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Bisubstrate Inhibitors of Biotin Protein Ligase in *Mycobacterium tuberculosis* Resistant to Cyclonucleoside Formation

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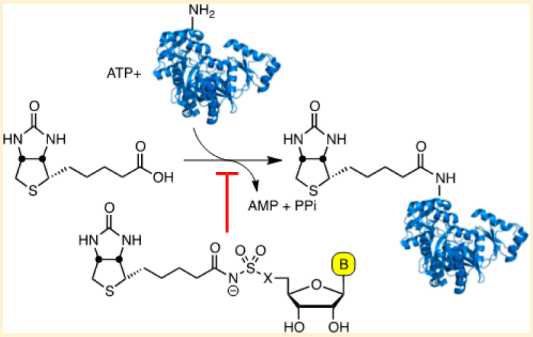
Supporting Information

ABSTRACT: *Mycobacterium tuberculosis* (*Mtb*), the etiological agent of tuberculosis, is the leading cause of bacterial infectious disease mortality. Biotin protein ligase (BirA) globally regulates lipid metabolism in *Mtb* through the posttranslational biotinylation of acyl coenzyme A carboxylases (ACCs) involved in lipid biosynthesis and is essential for *Mtb* survival. We previously developed a rationally designed bisubstrate inhibitor of BirA that displays potent enzyme inhibition and whole-cell activity against multidrug resistant and extensively drug resistant *Mtb* strains. Here we present the design, synthesis, and evaluation of a focused series of inhibitors, which are resistant to cyclonucleoside formation, a key decomposition pathway of our initial analogue. Improved chemical stability is realized through replacement of the adenosyl N-3 nitrogen and C-5′ oxygen atom with carbon as well as incorporation of a bulky group on the nucleobase to prevent the required *syn*-conformation necessary for proper alignment of N-3 with C-5′.



KEYWORDS: *Adenylate-forming, adenylation, antibiotic, biotin protein ligase, isothermal titration calorimetry, tuberculosis*

Tuberculosis (TB), caused by members of the *Mycobacterium tuberculosis* (*Mtb*) complex, remains the leading source of bacterial infectious disease mortality.[1,2] Current treatment for susceptible *Mtb* strains requires at least 6 months of drug therapy using a combination of four drugs (i.e., isoniazid, rifampin, ethambutol, and pyrazinamide), which were discovered over 40 years ago.[3] The development and dissemenation of multidrug resistant (MDR) and extensively drug resistant (XDR) *Mtb* strains significantly limits our current treatment options. Therefore, there is a pressing need for new TB drugs, ideally with novel mechanisms of action.[4,5]

In *Mtb*, biotin protein ligase encoded by the gene *birA* globally regulates lipid metabolism through the posttranslational biotinylation of acyl coenzyme A carboxylases (ACCs), which catalyze the first committed step in lipid biosynthesis, and pyruvate coenzyme A carboxylase (PCC) an enzyme in the gluconeogenesis pathway that is necessary for growth on fatty acids.[6] Consequently, BirA represents an extremely attractive target for the development of new antitubercular agents. BirA is responsible for the covalent ligation of biotin onto biotin-dependent enzymes and accomplishes this through two partial reactions (Figure 1A) .[7] In the first half-reaction, BirA catalyzes the condensation of biotin **1** and ATP to produce biotinyl-5′-AMP (Bio-AMP, **2**) and pyrophosphate. In the second half-reaction, BirA binds a biotin-dependent protein **3** and then transfers the biotin group of **2** onto the ϵ-amino group of a conserved lysine residue in **3** to afford the biotinylated protein **4**.

We reported a rationally designed inhibitor of BirA, termed Bio-AMS (**9**, Figure 2), with potent enzyme inhibition and antitubercular activity against MDR and XDR *Mtb* strains.[6] Polyak, Abell, Wilce, and co-workers have also disclosed some novel 1,2,3-triazole-biotin nucleosides and biotin analogues that are selective inhibitors of the biotin protein ligase from *Staphylococcus aureus*.[8−10] Bio-AMS **9** is a bisubstrate inhibitor, which mimics the intermediate **2** by replacing the labile acyl-phosphate linkage with an acyl-sulfamide moiety. The related analogue **5**[11,12] with an acyl-sulfamate linkage, another typical acyl-phosphate bioisostere, decomposes through cyclonucleoside formation to afford *N*-biotinylsulfamic acid **7** and 3,5′-cyclo-5′-deoxyadenosine **8** (Figure 1B).[6] The greater stability of **9** is due to the decreased nucleofugality of the sulfamide. Herein, we report the design, synthesis, and evaluation of a systematic series of Bio-AMS analogues that are completely resistant to cyclonucleoside formation (Figure 2). In analogue **10**, substitution of the 5′-oxygen atom of **5** with a methylene group converts the labile acyl-sulfamate moiety to a chemically stable acyl-sulfonamide. In analogue **11**, the N-3 nitrogen atom

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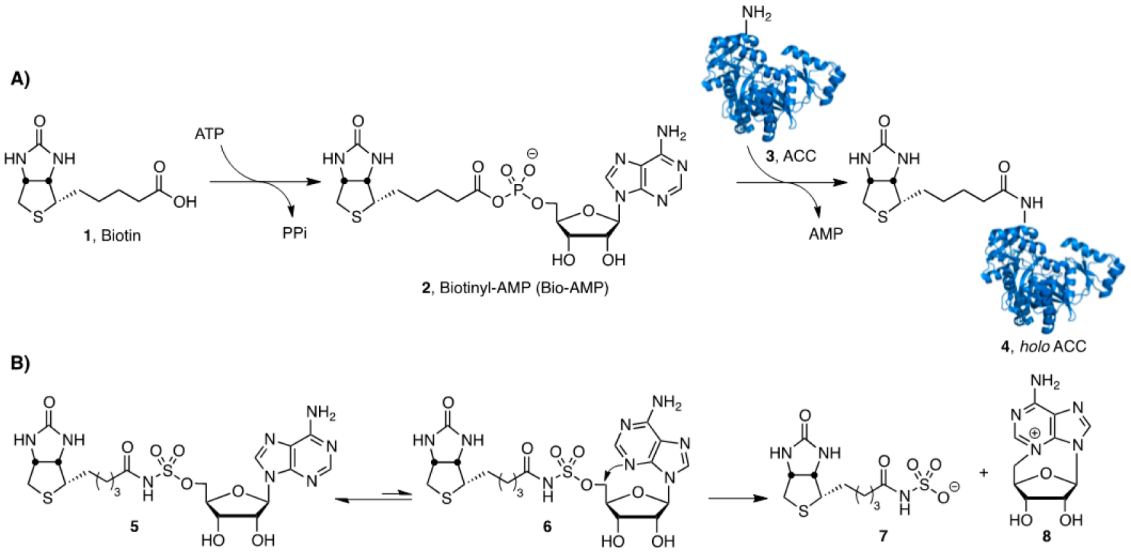


Figure 1. (A) BirA catalyzed biotinylation of ACC, a biotin dependent enzyme. (B) The prototypical bisubstrate inhibitor 5′-*O*-[*N*-(biotinyl)sulfamoyl]adenosine is in equibibrium between the anticonformation (**5**) around the glycosidic linkage and the *syn*-conformation (**6**), which can react intramolecularly to liberate *N*-biotinylsulfamic acid **7** and cycloadenosine **8**.

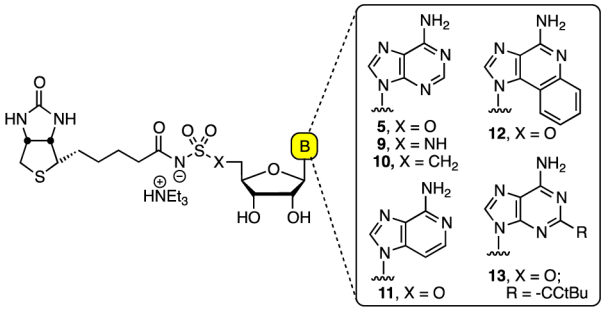


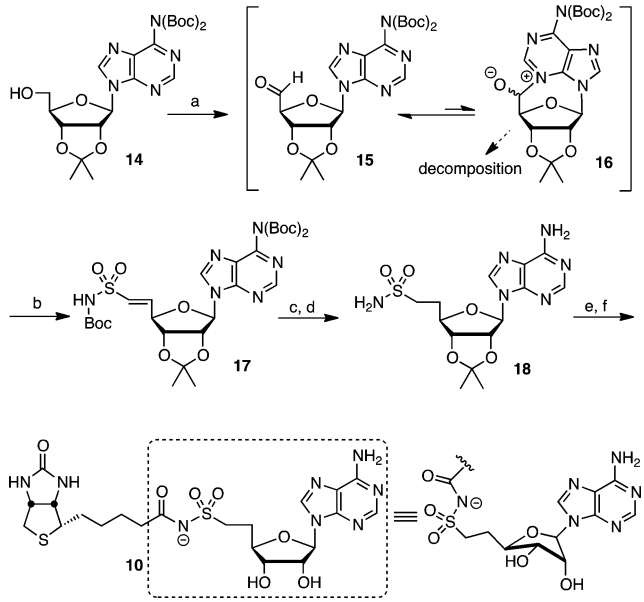
Figure 2. Structure of **5**, Bio-AMS **9**, and analogues **10**–**13**.

of adenine is replaced by a methine group thereby eliminating the internal nucleophile. We also conceived analogues **12** and **13** that prevent cyclonucleoside formation through the incorporation of bulky substituents on the adenine ring that hinder the required *syn*-conformation around the glycosidic linkage, necessary for proper alignment of N-3 with C-5′.

Synthesis of the noncleavable sulfonamide **10** was accomplished by Dess–Martin oxidation of protected adenosine **14**[13] to aldehyde **15** that is in equilibrium with hemiaminal **16** (Scheme 1). The N6-bis-Boc groups in **15** attenuate the nucleophilicity at N-3 suppressing hemiaminal formation and decomposition via depurination. After an aqueous workup the unstable crude aldehyde **15** was subjected to a modified Horner–Wittig olefination employing the dilithium salt of *N*-Boc-diphenylphosphorylmethanesulfonamide to afford exclusively the (*E*)-vinylsulfonamide **17**.[14,15] Catalytic hydrogenation of the alkene followed by chemoselective deprotection of the three Boc groups by treatment with trifluoroacetic acid (TFA) in dichloromethane furnished sulfonamide **18**. Biotinylation of **18** with (+)-biotin *N*-hydroxysuccinimide ester (biotin-NHS) mediated by Cs_2CO_3 and subsequent acetonide deprotection with 50% aqueous TFA afforded **10** (Scheme 1). Sulfonamide **10** exhibits excellent chemical stability with no degradation upon long-term storage as determined by HPLC analysis.

The 3-deaza analogue **11** was prepared from 5-amino-imidazole-5-carbonitrile riboside **19**[16,17] by an adaptation of the route described by Minnakawa and co-workers for the synthesis

Scheme 1. Synthesis of 10[a]



[a]Reagents and conditions: (a) Dess–Martin periodinane, CH_2Cl_2, 25 °C; (b) $Ph_2P(O)CH_2SO_2NHBoc$, LiHMDS, THF–DMF, −78 → 25 °C, 45% over 2 steps; (c) Pd/C, H_2, MeOH; (f) TFA, CH_2Cl_2, 0 °C, 87% over 2 steps; (e) biotin–NHS, Cs_2CO_3, DMF, 0 → 25 °C; (f) 50% aqueous TFA, 0 °C, 60% over 2 steps.

of 3-deazaadenosine (Scheme 2).[18−20] Sequential acetonide and TBS protection of **19** followed by Sandmeyer iodination provided **20**. The copper-free Sonogashira coupling of **20** with TMS-acetylene employing catalytic bis(benzonitrile)palladium chloride gave alkyne **21**. Treatment of **21** with 7 N methanolic ammonia at 110 °C in a sealed tube induced a formal aza-Bergmann cyclization to afford 3-deazaadenosine **22**. Removal of the TBS group from **22** was optimally accomplished under anhydrous conditions with KO*t*Bu in DMF.[21] Sulfamoylation[22] with NH_2SO_2Cl and NaH in DMF furnished **24**, which was biotinylated and deprotected using our optimized conditions to afford **11**.

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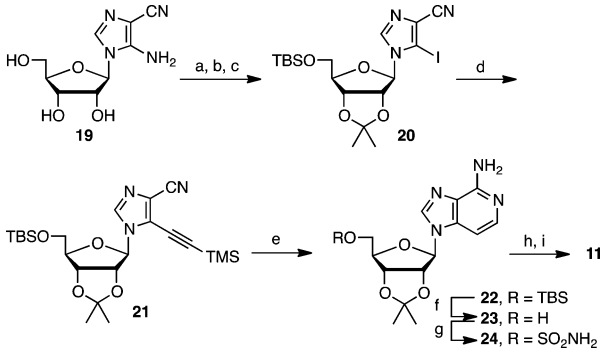
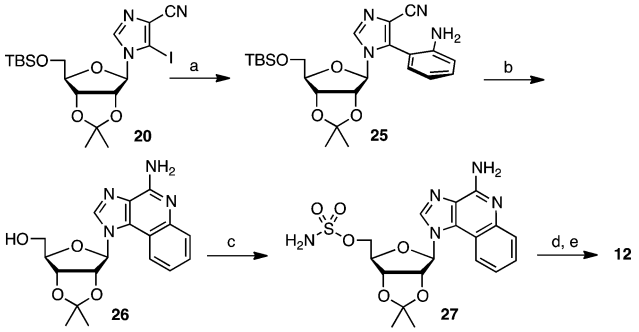
Scheme 2. Synthesis of 11[a]



[a]Reagents and conditions: (a) 2,2-dimethoxypropane, PTSA, acetone, 25 °C; (b) TBSCl, imidazole, DMF, 25 °C; (c) CH_2I_2, isoamylnitrite, $CHCl_3$, reflux, 55% over 3 steps; (d) $Pd(PhCN)_2Cl_2$, NEt_3, HC≡CTMS, CH_3CN, sealed tube, 100 °C, 70%; (e) 7N NH_3/MeOH, 110 °C sealed tube, 77%; (f) KO*t*Bu, DMF, 25 °C, 78%; (g) NH_2SO_2Cl, NaH, DME, 25 °C, 80%; (h) biotin−NHS, Cs_2CO_3, DMF, 0 → 25 °C; (i) 50% aqueous TFA, 0 °C, 65% over 2 steps.

The benzannulated analogue **12** was prepared from 5-iodoimidazole-4-carbonitrile riboside **20** in 5 steps as shown in Scheme 3. Initial Suzuki coupling of **20** with 2-amino-

Scheme 3. Synthesis of 12[a]

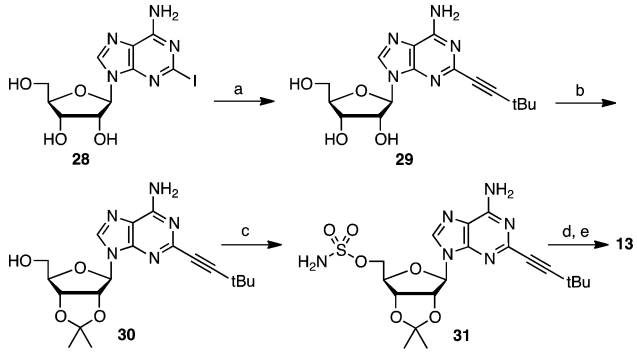


[a]Reagents and conditions: (a) $Pd(OAc)_2$, PPh_3, 2-aminophenylboronic acid hydrochloride, Na_2CO_3, DME−H_2O, 90 °C, 75%; (b) NaOMe, MeOH, reflux, 92%; (c) NH_2SO_2Cl, DMA, 88%. (d) biotin−NHS, Cs_2CO_3, DMF, 0 → 25 °C; (e) 50% aqueous TFA, 0 °C, 62% over 2 steps.

phenylboronic acid furnished heterobiaryl **25** as an approximately 1:2 mixture of atropisomers.[23] Cyclization, likely via an intermediate imidate,[24] and attendant desilylation to **26** was effected by refluxing **25** with NaOMe−MeOH. Sulfamoylation was performed under base-free conditions[25] with NH_2SO_2Cl in dimethylacetamide (DMA) to provide **27** in excellent yield. Biotinylation of **27** and subsequent TFA mediated deprotection of the acetonide gave **12**.

The 2-alkynyl analogue **13** was synthesized from 2-iodoadenosine **28**, prepared in 5 steps from guanosine (Scheme 4).[26] Sonogashira coupling was then performed to introduce the 3,3-dimethylbut-1-ynyl group directly onto unprotected **28** as attempts using the acetonide protected nucleoside were sluggish and low yielding.[26] The alkynylated intermediate **29** was converted to acetonide **30**, sulfamoylated to **31**, and biotinylated and deprotected using our optimized conditions to afford **13**.

Scheme 4. Synthesis of 13[a]



[a]Reagents and conditions: (a) $Pd(PPh_3)_2Cl_2$, CuI, NEt_3, 3,3-dimethyl-1-butyne, DMF, 88 °C, 77%; (b) 2,2-dimethoxypropane, PTSA, acetone, 25 °C, 85%; (c) NH_2SO_2Cl, Et_3N, DMF, 0 → 25 °C, 80%. (d) biotin−NHS, Cs_2CO_3, DMF, 0 → 25 °C; (e) 50% aqueous TFA, 0 °C, 65% over 2 steps.

As a result of the tight-binding nature of Bio-AMS **9** and analogues **10**−**13** and our inability to discriminate their potency by a coupled kinetic assay,[6] we turned to isothermal titration calorimetry (ITC) to evaluate the binding affinities of **9**−**13** to BirA (Table 1). ITC is an extremely useful technique to determine the dissociation constant (K_D) of ligand−protein interactions and additionally provides the thermodynamic binding parameters.[27] For ligands **9**−**12** that bound with subnanomolar affinity, we employed competitive displacement experiments with 250−500 μM biotin (K_D for BirA−biotin is 0.94 μM)[7] to determine ΔG, K_D, and n (the number of binding sites per monomer, see Supporting Information for details).[28] The binding enthalpy ΔH was obtained by stoichiometric titration of BirA in which the ratio of ligand/K_D was greater than 1000, and the entropy term $T\Delta S$ was then calculated from the Gibbs free energy equation. All compounds bind tightly with K_D values ranging from ≤0.04 to 11 nM and binding affinity is solely driven by a large favorable enthalpy. 3-Deaza analogue **11** is the most potent analogue with a K_D of 40 pM and an astonishing ΔH of −21.2 kcal/mol due to the large number of favorable electrostatic H-bond interactions between this bisubstrate inhibitor and both substrate binding pockets within the BirA active site.[6] The high potency demonstrates deletion of the polar N-3 atom is well tolerated. The increase in the enthalpy term relative to Bio-AMS **9** is likely due to a lower desolvation penalty. Replacement of the 5′-nitrogen atom of **9** with a CH_2 moiety in sulfonamide **10** results in a slight attenuation in potency providing a K_D of 0.10 nM. Benzannulated analogue **12** is remarkably potent with a K_D of 0.23 nM with the loss of enthalpy (relative to **9**) being compensating by a more favorable entropy term, presumably due to a greater entropy of desolvation. Alkyne analogue **13** is the least potent of the four compounds studied and displays a K_D of only 11 nM with an enthalpy term of −13.5 kcal/mol. This result is readily reconciled based on the cocrystal structure of **9**.[12] However, the findings that both analogues **12** and **13** retain such good binding affinities indicate that the enzyme is sufficiently flexible to accommodate these bulky substituents as has been observed previously for MbtA, a functionally related adenylating enzyme in *M. tuberculosis*.[29,30]

The antitubercular activity of **9**−**13** was next evaluated against *Mycobacterium tuberculosis* H37Rv (Table 1). The minimum concentration that inhibited greater than 90% of cell

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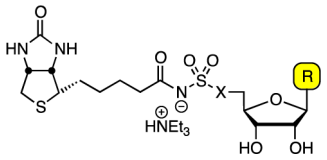
Table 1. Binding Affinities to BirA and Antitubercular Activity of Analogues 9−13[a,b]



Compound	X	R	K_D, nM[a]	ΔG, kcal/mol	ΔH, kcal/mol	$-T\Delta S$, kcal/mol	MIC, μM[b]
9[c]	NH		≤ 0.04	≤ -14.0	-17.5 ± 0.1	≤ 3.5	0.78
10	CH_2		0.10 ± 0.02	-13.4 ± 0.1	-18.0 ± 0.2	4.6 ± 0.2	3.12
11	O		0.04 ± 0.01	-14.0 ± 0.2	-21.2 ± 0.1	7.2 ± 0.2	1.56
12	O		0.23 ± 0.04	-12.9 ± 0.1	-15.6 ± 0.1	2.7 ± 0.1	>100
13	O	 R' = C≡CtBu	11.0 ± 1.0	-10.7 ± 0.1	-13.5 ± 0.1	2.8 ± 0.1	>100

[a]Competitive ITC experiments to determine K_D and ΔG were performed, and direct titration experiments were done in duplicate. All analogues showed *n* values of 1 ± 0.03. [b]Minimum inhibitory concentrations (MIC) that resulted in >90% growth inhibition of *M. tuberculosis* H37Rv were determined by a broth microdilution assay in GAS medium. Experiments were performed twice independently in triplicate. The MIC is defined as the lowest concentration of inhibitors that prevented growth, as determined by measuring the end point OD_{600} values (see Supporting Information for details). [c]The earlier reported[6] K_D of this compound is incorrect since competitive ITC was not performed, and thus, the potency was vastly underestimated. The previous ΔH value is also incorrect as the active enzyme concentration was not determined. In all experiments described above, the active enzyme concentration was determined by active site titration as described in the Supporting Information.

growth (MIC) was determined in defined glycerol-alanine salts (GAS) medium lacking biotin. Consistent with the ITC results, 3-deaza **11** possesses the most potent antitubercular activity with an MIC of 1.56 μM followed by sulfonamide **10** with a MIC of 3.12 μM. Analogues **12** and **13** that contain significant modifications to the nucleobase are totally inactive (MIC >100 μM) despite their excellent to modest biochemical potency. We hypothesize that one of the ATP-binding cassette (ABC) transporters present in *Mtb*, which are responsible for the assimilation of nucleosides and cofactors, facillitates the uptake of these polar nucleoside analogues.[31] The observation that analogues **12** and **13** are devoid of whole-cell activity could be due to their inability to be recognized by these ABC transporters.

In conclusion, we have designed and synthesized a focused series of Bio-AMS analogues that are resistant to cyclonucleoside formation. All analogues **10**−**13** showed remarkable chemical stability and demonstrated high affinities to BirA as measured by isothermal titration calorimetry. Analogues **10** and **11** also displayed potent activity against *M. tuberculosis* H37Rv, comparable to that of Bio-AMS **9**. The lack of antitubercular activity of **12** despite its potent enzyme affinity suggests modifications to the nucleobase may hinder cellular uptake and/or accumulation.

■ ASSOCIATED CONTENT

Supporting Information

All synthetic procedures, ITC experimental methods, and *Mtb* whole-cell assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ACC, acyl coenzyme A carboxylase; biotin-NHS, (+)-biotin *N*-hydroxysuccinimide ester; GAS, glycerol-alanine salts medium; ITC, isothermal titration calorimetry; MIC, minimum inhibitory concentration; MDR, multidrug resistant; *Mtb*, *Mycobacterium tuberculosis*; TB, tuberculosis; XDR, extensively drug resistant

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