁻¹⁹ including *M. bovis* BCG and *M. tb*.^{11, 14, 20, 21} Accordingly, potent PDF inhibitors like the *N*-formyl hydroxylamine **BB-3497**,²² as well as *N*-alkyl urea reverse hydroxamate **LBK-611** and its benzimidazole (**Cpd-15**) and benzoxazole (**Cpd-16**) bioisosteric derivatives^{14, 21} have been shown to efficiently inhibit *M. tb* growth under *in vitro* condition with MIC ranging from 0.05 to 0.2 $\mu\text{g/mL}$; while, the hydroxamic acid pseudopeptide **actinonin** showed a moderate antimycobacterial activity (MIC = 56 $\mu\text{g/mL}$) (**Figure 1A**).²² Moreover, **BB-3497** and **actinonin** also displayed a strong synergistic effect in combination with first-line drugs isoniazid (INH) and rifampicin (RIF) resulting in a MIC drop of around 4-fold for these two PDF inhibitors against *M. tb* growth.²²

Given all these findings, we report here the synthesis of a series of hydroxamic acid, hydrazide, and *O*-alkyl/*O*-acyl protected hydroxamic acid derivatives. A part of the structure of these compounds resemble the INH with addition of a bridge between the aryl group (either pyridyl as found in INH or substituted phenyl) composed by one or two C-C conjugated double bonds and a hydroxamic acid or hydrazide moiety (**Figure 1B**). Forty-five analogs have been synthesized and screened for their antimycobacterial activity, and they showed moderate-to high efficacy against *M. tb*, *M. abscessus* and *M. marinum*. The results obtained with the best growth inhibitor were next corroborated by *in silico* molecular docking studies.

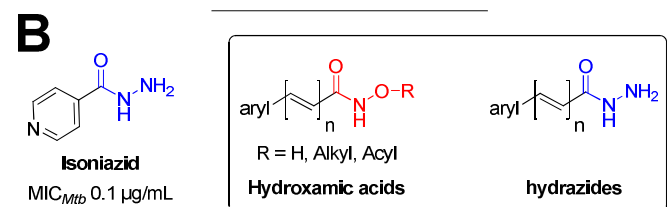
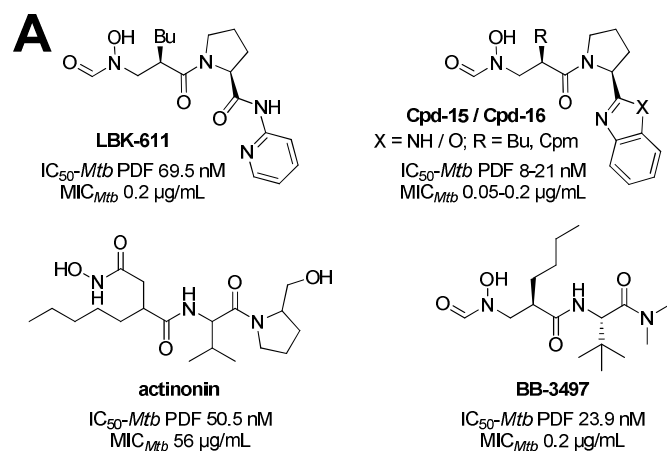
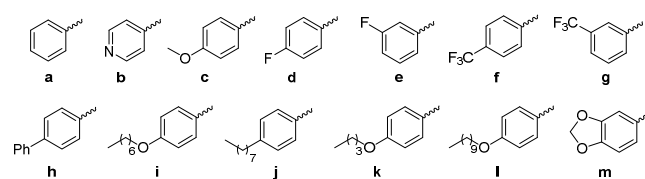


Figure 1. (A) Structures of reported PDF inhibitors together with their respective IC_{50} values towards *M. tb* PDF as well as their MIC values against *M. tb* growth.^{14, 21, 22} (B) First-line TB drug Isoniazid, and chemical structures of hydroxamic acids (R = H), *O*-alkyl/*O*-acyl protected derivatives (R = Alkyl, Acyl), and hydrazides.

2. Results and Discussion

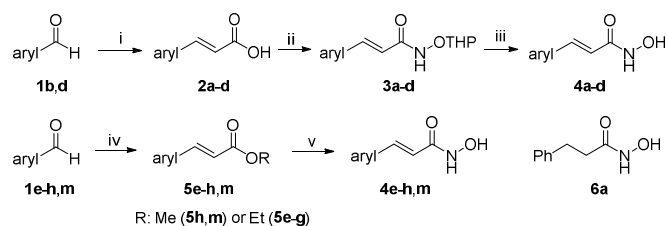
2.1. Design and synthesis of hydroxamic acids, hydrazides and *O*-alkyl/*O*-acyl protected hydroxamic acid derivatives

To simplify the schemes and compound structures presented onwards, a default designation for the aryl group substituents will be followed according to **Scheme 1**.



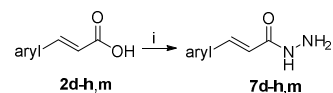
Scheme 1. Default designation of aryl group substituents **a-m**.

For the synthesis of hydroxamic acids **4a-d**, the appropriate cinnamic acid analogue was synthesized *via* a Knoevenagel reaction using malonic acid in pyridine and pyrimidine in a catalytic amount, and then coupled with the THP-protected hydroxylamine using EDC and HOBt. Finally, deprotection with 4N HCl in dry Et_2O yielded hydroxamic acids **4a-d** (**Scheme 2**). This method was preferred for compounds **4a-d** to the direct coupling with hydroxylamine as the purification and isolation of the final hydroxamic acids in the latter method was unsuccessful due to the poor solubility and high hydrophilicity of the products, especially for compound **4b**. For hydroxamic acids **4e-h,m**, the corresponding methyl or ethyl ester was synthesized *via* a Wittig reaction with the appropriate ylide and was then treated with 50% aqueous solution of hydroxylamine in the presence of KOH in methanol to yield the desired hydroxamic acids. To verify the importance of the conjugation between the aryl group and the hydroxamic group, the saturated analogue of **4a**, hydroxamic acid **6a**, was also prepared from ethyl 3-phenylpropanoate.²³



Scheme 2. Synthesis of hydroxamic acids of cinnamyl analogues **4a-h,m**. Reagents and conditions: (i) Malonic acid, pyridine, piperidine, 65-91% yield; (ii) NH_2OTHP , EDC, HOBt, dry DCM, r.t., o.n., 22-73% yield; (iii) 4N HCl in dry Et_2O , 56-80% yield; (iv) $\text{Ph}_3\text{P}=\text{CHCOOR}$, dry DCM or THF, r.t., 92-98% yield; (v) 50% aq. NH_2OH , sol. KOH in MeOH, THF, 0 $^\circ\text{C}$, then r.t., 2.5h, 51-88% yield.

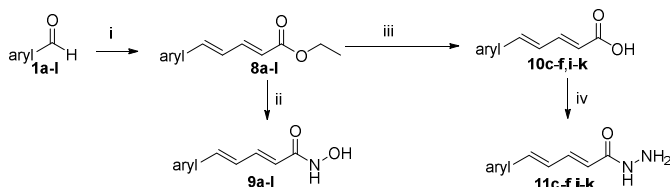
In a way to study the effect of hydrazides analogous to isoniazid on mycobacteria, compounds **7d-h,m** were also prepared (**Scheme 3**). Substituted cinnamic acids **2d,h,m** were coupled with hydrazine using EDC and HOBt to yield hydrazides **7d,h,m**, while cinnamic acids **2e-g** were coupled with Boc-hydrazine and were then deprotected using 4N HCl in dioxane.



Scheme 3. Synthesis of hydrazides **7d-h,m**, analogues of hydroxamic acids **4d-h,m**. Reagents and conditions: (i) For **7d,h,m**: EDC, HOBt, MeCN, then $(\text{NH}_2)_2\text{H}_2\text{O}$, cyclohexene, MeCN, and for **7e-g**: EDC, HOBt, MeCN, then BocNHNH₂, cyclohexene, MeCN, then 4N HCl in dioxane, 32-82% yield.

To enhance the lipophilicity of the hydroxamic acids, we decided to extend the conjugation by adding a second double bond between the aryl group and the hydroxamic acid moiety. As a first step, a Wadsworth-Horner-Emmons olefination reaction of the substituted aromatic aldehydes **1a-d,h-l** with triethyl 4-phosphonocrotonate yielded the unsaturated esters **8a-d,h-l**.²⁴ For

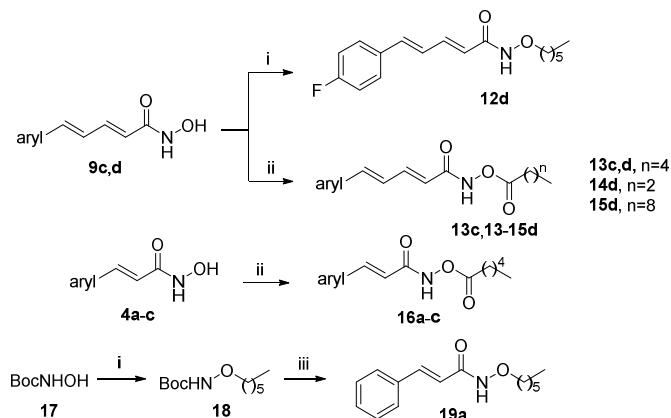
esters **8e-g** a different synthetic route was followed, as the Wadsworth-Horner-Emmons olefination reaction yielded poor results and inseparable mixtures of starting material and products. In the case of aldehydes **1e-g** first a Wittig reaction with $\text{Ph}_3\text{P}=\text{CHCH}=\text{O}$ took place yielding a cinnamaldehyde analogue that was purified by column chromatography, and then a second quantitative Wittig reaction with $\text{Ph}_3\text{P}=\text{CHCOOEt}$ gave the final unsaturated esters in moderate 2-step yield, but in high purity. Esters **8a-k** were then treated with a 50% aqueous solution of NH_2OH in the presence of a methanolic solution of KOH to obtain the desired hydroxamic acids **9a-k** (Scheme 4). For hydroxamic acid **9l** the use of the KOH/MeOH solution did not yield the desired product, and DBU was used instead. Isolation and purification of all the final hydroxamic acids **9a-l** was successful thanks to their improved lipophilicity and solubility. In order to prepare corresponding hydrazides, esters **9c-f,i-k** were hydrolyzed using NaOH in 1,4-dioxane to yield the unsaturated acids **10c-f,i-k**. To prepare the hydrazides, two routes were applied, depending on the difficulty to isolate and purify the final products. For hydrazides **11c,i,j**, the corresponding acids **10c,i,j** were coupled with hydrazine using EDC and HOBt. Hydrazides **11d-f,k** were synthesized *via* a two-step reaction, by coupling acids **10d-f,k** with BocNHNH₂, followed by column chromatography to purify the intermediates and deprotection using 4 HCl in dioxane.



Scheme 4. Synthesis of hydroxamic acids **9a-l** and hydrazides **11c-f,i-k**, bearing two conjugated double bonds. Reagents and conditions: i) For **8a-d,h-l**: triethyl 4-phosphonocrotonate, LiOH·H₂O, dry THF, reflux or for **8e-g**: $\text{Ph}_3\text{P}=\text{CHCH}=\text{O}$, dry DCM, r.t., column chromatography, then $\text{Ph}_3\text{P}=\text{CHCOOEt}$, dry DCM, r.t., 30-87% yield; ii) For **9a-k**: 50% aq. NH_2OH , sol. KOH in MeOH, THF, 0 °C, then r.t. o.n., and for **9l**: 50% aq. NH_2OH , DBU, r.t. o.n., 35-93% yield; iii) NaOH, 1,4-dioxane, 40-99% yield; iv) For **11c,i,j**: EDC, HOBt, MeCN, then $(\text{NH}_2)_2\cdot\text{H}_2\text{O}$, cyclohexene, MeCN, and for **11d-f,k**: EDC, HOBt, MeCN, then BocNHNH₂, cyclohexene, MeCN, then 4N HCl in dioxane, 38-87% yield.

We further decided to investigate the effect of the lipophilicity of the hydroxamic acid compounds by synthesizing several *O*-alkyl and *O*-acyl protected derivatives (Scheme 5). For the synthesis of the *O*-alkyl protected hydroxamic acid **12d**, hydroxamic acid **9d** was treated with *n*-hexylbromide and DBU in acetonitrile under reflux. The same conditions were applied on *N*-Boc-hydroxylamine to yield compound **18**. Deprotection, followed by coupling with cinnamic acid using EDC, HOBt and Et₃N gave the *O*-hexyl hydroxamic acid **19a**.

For the synthesis of the *O*-acyl protected hydroxamic acids **13d**, **14d**, **15d**, we coupled hydroxamic acid **9d** with hexanoic, butyric and decanoic acid, correspondingly, using EDC and HOBt in the presence of Et₃N. For the synthesis of the *O*-hexanoyl protected hydroxamic acids **13c** and **16a-c**, a different route was followed with the use of hexanoyl chloride and DIPEA, starting with the corresponding hydroxamic acids **9c** and **4a-c**, resulting in slightly better yields. However, in all cases *O*-acyl protected hydroxamic acids demanded multiple chromatographic separations in order to obtain the desired products in pure form.



Scheme 5. Synthesis of substituted hydroxamic acids and hydrazides. Reagents and conditions: (i) *n*-hexylbromide, DBU, MeCN, reflux, o.n., 25% yield; (ii) For **13d,14d,15d**: hexanoic acid (for **13d**), butyric acid (for **14d**) or decanoic acid (for **15d**), EDC, HOBt, dry DCM, 0 °C, r.t., o.n., and for **13c,16a-c**: hexanoyl chloride, DIPEA, dry DCM, r.t. o.n., 23-77% yield; (iii) 4N HCl in Et₂O; then cinnamic acid, EDC, HOBt, Et₃N, dry DCM, r.t., o.n., 50% yield.

2.2. Antibacterial activity

Following a lung infection, *M. tb*^{25, 26} and *M. abscessus*^{27, 28} can survive and replicate intracellularly within macrophages. So, from a therapeutic view, it is crucial that new drugs should solely impair intracellular pathogen growth without displaying a cytotoxic effect on the host cells. In this context, all final molecules were first tested for their toxicity against primary murine macrophage cells, *i.e.* bone marrow-derived macrophages (BMDM),²⁹ based on the concentration required to elicit a 50% decrease in cell viability, *i.e.*, CC₅₀³⁰ (Table 1). Among the 23 hydroxamic acids, 18 exhibited a high toxicity towards murine BMDM with a CC₅₀ < 34 μg/mL. In contrast, 9 out of 13 hydrazides (*i.e.*, **7e-h**, **7m**, **11c-f**) as well as 2 *O*-alkyl (*i.e.*, **12d** and **19a**) and 3 *O*-acyl protected hydroxamic acid derivatives (*i.e.*, **14d**, **15d** and **16c**) were not toxic (CC₅₀ ≥ 123 μg/mL).

Despite their toxicity against mammalian cells, drug susceptibility testing of all synthesized derivatives against the three pathogenic mycobacterial strains was further assessed. The corresponding MIC₅₀/MIC₉₀ values for each compound, as determined by the REMA assay³¹⁻³⁵ are reported in Table 1.

With regards to *M. tb*, all the hydroxamic acid derivatives **4a-m**, **9b-c**, **9e-g** active against this pathogen were unfortunately toxic (Table 1). The presence of the deactivating electron withdrawing fluorine atom at *m*-position or as a CF₃ substituent at *p*- or *m*-position of the phenyl group in **4e-g** (MIC₅₀ around 12.8-14.4 μg/mL / MIC₉₀ >100 μg/mL) and **9e** (MIC₅₀ = 7.6±0.31 μg/mL / MIC₉₀ >100 μg/mL), may be responsible for the increase in both the antibacterial potency as well as the toxicity of the corresponding molecules. It must be noted that, except **9k** which exhibited a weak antibacterial activity (MIC₅₀ = 38.6±1.1 μg/mL / MIC₉₀ >100 μg/mL), the other lipophilic hydroxamic acids **9h-i,l** were inactive (MIC > 100 μg/mL).

Among the hydrazide derivatives, the most promising and nontoxic (*i.e.*, CC₅₀ ≥120 μg/mL) anti-TB compounds were **7h** (MIC₅₀ = 13.1±0.51 μg/mL / MIC₉₀ = 22.0±0.65 μg/mL), and **11c** (MIC₅₀ = 18.1±0.44 μg/mL / MIC₉₀ = 46.7±0.53 μg/mL). Interestingly, these two hydrazides were bearing an activating electron donating group: a biphenyl and a *p*-MeO group as aryl substituent for **7h** and **11c**, respectively. In contrast, the presence of fluorine atom or a CF₃ substituent as in **7e-g**, and **11d,f** resulted in a lower antibacterial activity of the corresponding compounds.

Finally, only the nontoxic *O*-acyl protected hydroxamic acid derivatives **14d** (MIC₅₀ = 11.9±0.45 µg/mL / MIC₉₀ = 59.3±0.66 µg/mL), **15d** (MIC₅₀ = 20.3±0.33 µg/mL / MIC₉₀ = 34.7±1.7 µg/mL) and **16c** (MIC₅₀ = 32.8±1.4 µg/mL / MIC₉₀ = 61.4±0.71 µg/mL) exhibited promising antibacterial activity against *M. tb*. It must be noted that compounds **12d** and **19a**, both bearing an *O*-hexyl substituent were nontoxic but also inactive against the bacilli.

Regarding *M. marinum*, nearly all compounds exhibited antibacterial activity. With MIC₅₀ values comparable to INH ranging from 3.3 to 7.5 µg/mL, the most potent hydroxamic acid growth inhibitors were compounds **4a,d-g**, **6a** and **9a,c-e,g**. The best nontoxic inhibitors were hydrazide **11c** as well as the *O*-decanoyl protected derivative **15d**, for which both MIC₅₀/MIC₉₀ values are in the same order of magnitude in the 12.6-17.3 µg/mL range.

Table 1. Antibacterial activities of the 45 new derivatives compared to standard antimicrobial agents against three pathogenic mycobacterial strains.^a

| Cpds | MIC ₅₀ / MIC ₉₀ (µg/mL) | | | | CC ₅₀ (µg/mL) ^b |
|------------------|---|-----------------------------|-----------------------------|-----------------------------------|--|
| | <i>M. abscessus</i> CIP 104536 ^T | | <i>M. marinum</i> M | <i>M. tb</i> mc ² 6230 | |
| | Smooth | Rough | | | |
| actinonin | >100 | >100 | >100 | 53.5±1.6 / 60.4±2.1 | NT ^c |
| 4a | >100 | >100 | 6.7±0.22 / 32.4±1.7 | 28.8±1.9 / 40.7±1.4 | <10 |
| 4b | >100 | >100 | 75.0±1.2 / >100 | 35.1±0.58 / 42.9±1.3 | <10 |
| 4b*HCl | >100 | >100 | 12.6±0.63 / 54.0±2.5 | 32.1±1.1 / 33.6±2.1 | <10 |
| 4c | >100 | >100 | 58.2±1.8 / 89.5±3.7 | 23.1±0.51 / 24.3±0.99 | <10 |
| 4d | >100 | >100 | 3.5±0.13 / 23.9±0.56 | 47.3±0.86 / 55.4±1.9 | <10 |
| 4e | 12.2±0.72 / >100 | 7.6±0.60 / 68.4±1.9 | 3.3±0.13 / 25.9±0.68 | 13.5±1.1 / >100 | <10 |
| 4f | 37.2±2.6 / 39.6±0.24 | 6.3±0.44 / 21.9±0.13 | 14.1±0.71 / >100 | 14.4±1.0 / >100 | <10 |
| 4g | 72.5±0.82 / 74.8±3.2 | 7.8±0.24 / 16.0±0.29 | 7.1±0.13 / 47.7±1.4 | 12.8±0.14 / >100 | <10 |
| 4h | >100 | >100 | 20.8±0.89 / 68.6±1.3 | 20.6±0.56 / >100 | 16±0.8 |
| 4m | >100 | >100 | >100 | 31.9±0.89 / >100 | 34±1.6 |
| 6a | >100 | >100 | 11.9±0.38 / 89.3±4.5 | >100 | 95±5.4 |
| 9a | >100 | >100 | 14.9±0.29 / 91.4±3.6 | >100 | <10 |
| 9b | >100 | >100 | 26.9±0.30 / >100 | 19.4±1.3 / 27.6±1.6 | <10 |
| 9c | >100 | >100 | 14.8±0.37 / 62.1±2.5 | 48.0±1.2 / >100 | <10 |
| 9d | >100 | >100 | 18.1±0.45 / >100 | >100 | <10 |
| 9e | 36.8±1.5 / >100 | 6.0±0.25 / 19.0±0.62 | 7.5±0.31 / 39.31.3 | 7.6±0.31 / >100 | <10 |
| 9f | >100 | 5.5±0.30 / 10.9±0.19 | >100 | 28.8±1.6 / 80.6±1.4 | <10 |
| 9g | 52.3±2.0 / >100 | 4.4±0.27 / 7.0±0.16 | 10.1±0.43 / >100 | 50.5±3.1 / >100 | <10 |
| 9h | 31.8±0.81 / >100 | 6.9±0.11 / 7.5±0.30 | >100 | >100 | >125 |
| 9i | >100 | >100 | 48.3±1.1 / 68.3±2.0 | >100 | 67±6.3 |
| 9j | >100 | >100 | 49.4±1.8 / >100 | >100 | <10 |
| 9k | 27.2±0.79 / 59.3±2.1 | >100 | >100 | 38.6±1.1 / >100 | >125 |
| 9l | >100 | >100 | >100 | >100 | >125 |
| 7d | >100 | >100 | 60.5±1.8 / 80.4±3.5 | >100 | <10 |
| 7e | >100 | >100 | 41.1±1.3 / >100 | 65.8±3.5 / >100 | >125 |
| 7f | >100 | 46.0±0.86 / 46.8±4.1 | >100 | 20.4±0.38 / 44.2±3.9 | >125 |
| 7g | >100 | 51.4±0.43 / 55.7±0.72 | >100 | 40.5±0.34 / 65.7±0.85 | >125 |
| 7h | >100 | 18.9±0.22 / >100 | 22.2±0.70 / 71.5±2.2 | 13.1±0.51 / 22.0±0.65 | 125±10.6 |
| 7m | >100 | >100 | >100 | >100 | 123±12 |
| 11c | >100 | >100 | 12.6±0.33 / 13.3±0.15 | 18.1±0.44 / 46.7±0.53 | 120±15.2 |
| 11d | >100 | >100 | 20.6±0.11 / >100 | 27.4±1.1 / >100 | >125 |
| 11e | >100 | >100 | >100 | >100 | >125 |

Hydroxamic acid derivatives

Hydrazide derivatives

| | | | | | | |
|--|------------|---------------------|----------------------|------------------------------|------------------------------|-------------------------|
| | 11f | >100 | 5.7±0.32 / 19.4±0.61 | >100 | 23.7±1.3 / >100 | >125 |
| | 11i | 26.6±2.2 / >100 | 9.7±0.22 / 26.1±0.77 | >100 | 27.0±0.60 / 29.1±0.86 | 64±3.0 |
| | 11j | 57.3±1.8 / >100 | 11.4±0.10 / >100 | 42.0±1.2 / >100 | 23.2±1.1 / 24.5±2.2 | 30±1.5 |
| | 11k | >100 | >100 | >100 | >100 | 65±4.7 |
| O-alkyl/O-acyl protected hydroxamic acid derivatives | 12d | >100 | >100 | 37.1±1.5 / >100 | >100 | >125 |
| | 13c | >100 | >100 | 26.8±0.80 / 54.5±1.7 | 13.8±0.78 / 22.9±1.2 | <10 |
| | 13d | >100 | >100 | 19.8±0.67 / 92.9±3.8 | 21.6±0.72 / 40.7±1.5 | <10 |
| | 14d | >100 | >100 | 7.9±0.18 / 22.4±0.69 | 11.9±0.45 / 59.3±0.66 | >125 |
| | 15d | >100 | >100 | 13.2±0.14 / 17.3±0.63 | 20.3±0.33 / 34.7±1.7 | >125 |
| | 16a | 63.4±1.1 / 67.7±3.3 | >100 | 13.0±0.51 / 24.7±1.0 | 27.0±1.6 / 62.1±2.4 | 17±2.4 |
| | 16b | >100 | >100 | 15.9±0.64 / 17.4±0.53 | 63.9±4.6 / 65.7±2.0 | 51±3.3 |
| | 16c | 63.0±1.1 / 76.5±1.0 | >100 | 21.2±0.75 / 46.7±2.4 | 32.8±1.4 / 61.4±0.71 | >125 |
| | 19a | >100 | >100 | 24.8±0.93 / >100 | >100 | 100±5.0 |
| | | AMK | 2.3±0.12 / 3.4±0.117 | 4.3±0.15 / 5.9±0.26 | 0.62±0.026 / 1.7±0.039 | 0.24±0.010 / 0.37±0.016 |
| | INH | NA | NA | 2.8±0.13 / 9.2±0.46 | 0.07±0.003 / 0.11±0.005 | >20 ^d |

^a Minimum inhibitory concentration leading to either 50% or 90% growth inhibition (MIC₅₀/MIC₉₀) as determined by the resazurin microtiter assay (REMA). ^b Cytotoxic concentration of compound leading to 50% cell toxicity (CC₅₀) determined on murine Bone marrow-derived macrophage (BMDM). ^c NT: nontoxic up to 400 mg/kg when administered to mice,³⁶ and nontoxic to normal tissues up to > 193 µg/mL (*i.e.*, >500 µM).³⁷ ^d Data from³⁸. INH: isoniazid; AMK: amikacin. NA: not active. All values are expressed as mean ± SD of at least two independent assays.

In the case of *M. abscessus* S, most of the tested compounds were inactive (MIC > 100 µg/mL). Among the nontoxic inhibitors, the MIC₅₀/MIC₉₀ values were indicative of a moderate antibacterial activity for **9k** (MIC₅₀ = 27.2±0.79 µg/mL / MIC₉₀ = 59.3±0.2.1 µg/mL), or a weak activity for **9h** (MIC₅₀ = 31.8±0.81 µg/mL / MIC₉₀ >100 µg/mL) and **16c** (MIC₅₀ = 63.0±1.1 µg/mL / MIC₉₀ = 76.5±1.0 µg/mL). In contrast, *M. abscessus* R variant was 1.5- to 18-times more sensitive to the same derivatives than the S morphotype. The best antibacterial inhibitors were hydroxamic acid **9h** (MIC₅₀ = 6.9±0.11 µg/mL / MIC₉₀ = 7.5±0.30 µg/mL), hydrazides **7h** (MIC₅₀ = 18.9±0.22 µg/mL / MIC₉₀ > 100 µg/mL), and **11f** (MIC₅₀ = 5.7±0.32 µg/mL / MIC₉₀ = 19.4±0.61 µg/mL). Interestingly, this latter compound was found to impair the growth of *M. abscessus*, with no antibacterial activity against *M. marinum* or *M. tb*.

Regarding **actinonin**, and in agreement with previous studies,^{14, 22} this PDF-inhibitor exhibited only a poor antibacterial activity against all tested mycobacteria (**Table 1**).

2.3. Molecular Docking Analysis

Given the fact that compounds bearing hydroxamic acid-chelating or *N*-formyl hydroxylamine-chelating groups were known to display potent inhibitory activity towards PDF enzyme,¹⁵⁻¹⁹ hydrazide **7h** which displays the most promising antibacterial activity against the three pathogens, was selected for *in silico* molecular docking experiments using the reported crystal structure of *M. tb* PDF complexed with **NVC** inhibitor in its active site (PDB entry code: 3E3U).²¹ Such docking study will allow to predict the putative binding mode of this latter inhibitor in the active site of the enzyme.

The best scoring position obtained (*i.e.*, lowest energy complex) showed that compound **7h** would be located inside the *M. tb* PDF active site with a predicted binding energy value $\Delta E_{7h} = -7.6$ kcal/mol. Moreover, a high level of concordance between the most favorable docked conformation of **7h** and the crystalized **NVC** inhibitor (**Figure 2A,B**) was observed. In particular, the keto

hydrazide moiety of **7h** was nearly superimposable to the hydroxamic acid group of **NVC** (<0.6 Å), and located at a distance of 2.6 (C=O) and 2.9 Å (NH-NH₂) from the Ni²⁺ ion, thus favoring the formation of a stable chelate with this transition metal ion.

The docked **7h**-*M. tb* PDF complex was further subjected to interactions analysis using Ligplot+ v.1.4,³⁹ to schematically depicts the hydrogen bonds and hydrophobic interactions between the ligand (*i.e.*, hydrazide **7h**), and the active site residues of the protein (*i.e.*, *M. tb* PDF) during the binding process. In the obtained binding mode, the bulky biphenyl group of **7h** may be involved in π -stacking interactions with Val⁵⁰, Gly⁵¹, Gly¹⁰⁵ and Leu¹⁴¹; while the keto hydrazide would be stabilized by 2 H-bonding with Leu¹⁰⁷ and Glu¹⁴⁹ residues and interact with Gln⁵⁶ and Leu⁵² residues (**Figure 2C**). As previously observed with the **NVC**-*M. tb* PDF complex,²¹ the Ni²⁺ metal ion present inside the enzyme active site would be coordinated by Cys¹⁰⁶, His¹⁴⁸ and His¹⁵² residues, as well as by the bidentate keto hydrazide moiety, resulting in a square pyramidal coordination.

From these findings, **7h** may thus bind to *M. tb* PDF in a very similar orientation and with clear overlapping areas of interaction than the previously reported *M. tb* PDF-NVC bound inhibitor.²¹ Specifically, the residues involved in H-bonding and hydrophobic contacts in the above mentioned complex structure are also present in stabilizing **7h**, therefore reinforcing the accuracy of our model. Taken together, this network of interactions presumably allows the formation of a stable and productive binding mode and might provide a clear picture of the inhibition of *M. tb* PDF by **7h**.

2.4. Validation of *M. tb* PDF as vulnerable target of **7h**

Since the Rv0429c *M. tb* PDF is conserved in *M. marinum* (*i.e.*, MMAR_0744, 85.8% sequence identity) and in *M. abscessus* (*i.e.*, MAB_4187, 80.7% sequence identity), the observed binding mode for **7h** would suggest that this compound, as well as other hydrazide analogs, might be able to interact with the metal ion inside the PDF protein's active site, therefore resulting in its enzyme activity inhibition and consequently to bacterial death.

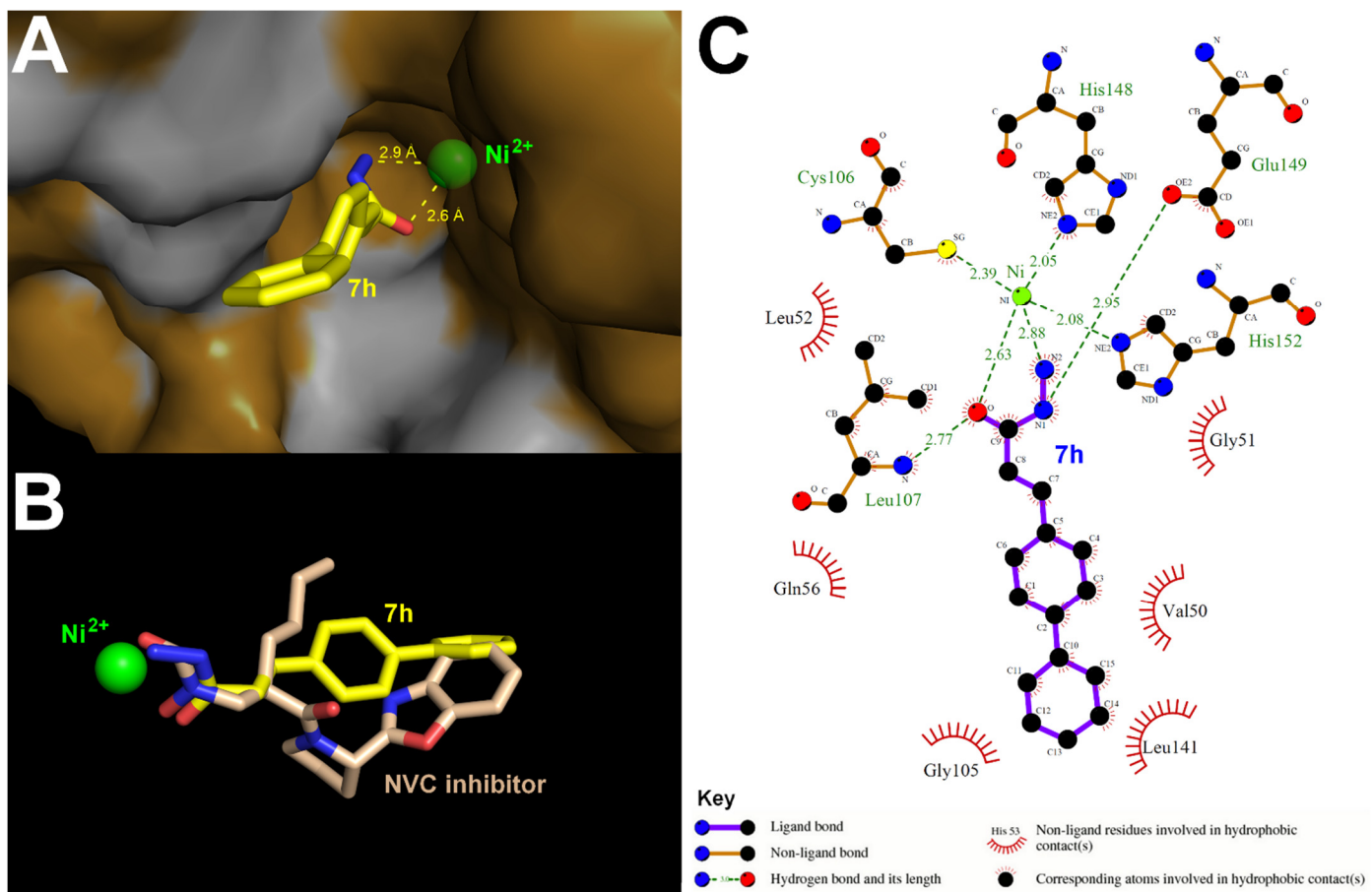


Table 2. Variation of MIC ($\mu\text{g/mL}$) of **7h** and **actinonin** against *M. tb*-PDF-overexpression mutant strain.^a

| Cpds / <i>M. smegmatis</i> strains | MIC ₅₀ / MIC ₉₀ ($\mu\text{g/mL}$) | MIC ₅₀ / MIC ₉₀ ratio mutant vs. WT |
|---|--|---|
| 7h | | |
| <i>M. smegmatis</i> WT | 9.6 \pm 0.60 [§] / 53.6 \pm 0.10 [†] | 1.0 / 1.0 |
| <i>M. smegmatis</i> _pMyC empty vector | 7.2 \pm 0.53 / 43.8 \pm 0.36 | 0.75 / 0.82 |
| <i>M. smegmatis</i> _pMyC::PDF _{Mtb} | 56.6\pm3.3[§] / >200[†] | 5.9 / >3.7 |
| actinonin | | |
| <i>M. smegmatis</i> WT | 4.6 \pm 0.25 [†] / 24.7 \pm 2.3 | 1.0 / 1.0 |
| <i>M. smegmatis</i> _pMyC empty vector | 4.3 \pm 0.27 / 29.0 \pm 1.4 | 0.95 / 1.2 |
| <i>M. smegmatis</i> _pMyC::PDF _{Mtb} | 13.5\pm0.53[†] / >200 | 3.0 / >8.1 |

^a Minimum inhibitory concentration leading to either 50% or 90% growth inhibition (MIC₅₀/MIC₉₀) as determined by the resazurin microtiter assay (REMA). Values are mean \pm SD of two independent assays performed in triplicate. MIC values with a common symbol are significantly different ([§], [†], ^{||}; *p*-value<0.001; ANOVA followed by Fisher's test).

Given the essentiality of the PDF enzyme for *M. tb* growth,¹³ we decided to confirm this enzyme as a potential target of compound **7h**. Gene encoding the Rv0424c PDF protein was cloned and overexpressed in *M. smegmatis*, a rapidly-growing

mycobacteria often used as an alternative for *M. tb*,⁴⁰ using the pMyC::PDF_{Mtb} inducible plasmid, where gene was cloned under the control of an acetamide promoter.⁴¹ The overexpression of *M. tb* PDF protein in *M. smegmatis* was confirmed by Western blotting as compared to the parental strain (WT) (Figure S1). As depicted in Table 2, a significant increase in MIC₅₀ values by 3.0- and 5.9-fold for **7h** and **actinonin**, respectively, as well as in MIC₉₀ values (>200 $\mu\text{g/mL}$) was reached for the *M. smegmatis*_pMyC::PDF_{Mtb} overexpression mutant as compared to the respective pMyC vector control and wild-type strain. In contrast, no significant changes in the efficacy of the standard drugs Isoniazid, Rifampicin or Kanamycin were observed (Table S1). These results clearly suggest that *M. tb* PDF is responsible for the decreased susceptibility to the hydrazide **7h**, thus confirming this protein as a potential target of our compound.

3. CONCLUSION

In summary, out of the 45 hydroxamic acid and hydrazide derivatives synthesized and evaluated for their antibacterial activity, different affinity and/or selectivity were observed. The most promising growth inhibitors that may be considered as lead structures are hydroxamic acids **4e** and **9e** which exhibit the highest antibacterial potency against all mycobacteria, while also highly toxic towards murine macrophages.

Among the nontoxic derivatives, the best growth inhibitors are hydroxamic acid **9h** potent against *M. abscessus* R and S only; hydrazide **7h** active against *M. abscessus* R, *M. marinum* and *M.*

tb; and the *O*-acyl hydroxamic acid derivatives **14d** and **15d** which exhibit promising antibacterial activity against both *M. marinum* and *M. tb*. Since these two latter compounds were based on hydroxamic acid **9d**, that was highly toxic and inactive against *M. tb*, introduction of the butanoyl and decanoyl group at the most promising hydroxamic acids **4e** and **9e** might enhance their respective anti-TB activity and above all minimize their individual toxicity.

Significant increased resistance level when overexpressing the *M. tb* PDF protein in *M. smegmatis* thus confirm the assertion that this enzyme is an effective target of **7h**. *In silico* molecular docking study have also brought useful and informative data regarding the potential binding mode of compound **7h** inside the *M. tb* PDF enzyme's active site, therefore providing some clues for developing new lead compounds as potent antibacterial agents.

Based on these encouraging results, we hope that by applying appropriate chemical modifications to the most interesting compounds reported in this study, we can synthesize novel nontoxic and above all more potent anti-mycobacterial agents.

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Appendix A. Supplementary data

Supplementary data to this article can be found online.

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