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Inhibition of *Mycobacterium tuberculosis* methionine aminopeptidases by bengamide derivatives

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Abstract

Methionine aminopeptidase (MetAP) carries out an essential function of protein N-terminal processing in many bacteria and is a promising target to develop novel antitubercular agents. Natural bengamides potentially inhibit proliferation of mammalian cells by targeting MetAP enzymes, and the X-ray structure of human type 2 MetAP in complex with a bengamide derivative revealed the key interactions at the active site. By preserving the interactions with the conserved residues inside the binding pocket while exploring the differences between bacterial and human MetAPs around the binding pocket, seven bengamide derivatives were synthesized and evaluated for inhibition of *Mt*MetAP1a and *Mt*MetAP1c in different metalloforms, inhibition of growth of *M. tuberculosis* in replicating and non-replicating states, and inhibition of growth of human K562 cells. Potent inhibition of *Mt*MetAP1a and *Mt*MetAP1c and modest growth inhibition of *M. tuberculosis* were observed for some of these derivatives. X-ray structures of *Mt*MetAP1c in complex with two of the derivatives provided the valuable structural information for improvement of these inhibitors for potency and selectivity.

Keywords

antibacterial; drug discovery; enzyme inhibition; hydrolases; metalloenzymes

Introduction

Methionine aminopeptidase (MetAP) exists in every cell and carries out a cotranslational modification by removing the N-terminal methionine residue from many nascent proteins.^[1] MetAP is divided into two subtypes, namely type 1 and type 2. Eukaryotic cells have both subtypes, and prokaryotic cells have only one. Deletion of either of the two MetAP genes in *Saccharomyces cerevisiae* rendered a slow growth phenotype, and lethality was observed when both genes were deleted.^[2] Human type 1 and type 2 MetAPs play important roles in cell proliferation, as well. For example, bengamides, a class of natural products that were isolated from marine sponge,^[3] showed nanomolar potency against cancer cell lines (**1** and **2** in Fig. 1),^[4, 5] and by a proteomic approach, human MetAPs were identified as the cellular

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PDB ID Codes: Coordinates and structure factors for *Mt*MetAP1c complexed to **9** and **10** have been deposited in the Protein Data Bank under the access codes 3PKB and 3PKA, respectively.

targets.^[6] Bengamides arrested cells at G1 and G2/M phases of the cell cycle,^[4, 7] and inhibition of MetAPs led to regulation of c-Src nonreceptor tyrosine kinase activity.^[8] An anticancer clinical trial was carried out using a synthetic bengamide derivative LAF389 (3).^[9]

In contrast to eukaryotic cells, most bacteria have a single MetAP gene, which codes for a type 1 MetAP. Deletion of this gene is lethal in *Escherichia coli*,^[10] and *Salmonella typhimurium*,^[11] suggesting that MetAP is an essential enzyme and a promising target to develop broad-spectrum antibiotics.^[12] Recently, we demonstrated that a few MetAP inhibitors displayed significant antibacterial activity on *E. coli* and *Bacillus subtilis* through MetAP inhibition.^[13] Multiple MetAPs are not common in bacteria, but with more genomic sequences reported, two or more putative MetAP genes are identified in a small number of bacteria. So far, twenty genomes of mycobacteria have been sequenced, and putative MetAP proteins in each mycobacterial genome, ranging from two to four, have been identified by sequence analysis. For example, *Mycobacterium tuberculosis* has two MetAP genes (*mapA* and *mapB* in H37Rv genome and *map_1* and *map_2* in CDC1551 genome), and both belong to type 1 MetAP with high homology to *E. coli* MetAP.

For most of these putative MetAPs, little is known about their biochemical properties beyond their sequences. The protein from *mapB* gene of *M. tuberculosis*, named *MtMetAP1c*, was purified, and its structures in apoform and in complex with methionine were reported.^[14] The structural analysis revealed an SH3 binding motif in its N-terminus, and potential interaction with ribosome through this motif to facilitate cotranslational methionine excision was proposed.^[14] We recently further characterized this enzyme for metal activation and inhibition and described three X-ray structures of *MtMetAP1c* with different inhibitors bound.^[15] Interestingly, the other MetAP (from *mapA* gene) of *M. tuberculosis*, named *MtMetAP1a*, is shorter at the N-terminus and has no such SH3 binding motif. When purified, *MtMetAP1a* could be activated by divalent metals and inhibited by small molecules.^[16] The mRNA transcripts of both *MtMetAP1a* and *MtMetAP1c* have been analyzed, and they showed different levels in log phase and stationary phase, leading to the conclusion that the two MetAPs may perform the important function in different growth phases of *M. tuberculosis*.^[17] *MtMetAP1a* gene expressed more in log phase, while *MtMetAP1c* gene showed a higher level in stationary phase. The high level of *MtMetAP1a* in log phase is consistent with the recent studies that knockdown of *MtMetAP1a* gene by antisense RNAs resulted in decreased viability of *M. tuberculosis*, while knockdown of *MtMetAP1c* gene showed little effect on growth.^[18] Although *MtMetAP1c* may not play a major role during growth phase, its high level in stationary phase may suggest its potential role during dormancy. Based on comparison of mycobacterial genomes, it was predicted that both *MtMetAP1a* and *MtMetAP1c* are essential for *M. tuberculosis* survival *in vivo* and pathogenicity.^[19] In comparison to most prokaryotes, *M. tuberculosis* is unique in that it has to evade and propagate within human macrophages. Question is whether *MtMetAP1c* plays a role in the survival of *M. tuberculosis* within mammalian cells.^[18] The special characteristics of mycobacterial life cycle may require more than one MetAP to carry out the important cotranslational modification.

Tuberculosis (TB) is a deadly disease caused by mycobacterial infection, and *M. tuberculosis* is the major TB pathogen of humans. Now, multidrug-resistant and extensively drug-resistant TB is happening at an alarming rate.^[20] To overcome the drug resistance, new antibiotics with novel mechanism of action are urgently needed. Therefore, MetAP is a promising target for developing novel drugs against TB-causing drug resistance bacteria.^[12] We report here several newly designed MetAP inhibitors based on natural bengamides that not only inhibited the purified tubercular MetAP enzymes but also showed initial antitubercular activity. Additionally, X-ray structures of *MtMetAP1c* complexed with two

such inhibitors revealed the binding mode of these bengamide derivatives and provided the structural basis for further improvement of MetAP inhibitors.

Chemistry

Design of bengamide derivatives

MetAP is a metal-dependent enzyme and requires a divalent metal for catalysis, such as Co^{II} , Mn^{II} , Ni^{II} and Fe^{II} .^[21, 22] The active site is a shallow and mostly hydrophobic pocket to accommodate the terminal methionine residue with two metal ions sitting at the bottom, as revealed in several X-ray structures.^[23] Bengamides are inhibitors of both human type 1 and type 2 MetAPs with micromolar potency,^[8] and their bound conformation at the active site was illustrated by the only reported X-ray structure of the bengamide derivative LAF153 (**11**) in complex with human type 2 MetAP (Figs. 2A and 3A, pdb code 1QZY).^[6] In the dimetalated structure, the triol moiety of **11** coordinates with two Co^{II} ions to form two tetrahedron coordination geometries, which is reminiscent of the binding of transition-state inhibitors, such as methionine phosphonate (**13**, Fig. 2B, pdb code 1C23)^[24] and the bestatin analog (**14**, pdb code 3MAT).^[25] Possibly, the spatial arrangement of three hydroxyl groups in **11** uniquely satisfies the coordination requirement and confers the high affinity. On one side of the triol moiety, the t-butylalkene substituent occupies the site reserved for the terminal methionine, and on the other side, the caprolactam ring beyond the amide bond interacts with residues towards the opening of the active site pocket (Fig. 3A).

Among MetAPs of both prokaryotic and eukaryotic origins, most residues in the active site pocket are conserved, making the interactions of an inhibitor with the residues and with the metal ions more predictable. However, variation around the opening of the pocket is significant, because of the different lengths and residues in the N-terminal extension of human and bacterial MetAPs.^[23] The caprolactam moiety of bengamides was believed to be essential for their potency on human MetAPs, and synthetic effort for anticancer therapy has been directed towards modification of this moiety by introducing different functional groups on the ring while keeping this seven-membered ring structure.^[4, 5, 26] Our purpose is to design bengamide derivatives for strong inhibition of tubercular MetAPs and weak or no inhibition of human MetAPs. Therefore, we kept the triol moiety and the t-butylalkene substituent as the bengamide core structure for interaction with the two metal ions and with the hydrophobic methionine binding pocket. At the same time, we eliminated the caprolactam moiety and replaced it with different amide moieties, attached to the core structure, to explore the additional interactions near the opening of the active site pocket. Initially, seven such derivatives (**4-10**, Fig. 1) were designed, synthesized and evaluated as potential antitubercular agents.

Synthesis of bengamide derivatives

Commercially available **15** was converted to the common intermediate **16** in several steps, according to the reported synthetic procedure with minor modifications (Scheme 1).^[27] Coupling of the lactone **16** with different amines was accomplished by refluxing in isopropanol. Final deprotection produced the target compounds **4-10** through the intermediate **17** in yields ranging from 9% to 25% for the two steps.

Results and discussion

Inhibition of *Mt*MetAP1a and *Mt*MetAP1c

Both *Mt*MetAP1a and *Mt*MetAP1c belong to type 1 MetAP and share high sequence homology.^[16] When purified as apoenzymes, both can be activated by divalent metals, including Co^{II} , Mn^{II} , Ni^{II} , and Fe^{II} .^[15, 16] Because inhibitors of a metalloenzyme often

interact directly with the metal ions at the active site, inhibitors can display marked difference in inhibitory potency on different metalloforms of the enzyme, as we have shown for *E. coli* MetAP.^[28] For therapeutic application of the inhibitors, it is critical that these inhibitors can effectively inhibit the cellular enzyme in its native metalloform. We have shown that *E. coli* MetAP uses Fe^{II} for catalysis in *E. coli* cells, and inhibitors of *E. coli* MetAP with selectivity for the Fe^{II}-form displayed antibacterial activity.^[29] We demonstrated that *Mt*MetAP1c uses Fe^{II} in *E. coli* cells,^[15] but the native metalloform for *Mt*MetAP1a and *Mt*MetAP1c in *M. tuberculosis* remains unknown. Therefore, we tested the bengamide derivatives for their inhibitory activity on *Mt*MetAP1a and *Mt*MetAP1c activated by each of the activating metals (Table 1).

All seven derivatives **4-10** showed micromolar or submicromolar potency on the Mn^{II}-form of *Mt*MetAP1c. Considering that the IC₅₀ values were close to the concentration of *Mt*MetAP1c used (0.5 μM), which was limited by sensitivity of the assay, the actual potency for some of them could be higher. All compounds showed almost no activity against the Ni^{II}-form of *Mt*MetAP1c, and their potency on the Co^{II} or Fe^{II}-form was also weak, with **6, 7, 9** and **10** showed relatively higher potency. Although these synthetic bengamides clearly were selective for the Mn^{II}-form of *Mt*MetAP1c, the metalloform selectivity on *Mt*MetAP1a was not as noticeable. The inhibition of the Ni^{II}-form of *Mt*MetAP1a was also weaker for all of the bengamides, but **6, 7** and **10** all showed considerable potency on the Co^{II}, Mn^{II}, and Fe^{II}-forms of *Mt*MetAP1a. Structurally, **6, 7, 8** and **10** all have aromatic rings in their amide moiety. It is possible that these substituents can be accommodated at the active site, and they provide additional binding interactions.

Inhibitors of *Mt*MetAP1a and *Mt*MetAP1c were reported only recently.^[15, 16, 18] Some of these bengamide derivatives with micromolar potency are promising lead compounds with a new scaffold for further structural modifications. It is not known which metal(s) *Mt*MetAP1a and *Mt*MetAP1c use as their native metal. Characterization of the metalloform-selective inhibition of these bengamides and discovery of metalloform selective inhibitors will provide the necessary research tools to reveal the metal requirement for catalysis of MetAP in *M. tuberculosis* cells. Clearly, therapeutic MetAP inhibitors need to effectively inhibit cellular MetAPs with their native metal in place.

X-ray structures of *Mt*MetAP1c in complex with **9** and **10**

The previous structure of human type 2 MetAP revealed the binding mode of **11**.^[6] To confirm that the newly synthesized bengamide derivatives bind to tubercular MetAPs in the same way as designed, we obtained two X-ray structures of *Mt*MetAP1c in complex with either **9** or **10** at high resolution (both at 1.25 Å, Figs. 2C, 3B, and 3C). The overall fold of the new *Mt*MetAP1c structures was the same as our previously obtained structures of the same enzyme in complex with other inhibitors,^[15] and two Mn^{II} ions and the new bengamide inhibitors bound at the active site pocket. Similar to **11**, both the bengamides **9** and **10** used their triol moiety to coordinate with the two active site metal ions and the *t*-butylalkene chain to occupy the hydrophobic S1 site.

Our strategy in designing the bengamide derivatives for selectivity was to substitute the caprolactam ring in **11** with different amide moieties to explore different interactions at the opening of the active site pocket. Indeed, the significant difference in binding was observed at the amide moiety. While the amide moiety in **9** was shorter and took the similar position to that of the caprolactam ring in **11** (Fig. 3B), the trimethylphenyl group in **10** made a sharp turn, reached the shallow cavity at the opening unique to *Mt*MetAP1c, and fitted snugly in the cavity (Fig. 3C). With this structural information on binding, additional substitutions on

the phenyl ring could be introduced to explore the interactions with different residues in the cavity, in order to improve both potency and selectivity.

Growth inhibition of *M. tuberculosis* at replicating state and nonreplicating state

All seven newly synthesized bengamide derivatives were evaluated for their antitubercular activity against *M. tuberculosis* strain H37Rv (replicating phenotype, R-TB) by using microplate Alamar Blue assay^[30, 31] and *M. tuberculosis* strain H37Rv-CA-lux AB (nonreplicating persistent phenotype, NRP-TB) by using low oxygen recovery assay.^[32] Among the compounds tested (Table 1), **10** exhibited the best antitubercular activity against both replicating *M. tuberculosis* (MIC = 50.6 μ M; 0.08 μ M for rifampin and 0.24 μ M for isoniazid, respectively) and nonreplicating *M. tuberculosis* (MIC = 107.4 μ M; 1.96 μ M for rifampin and >128 μ M for isoniazid). Notably, **10** was also one of the best inhibitors of *M*MetAP1a and *M*MetAP1c. It is interesting that the bengamides that displayed antitubercular activity in the microplate Alamar Blue assay also displayed the activity in the using low oxygen recovery assay (Table 1), possibly due to inhibition of both *M*MetAP1a and *M*MetAP1c by these bengamides in cells.

Growth inhibition of human K562 cells

To develop antitubercular bengamide derivatives, we want to minimize inhibition of human MetAPs to reduce potential toxicity. To evaluate the antiproliferative effect of these derivatives, we tested their effect on growth of human K562 cells, which was derived from leukemia cells (Table 1). Inhibitors **4**, **5**, **8** and **9** showed no or weak activity at 333 μ M, the highest concentration tested, and inhibitors **6**, **7** and **10** showed low and reproducible activity. Interestingly, **10** was the best growth inhibitor of *M. tuberculosis*, and it was also the most active against growth of human cells. Although the initial results from the limited number of bengamide derivatives are encouraging, significant improvement on potency and selectivity is required.

Conclusions

Natural bengamides with demonstrated antiproliferative potency on mammalian cells provide a unique scaffold to develop a new class of MetAP inhibitors for antibacterial and antitubercular therapeutics. Based on analysis of the binding mode of a bengamide derivative **11** on human type 2 MetAP, we replaced the caprolactam moiety in natural bengamides with different amide moieties to take advantage of the differences at the inhibitor binding pocket, aiming to obtain inhibitors with high potency for *M. tuberculosis* MetAPs and reduced or no potency on human MetAPs. Some of these newly designed and synthesized bengamide derivatives showed modest but reproducible activity against both replicating and non-replicating *M. tuberculosis*. Evaluation of these derivatives on human K562 cells detected their growth inhibition, indicating their activity on mammalian MetAPs has not been completely eliminated. Importantly, the structural information of two such inhibitors (**9** and **10**) in complex with *M*MetAP1c provided the valuable structural information of these inhibitors at the active site and will guide the development of further bengamide derivatives with better potency and selectivity on tubercular MetAPs.

Experimental Section

General Methods

All chemicals were reagent-grade and were used without further purification. Solvents were of analytical grade. ¹H (500 MHz) and ¹³C (125 MHz) were obtained on a Bruker Avance II 500. Chemical shifts were reported in δ (ppm) using δ 7.26 signal of CDCl₃ (¹H NMR), δ 77.23 signal of CDCl₃ (¹³C NMR), or δ 2.50 signal of DMSO-*d*₆ (¹H NMR), δ 40.60 signal

of DMSO- d^6 (^{13}C NMR) as the internal standard. In the case of multiplets, the signals are reported as intervals. Signals were abbreviated as s, singlet; d, doublet; m, multiplet. All tested compounds were at least 95% pure on the basis of HPLC-MS using a LTQ Orbitrap mass spectrometer (Thermo-Fisher Scientific) coupled to an Accela HPLC system (Thermo-Fisher Scientific). High resolution MS data were obtained on either a Thermo Finnigan MAT-95 XP or a Waters/Micromass LCT. All reactions were routinely checked by thin-layer chromatography (TLC) on silica gel 60F254 (Merck) and visualized with 5% ethanolic phosphomolybdic acid solution and heat. Organic solutions were dried over anhydrous MgSO_4 , filtered, and concentrated with a Buchi rotary evaporator at reduced pressure. Yields are of purified product and were not optimized.

General Procedure for the Preparation of 4-10

A solution of **16** (0.1 g, 0.35 mmol), and an appropriate amine (0.9 mmol) in isopropanol (5 mL) was stirred at reflux for 10 h. When the amine was HCl salt, 1.4 mmol of 2-ethylhexanoate was added.^[33] The solvent was removed, and the residue was dissolved in ethyl acetate (20 mL), washed with 10% *aq.* citric acid solution (2×15 mL) and *aq.* NaHCO_3 (20 mL) successively. The organic layer was dried over MgSO_4 , filtered, concentrated to give crude acetone intermediate **17**. The intermediate **17** was dissolved in methanol (3 mL). 3 N *aq.* HCl in methanol (1 mL) was added dropwise, and the mixture was stirred at r.t. for 4 h. The solution was basified to pH 8-9 with *aq.* NaHCO_3 and the organic solvent was removed. The residue was diluted with H_2O (10 mL), extracted with EtOAc (2×15 mL). The combined organic layers were dried over MgSO_4 , concentrated, and purified by column chromatography (hexane/ethyl acetate) to give the desired product **4-10**.

(2R,3R,4S,5R,E)-3,4,5-trihydroxy-N-((1-isobutyrylpiperidin-3-yl)methyl)-2-methoxy-8,8-dimethylnon-6-enamide (**4**)

Yield for two steps: 9%. ^1H NMR (CDCl_3 , 500 MHz) δ 1.06 (s, 9H), 1.13 (m, 6H), 1.53-1.41 (m, 2H), 1.90-1.67 (m, 4H), 2.84-2.75 (m, 2H), 3.46-3.11 (m, 3H), 3.56 (s, 3H), 3.30-3.62 (m, 3H), 3.88-3.86 (m, 1H), 4.20-4.17 (m, 1H), 5.50-5.42 (m, 1H), 5.86-5.82 (m, 1H), 7.23 (s, 1H). ^{13}C NMR (CDCl_3 , 125 MHz) δ 19.49, 25.13, 31.11, 31.29, 33.02, 35.72, 41.35, 46.37, 60.40, 72.01, 72.52, 74.55, 82.05, 82.60, 99.99, 123.34, 145.63, 172.18, 175.84. HRMS (ESI-TOF) m/z 451.2784 (calcd for $\text{C}_{22}\text{H}_{40}\text{N}_2\text{O}_6\text{Na}$ 451.2784).

(2R,3R,4S,5R,E)-N-((1-(cyclopropanecarbonyl)piperidin-3-yl)methyl)-3,4,5-trihydroxy-2-methoxy-8,8-dimethylnon-6-enamide (**5**)

Yield for two steps: 18%. ^1H NMR (CDCl_3 , 500 MHz) δ 0.78-0.77 (m, 2H), 0.97-0.95 (m, 2H), 1.06 (s, 9H), 1.57-1.33 (m, 2H), 1.87-1.74 (m, 7H), 3.47-3.26 (m, 2H), 3.51 (s, 3H), 3.87-3.78 (m, 2H), 4.30-4.16 (m, 2H), 5.48-5.40 (m, 1H), 5.86-5.85 (m, 1H), 7.29 (s, 1H). ^{13}C NMR (CDCl_3 , 125 MHz) δ 7.40, 10.92, 24.63, 28.40, 29.42, 33.03, 35.66, 41.35, 45.52, 46.16, 46.55, 60.20, 72.25, 74.66, 82.3, 99.99, 123.29, 145.68, 172.12, 172.27. HRMS (ESI-TOF) m/z 449.2606 (calcd for $\text{C}_{22}\text{H}_{38}\text{N}_2\text{O}_6\text{Na}$ 449.2628).

(2R,3R,4S,5R,E)-N-(3-(furan-2-yl)-3-phenylpropyl)-3,4,5-trihydroxy-2-methoxy-8,8-dimethylnon-6-enamide (**6**)

Yield for two steps: 16%. ^1H NMR (CDCl_3 , 500 MHz) δ 1.01 (s, 9H), 2.15-2.14 (m, 1H), 2.37-2.35 (m, 1H), 3.33-3.27 (m, 2H), 3.47 (s, 3H), 3.57-3.52 (m, 2H), 3.69-3.67 (m, 1H), 3.78-3.77 (m, 1H), 3.99-3.98 (m, 1H), 4.21-4.196 (m, 2H), 5.44-5.39 (dd, 1H), 5.85-5.81 (d, 1H), 6.07 (s, 1H), 6.29 (s, 1H), 6.86 (s, 1H), 7.25-7.22 (m, 3H), 7.33-7.30 (m, 3H). ^{13}C NMR (CDCl_3 , 125 MHz) δ 14.16, 29.46, 29.70, 31.62, 33.03, 34.25, 37.67, 43.17, 59.89, 72.42, 74.57, 81.13, 105.80, 110.16, 123.20, 127.03, 127.70, 128.76, 141.57, 145.91, 156.68, 172.53. HRMS (ESI-TOF) m/z 468.2357 (calcd for $\text{C}_{25}\text{H}_{35}\text{NO}_6\text{Na}$ 468.2362).

(2R,3R,4S,5R,E)-N-(3-(4-fluorophenyl)-3-(furan-2-yl)propyl)-3,4,5-trihydroxy-2-methoxy-8,8-dimethylnon-6-enamide (7)

Yield for two steps: 10%. ^1H NMR (CDCl_3 , 500 MHz) δ 1.07 (s, 9H), 2.16-2.13 (m, 1H), 2.37-2.35 (m, 1H), 3.04 (s, 1H), 3.35-3.27 (m, 2H), 3.52-3.51 (d, 1.9 Hz, 3H), 3.61-3.59 (m, 1H), 3.72-3.70 (dd, 1H), 3.82-3.96 (m, 1H), 4.02-3.99 (m, 1H), 4.17-4.15 (m, 1H), 4.24-4.22 (m, 1H), 5.47-5.42 (dd, 1H), 5.88-5.84 (d, 1H), 6.10-6.09 (m, 1H), 6.33-6.32 (m, 1H), 6.90-6.80 (m, 1H), 7.04-7.01 (m, 2H), 7.24-7.23 (m, 2H), 7.35 (d, 1H). ^{13}C NMR (CDCl_3 , 125 MHz) δ 29.42, 33.04, 34.38, 37.58, 42.38, 60.20, 72.38, 81.19, 99.99, 105.88, 110.21, 115.50, 123.18, 129.16, 141.77, 145.99, 160.83, 162.78, 172.52. HRMS (ESI-TOF) m/z 486.2253 (calcd for $\text{C}_{25}\text{H}_{34}\text{FNO}_6\text{Na}$ 486.2268).

(2R,3R,4S,5R,E)-N-(2,3-dihydro-1H-inden-2-yl)-3,4,5-trihydroxy-2-methoxy-8,8-dimethylnon-6-enamide (8)

Yield for two steps: 25%. ^1H NMR (CDCl_3 , 500 MHz) δ 1.05 (s, 9H), 2.89-2.83 (m, 2H), 3.41-3.36 (m, 2H), 3.49 (s, 3H), 3.61-3.60 (d, 1H), 3.75-3.74 (d, 1H), 3.83-3.82 (d, 1H), 4.26-4.23 (m, 1H), 4.79-4.77 (m, 1H), 5.46-5.42 (dd, 1H), 5.88-5.84 (d, 1H), 7.08-7.07 (d, 1H), 7.24-7.21 (m, 4H). ^{13}C NMR (CDCl_3 , 125 MHz) δ 29.42, 33.05, 40.00, 50.14, 59.79, 72.36, 72.44, 74.59, 81.19, 123.19, 124.80, 126.97, 140.40, 145.98, 172.29. HRMS (ESI-TOF) m/z 400.2082 (calcd for $\text{C}_{21}\text{H}_{31}\text{NO}_5\text{Na}$ 400.2100).

(2R,3R,4S,5R,E)-N-(2-amino-2-oxoethyl)-3,4,5-trihydroxy-2-methoxy-8,8-dimethylnon-6-enamide (9)

Yield for two steps: 14%. ^1H NMR (CDCl_3 , 500 MHz) δ 1.01 (s, 9H), 3.51 (s, 2H), 3.79 (s, 3H), 3.83-3.92 (m, 2H), 4.16-4.21 (m, 1H), 4.25-4.30 (m, 1H), 5.37-5.45 (m, 1H), 5.83-6.01 (m, 1H). ^{13}C NMR (CDCl_3 , 125 MHz) δ 29.28, 42.72, 60.00, 66.29, 73.00, 74.30, 81.80, 99.71, 121.27, 145.62, 169.25, 170.71. HRMS (ESI-TOF) m/z 341.1689 (calcd for $\text{C}_{14}\text{H}_{26}\text{N}_2\text{O}_6\text{Na}$ 341.1689).

(2R,3R,4S,5R,E)-3,4,5-trihydroxy-N-(2-(mesityloxy)ethyl)-2-methoxy-8,8-dimethylnon-6-enamide (10)

Yield for two steps: 15%. ^1H NMR (CDCl_3 , 500 MHz) δ 7.53 (s, 1H), 6.85 (s, 2H), 5.88-5.85 (d, 1H), 5.48-5.44 (dd, 1H), 4.28-4.25 (m, 1H), 3.88-3.63 (m, 7H), 3.59 (s, 3H), 2.22 (s, 9H), 1.06 (s, 9H). ^{13}C NMR (CDCl_3 , 125 MHz) δ 16.15, 20.65, 29.44, 33.04, 39.46, 60.15, 70.10, 72.39, 72.67, 74.59, 80.89, 123.21, 129.61, 130.15, 133.66, 145.88, 152.74, 172.81. HRMS (ESI-TOF) m/z 446.2505 (calcd for $\text{C}_{23}\text{H}_{37}\text{NO}_6\text{Na}$ 446.2519).

Inhibition assay of *MtMetAP1a* and *MtMetAP1c*

Both *MtMetAP1a* and *MtMetAP1c* were expressed in *E. coli* and purified as apoenzymes, as previously described.^[15, 16] Both can be activated by Co^{II} , Mn^{II} , Ni^{II} , and Fe^{II} instantly. Enzymatic activity was monitored by fluorescence ($\lambda_{\text{ex}} = 360$ nm, $\lambda_{\text{em}} = 460$ nm) on a Spectramax Gemini XPS plate reader (Molecular Devices, Sunnyvale, CA), following hydrolysis of the fluorogenic substrate, methionyl aminomethylcoumarin (Met-AMC), at room temperature as described.^[22] All kinetic experiments were carried out on 384-well plates. For inhibition of *MtMetAP1a*, each well contained 80 μl of assay mixture with 50 mM MOPS (pH7.5), 100 μM Met-AMC, apoenzyme and metal ions (50 nM enzyme, 50 μM CoCl_2 ; 200 nM enzyme, 250 μM MnCl_2 ; 12.5 nM enzyme, 50 μM NiCl_2 ; or 50 nM enzyme, 10 μM FeCl_2 , 10 μM ascorbic acid). For inhibition of *MtMetAP1c*, each well contained 80 μl of assay mixture with 50 mM MOPS (pH7.5), 100 μM Met-AMC, apoenzyme (500 nM), and metal ions (50 μM CoCl_2 , 250 μM MnCl_2 , 50 μM NiCl_2 , or 10 μM FeCl_2 plus 10 μM ascorbic acid). The inhibitors were tested at six or more serially

diluted concentrations. IC₅₀ values were calculated from nonlinear regression curve fitting of percent inhibitions as a function of inhibitor concentrations.

***M. tuberculosis* MABA and LORA Assays**

Minimum inhibitory concentrations (MIC) against replicating and non-replicating cultures of *Mycobacterium tuberculosis* were determined using the microplate Alamar Blue assay (MABA [30, 31]) and low oxygen recovery assay (LORA [32]), respectively. The former was determined against *M. tuberculosis* H₃₇Rv ATCC 27294 (American Type Culture Collection) following 7 days incubation with test samples. The latter was determined against low oxygen adapted *M. tuberculosis* H₃₇Rv luxAB carrying a luciferase reporter gene following 10 days incubation under low oxygen followed by 28 hours of normoxic recovery. Both assays were conducted in microplate format in 7H12 medium.^[30] The MIC was defined as the lowest concentration effecting a reduction of 90% in fluorescence (MABA) or luminescence (LORA) relative to untreated controls.

Crystallization and data collection

Crystals of the enzyme-inhibitor complexes were obtained independently by a hanging-drop vapor-diffusion method at room temperature. Each of the inhibitors (**9** or **10**; 100 mM in DMSO) was added to concentrated metalated enzyme (10 mg/mL, 0.32 mM protein; 2 mM metal) in 50 mM Tris, pH 8.0, 150 mM NaCl and the molar ratio of inhibitor to *Mt*MetAP1c was 5:1 or 10:1. The enzyme/inhibitor mixture was mixed with a reservoir buffer in a 1:1 ratio. The reservoir buffer was 100 mM Bis-Tris (pH 5.5), 1.3 M (NH₄)₂SO₄, and 10% glycerol. Diffraction data were collected at the Advanced Photon Source, Argonne National Laboratory (beamlines 19ID and 19BM) and processed with HKL3000.^[34] Both crystals belong to space group *P*6₃. One molecule is in the asymmetric unit.

Structural solution and refinement

The structures were solved by molecular replacement with MolRep^[35] in CCP4^[36] with CCP4i interface,^[37] using our previously published *Mt*MetAP1c structure (PDB code 3IU7)^[15] as the search model. The structure was refined with REFMAC5^[38] with iterative model building using WinCoot.^[39] The refinement was monitored with 5% of the reflections set aside for *R*_{free} factor analysis throughout the whole refinement process. Electron density was clear for almost all residues, and residues from the second (P2) in the native protein to the end (L285) were modeled. Comparison of structures and generation of structural drawings were carried out by using PyMOL.^[40] Statistic parameters in data collection and structural refinement are shown in Table 2. Atomic coordinates and structure factors for the two structures have been deposited in the Protein Data Bank.

Human K562 growth inhibition assay

K562 cells from ATCC were cultured as suspension in RPMI 1640 medium, containing 10% newborn calf serum. Growth inhibition assay was carried out in a total volume of 120 µl in each well on 96-well white plates. Compounds were serially diluted in 12 concentrations (2 fold), and cells were seeded at 12,000 cells per well by dispensing suspended K562 cells in growth medium with an 8-channal automated MultiDrop liquid dispenser. The plates were incubated at 37 °C in a humidified 5% CO₂ incubator for 48 hrs. A modified luciferase activity assay was used to monitor the cell growth. A mixture of luciferase, luciferin, Triton X100, and MgCl₂ in a total volume of 80 µl was added to the cells in each well, and luminescence was determined on a Spectramax Gemini or Spectramax M5 plate reader. The IC₅₀ values were calculated from non-linear regression curve fitting of percent inhibitions as a function of inhibitor concentrations.

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Abbreviations

MetAP	methionine aminopeptidase
MtMetAP1a	<i>M. tuberculosis</i> methionine aminopeptidase type 1a
MtMetAP1c	<i>M. tuberculosis</i> methionine aminopeptidase type 1c
Met-AMC	methionyl aminomethylcoumarin
TB	tuberculosis

Figure 1. Chemical structures of natural bengamides (**1** and **2**) and their synthetic derivative (**3**). Compounds **4-10** are the newly designed and synthesized bengamide derivatives.

Figure 2.

Design of antitubercular bengamides. **A.** Coordination of **11** with two metal ions inside the active site pocket of human type 2 MetAP. Color scheme: carbon, yellow (inhibitor) or grey (protein); oxygen, red; nitrogen, blue. Two Co^{II} ions are shown as green spheres. Coordination between the metal ions and the heteroatoms of the inhibitor or protein residues is shown as dashed lines. **B.** Chemical structures of **11**, methionine (**12**), methionine phosphonate (MetP, **13**), and the bestatin analog (**14**). **C.** Stereo view of the binding of newly synthesized **9** (carbon, magenta) and **10** (carbon, cyan) at the active site, in comparison with **11** (carbon, yellow). Co^{II} (green) and Mn^{II} (orange) ions are shown as spheres.

Figure 3. Binding of bengamide derivatives at the active site pocket of human type 2 MetAP and *Mm*MetAP1c. **A.** Inhibitor **11** (carbon, yellow) with human type 2 MetAP. **B.** Inhibitor **9** (carbon, magenta) with *Mm*MetAP1c. **C.** Inhibitor **10** (carbon, cyan) with *Mm*MetAP1c. Co^{II} (green) and Mn^{II} (orange) ions are shown as spheres. Color scheme for enzyme surface: carbon, gray; oxygen, red; and nitrogen, blue.

Scheme 1.

Table 1
Inhibition of MetAP enzymes in different metalloforms and inhibition of growth of *M. tuberculosis* and human cells

Cmpd	Enzyme inhibition (IC ₅₀ in μM)										Growth inhibition of <i>M. tuberculosis</i> cells (MIC in μM or % inhibition at 128 μM) ^a		Growth inhibition of human K562 cells (IC ₅₀ in μM)
	M/MetAP1a				M/MetAP1c						MABA ^b	LORA ^c	
	Co	Mn	Ni	Fe	Fe	Co	Mn	Ni	Fe				
4	45	68	141	67	67	>250	1.3	>250	179		0%		>333
5	88	187	>250	110	110	>250	0.96	>250	>250		0%		>333
6	6.0	11	26	5.5	5.5	39	0.40	150	61		15%	8%	79.6
7	8.1	12	80	6.7	6.7	52	0.20	>250	68		48%	12%	96.5
8	31	11	140	18	18	>250	0.76	>250	201		29%	28%	>333
9	21	49	114	14	14	50	0.62	120	3.7		122	0%	>333
10	7.9	6.9	33	4.5	4.5	75	0.54	>250	42		53.9	106	37.8

^aMIC is minimum inhibitory concentration.

^bMABA, microplate Alamar Blue assay.

^cLORA, low oxygen recovery assay.

All values in this table are averages of at least triplicate determinations.

Table 2

X-ray data collection and refinement statistics

Inhibitor	9	10
<i>Cell Parameters</i>		
space group	$P6_3$	$P6_3$
a (Å)	106.0	106.0
b (Å)	106.0	106.0
c (Å)	50.3	50.4
α (deg)	90	90
β (deg)	90	90
γ (deg)	120	120
<i>X-ray Data Collection</i>		
Resolution range (Å)	50-1.25 (1.27-1.25) $[a]$	50-1.25(1.27-1.25)
Collected reflections	559,494	633,834
Unique reflections	85,942	89,260
Completeness (%)	97.0 (73.9)	100 (100)
I/σ (I)	40.0 (1.7)	36.8 (3.3)
R_{merge} (%)	5.3 (47.5)	5.7 (52.5)
<i>Refinement Statistics</i>		
R (%)	16.8	16.9
R_{free} (%)	18.1	18.8
R.m.s.d. bonds (Å)	0.032	0.033
R.m.s.d. angles (°)	2.49	2.51
No. of solvent molecules	211	223
$\langle B \rangle$ protein (Å ²)	13.1	12.2
$\langle B \rangle$ inhibitor (Å ²)	11.7	10.5
$\langle B \rangle$ water (Å ²)	19.4	19.0

$[a]$ Values given in parentheses correspond to the outer shell of data.