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Design, synthesis, and biological evaluation of α -hydroxyacyl-AMS inhibitors of amino acid adenylation enzymes

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Abstract

Biosynthesis of bacterial natural-product virulence factors is emerging as a promising antibiotic target. Many such natural products are produced by nonribosomal peptide synthetases (NRPS) from amino acid precursors. To develop selective inhibitors of these pathways, we have previously described aminoacyl-AMS (sulfamoyladenine) macrocycles that inhibit NRPS amino acid adenylation domains but not mechanistically-related aminoacyl-tRNA synthetases. To improve the cell permeability of these inhibitors, we explore herein replacement of the α -amino group with an α -hydroxy group. In both macrocycles and corresponding linear congeners, this leads to decreased biochemical inhibition of the cysteine adenylation domain of the *Yersinia pestis* siderophore synthetase HMWP2, which we attribute to loss of an electrostatic interaction with a conserved active-site aspartate. However, inhibitory activity can be regained by installing a cognate β -thiol moiety in the linear series. This provides a path forward to develop selective, cell-penetrant inhibitors of the biosynthesis of virulence factors to probe their biological functions and potential as therapeutic targets.

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Supplementary Material

Experimental procedures and analytical data for all new compounds and experimental procedures for biochemical assays are provided. Supplementary data associated with this article can be found, in the online version, at doi: XXXX

Graphical abstract

Keywords

Antibiotic; Adenylation; Non-ribosomal peptide synthetase; Rational design; Virulence

Antibiotic resistance is a major threat to human health and new antibacterials with novel mechanisms of action are urgently needed.^{1–4} Natural products play key roles in bacterial virulence and communication,^{5–8} and targeting their biosyntheses has emerged as a promising antibiotic strategy.^{9,10} Antibacterials that block virulence factor production, as opposed to processes that are essential for bacterial survival, are also attractive in that this may reduce the propensity for the development of antibiotic resistance.^{11–14} This is based on the hypothesis that drug-resistant bacteria may suffer a relative fitness cost to produce communally beneficial virulence factors for use by their drug-sensitive neighbors. Indeed, proof-of-concept for this hypothesis has been provided in coculture experiments using mutant *Pseudomonas aeruginosa* strains that mimic drug-sensitive variants and wild-type strains that mimic drug-resistant variants.^{15,16}

Many natural-product virulence factors are biosynthesized by non-ribosomal peptide synthetases (NRPS).^{17–23} These include a variety of siderophores, which are iron-chelating molecules used by pathogenic bacteria to capture iron, an essential nutrient, from the host organism.^{10,24–27} NRPS pathways involved in siderophore biosynthesis have been genetically validated as promising antibacterial targets using mutant strains in animal infection models.^{28–43} Recently, we have also pharmacologically validated siderophore biosynthesis as an effective antibacterial target in a mouse model of *Mycobacterium tuberculosis* infection.^{44,45} By definition, all NRPS include at least one amino acid adenylation domain. Thus, to develop inhibitors of NRPS as probes and potential therapeutic leads, we have previously designed an aminoacyl-AMS (5'-O-[N-aminoacylsulfamoyl]-adenosine) macrocycle **5a** as a selective inhibitor of amino acid adenylation domains (Figure 1).⁴⁶ However, although this macrocycle was a potent biochemical inhibitor of the cysteine adenylation domain of HMWP2 (high molecular weight protein 2), a component of the *Yersinia pestis* yersiniabactin siderophore synthetase, it did not inhibit yersiniabactin production in *Y. pestis* cell culture (unpublished results). This is in contrast to our earlier demonstration that salicyl-AMS, which inhibits the upstream salicylate adenylation enzyme YbtE, also blocks yersiniabactin production in cell culture.⁴⁵ We postulate that this lack of cellular activity in the macrocycle is due to its zwitterionic character, which may prevent cell penetration. Indeed, we recently showed that alanyl-AMS (**4a**) is less cell-penetrant in a variety of bacteria compared to the corresponding α -hydroxy analogue, lactyl-AMS (**4b**).⁴⁷

Thus, toward the development of selective, cell-penetrant inhibitors of NRPS amino acid adenylation domains, we report herein the synthesis and evaluation of a series of linear (**4b,d**) and macrocyclic (**5b**) α -hydroxyacyl-AMS analogues as inhibitors of HMWP2. While this α -amino-to- α -hydroxy modification results in a significant decrease in biochemical potency, inhibitory activity can be restored by installation of a β -thio group that corresponds to the cognate cysteine side chain recognized by HMWP2. This provides a path forward to develop selective, cell-penetrant NRPS inhibitors to probe the biological functions of bacterial natural products and the therapeutic potential of targeting virulence factor biosynthesis.

Amino acid adenylation domains belong to a mechanistic superfamily of enzymes that catalyze a two-step process involving initial activation of a carboxylic acid substrate (**1**) to form a tightly-bound acyl adenylate (acyl-AMP) intermediate (**2**), followed by nucleophilic attack to yield a carboxylate derivative (**3**, Figure 1a).⁴⁸ We^{44–46,49–52} and others^{53–70} have used this mechanistic information to develop potent inhibitors of a variety of bacterial adenylation enzymes using an acyl-sulfamoyladenosine (acyl-AMS) scaffold,⁷¹ which acts as a nonhydrolyzable mimic of the tightly-bound acyl-AMP intermediate (Figure 1b).

Bacterial NRPS amino acid adenylation domains and aminoacyl-tRNA synthetases, which are ubiquitous in both pathogens and the host, catalyze mechanistically identical reactions (Figure 1a), but have distinct protein folds and active-site interactions. Thus, to develop selective NRPS inhibitors, we have previously exploited conformational differences between ligands observed in cocrystal structures.⁴⁶ In aminoacyl-tRNA synthetases, the aminoacyl-AMP intermediate **2** adopts an extended, ‘transoid’ conformation (Figure 2a),^{72,73} whereas in NRPS adenylation domains and related enzymes, this same intermediate is bound in a C-shaped, ‘cisoid’ conformation (Figure 2b).^{74–77} Thus, while linear aminoacyl-AMS inhibitors⁵⁴ such as alanyl-AMS (**4a**, Figure 2c) inhibit both enzyme classes, installation of a macrocyclic constraint linking the C8 position of adenine and the β -carbon of the amino acid in *cyclo*-alanyl-AMS (**5a**) (Figure 2d) enforces the cisoid pharmacophoric conformation that is specific to NRPS amino acid adenylation domains and affords highly selective inhibition.⁴⁶ Notably, the alanyl-AMS-based macrocycle **5a** lacks a β -position side chain and was designed as a potential broad-spectrum inhibitor of NRPS amino acid adenylation domains.

Although the *cyclo*-alanyl-AMS macrocycle (**5a**) was a potent and selective biochemical inhibitor of the cysteine adenylation domain of the *Y. pestis* yersiniabactin synthetase component HMWP2,⁴⁶ it did not inhibit yersiniabactin production in *Y. pestis* cell culture (unpublished results). We posited that this was due to the inability of this zwitterionic molecule to penetrate the bacterial cell membrane. The structural requirements for bacterial cell permeability remain enigmatic,^{78–81} and our laboratory recently carried out a series of prospective compound accumulation studies using acyl-AMS analogues with diverse structural and physicochemical properties, across various bacterial species.⁴⁷ This study revealed that alanyl-AMS (**4a**) did not penetrate any of the bacteria tested (*Escherichia coli*, *Bacillus subtilis*, *Mycobacterium smegmatis*) above the limits of detection, while the corresponding α -hydroxy congener, lactyl-AMS (**4b**, Figure 1b), penetrated all three strains at significantly higher levels (28–53 μ M intracellular concentration vs. 100 μ M extracellular

concentration applied). Thus, we envisioned that the corresponding macrocycle *cyclo*-lactyl-AMS (**5b**) might also be developed as a cell-penetrant inhibitor of NRPS amino acid adenylation domains. Notably, Abell and Aldrich have previously reported α -hydroxyacyl-AMS inhibitors of pantothenate synthetase (PanC), although no cellular activity of these compounds (up to 250 μ M) has been observed.^{63–65}

To investigate this hypothesis, we synthesized a series of acyl-AMS analogues to assess systematically the impacts of the α -amino-to- α -hydroxy modification, the amino acid side chain, and the macrocyclic constraint upon inhibition of the cysteine adenylation domain of HMWP2. Thus, alanyl-AMS (**4a**, Figure 1b), lactyl-AMS (**4b**), and cysteyl-AMS (**4c**) were synthesized as previously described.^{47,82} The corresponding α -hydroxy-cysteyl-AMS analogue **4d** was synthesized from (2*S*)-methyl glycidate (**6**) by epoxide opening with tritylthiol⁸³ and alcohol protection to form protected hydroxycysteine **7**, followed by conversion to the corresponding *N*-hydroxysuccinimidyl (NHS) ester **8** (Scheme 1). Coupling to 2',3'-acetonide-protected 5'-*O*-sulfamoyladosine (**9**),⁸² prepared as previously described,⁸⁴ and global deprotection with acid afforded α -hydroxy-cysteyl-AMS (**4d**).

The macrocycle *cyclo*-alanyl-AMS (**5a**) was also synthesized as previously described.⁴⁶ To access the α -hydroxy analogue, *cyclo*-lactyl-AMS (**5b**), we envisioned a late-stage macrocyclization of a functionalized AMS precursor **10**, which could be accessed by Sonogashira coupling of α -hydroxy-pentynoate **11** with 8-iodoadenosine **12** (Scheme 2a). Synthesis of the pentynoate fragment **11** started from (2*S*)-methyl glycidate (**6**), which underwent regioselective epoxide opening with diethyl[(trimethylsilyl)ethynyl]aluminum,⁸⁴ followed by alkyne desilylation and TBS protection of the secondary alcohol (Scheme 2b). The alkyne **11** then underwent efficient Sonogashira coupling to 8-iodoadenosine **12**, prepared as previously described,⁴⁶ to provide 8-alkynyladosine **13**. Sulfamoylation of the adenosine 5'-hydroxyl group and hydrogenation of the alkyne provided cyclization precursor **10**. Saponification of the ester, macrocyclization of the resulting acid, and global deprotection under acidic conditions afforded *cyclo*-lactyl-AMS (**5b**).

Inhibition of the cysteine adenylation activity of recombinant HMWP2 (residues 1–1491) was evaluated using a coupled, continuous, spectrophotometric 7-methylthioguanosine (MesG) assay recently developed for adenylation domains by Aldrich and coworker (Supplementary Figure 1).⁸⁶ In contrast to the classical ATP–³²PP_i isotope exchange assay that we used previously to assess HMWP2 inhibition,⁴⁶ the MesG assay does not require radioactive reagents and has been applied to several other adenylate-forming enzymes, including those in NRPS pathways (EntE, VibE, BasE, MbtA, GrsA, FadD28).^{86,87} The MesG assay uses hydroxylamine as an acyl acceptor nucleophile, allowing turnover of the forward adenylation reaction by facilitating release of the acyl-AMP intermediate (**2**) and pyrophosphate (Figure 1a). The pyrophosphate is then hydrolyzed by a pyrophosphatase to form phosphate, which is, in turn, used by a purine nucleoside phosphorylase to convert MesG into ribose-1-phosphate and 7-methylthioguanine, a UV-active product that can be monitored spectrophotometrically. Using this assay with 30 nM HMWP2, the cognate α -aminoacyl-AMS inhibitor cysteyl-AMS (**4c**) exhibited an IC₅₀ of 40 $\pm$ 10 nM (Figure 3). This recapitulates the tight-binding inhibitor behavior^{88,89} that we observed previously with

the ATP-³²PP_i isotope exchange assay,⁴⁶ thus setting the stage for the use of the MesG assay to characterize our other analogues.

Analysis of the remaining linear and macrocyclic analogues revealed two important trends. First, replacement of the α -amino group with an α -hydroxy group led to decreased HMWP2 inhibition by 2–3 orders of magnitude (**4a** vs. **4b**, **4c** vs. **4d**, **5a** vs. **5b**). Second, inclusion of the cognate β -thio group corresponding to the cysteine sidechain recognized by HMWP2 resulted in increased HMWP2 inhibition by $\approx$ 2 orders of magnitude (**4a** vs. **4c**, **4b** vs. **4d**). The decreased inhibitory activity upon replacement of the α -amino group with an α -hydroxy group in both the linear analogues and the corresponding macrocycles can be rationalized by examination of the cocrystal structure of phenylalanine and AMP (presumably hydrolysis products of the cognate phenylalanyl-AMP reaction intermediate) bound to the phenylalanine adenylation domain of *Bacillus brevis* gramicidin S synthetase 1 (PDB: 1AMU) (Figure 4).⁷⁴ In the active site, the α -amino group of the amino acid is engaged in an electrostatic interaction with Asp-235. This aspartate is completely conserved in other amino acid adenylation domains, including the cysteine adenylation domain of HMWP2 (Asp-757).^{46,90} On the other hand, inclusion of the β -thiol side chain that is specifically recognized by the cysteine adenylation domain of HMWP2 increases potency in the linear series, presumably by filling the corresponding specificity pocket in the enzyme.^{46,90}

To assess the effects of the α -amino-to- α -hydroxy modification on selective inhibition of HMWP2 over aminoacyl-tRNA synthetases, we also evaluated the inhibitory activity of these analogues in a rabbit reticulocyte lysate *in vitro* translation assay, using translation of luciferase as a readout (Fig. 3).⁴⁶ Notably, in the linear series, the α -hydroxyacyl-AMS analogues were much weaker inhibitors than the corresponding α -aminoacyl-AMS analogues, by 2–3 orders of magnitude (**4a** vs. **4b**, **4c** vs. **4d**). This is consistent with engagement of the α -amino group by multiple hydrogen bonds in aminoacyl-tRNA synthetase cocrystal structures.^{72,91–94} In contrast, both of the macrocycles, *cyclo*-alanyl-AMS (**5a**) and *cyclo*-lactyl-AMS (**5b**), were inactive at the highest concentration tested (250 μ M), indicative of the high specificity of the macrocyclic design.

In conclusion, the advancement of selective adenylation enzyme inhibitors as novel antibacterial agents relies on their ability to permeate bacterial cell membranes to access these intracellular targets. To this end, it is essential to identify rational modifications to biochemically potent inhibitors that confer permeability while maintaining activity.⁷⁹ We have previously shown that lactyl-AMS (**4b**) penetrates Gram-positive *B. subtilis*, Gram-negative *E. coli*, and *M. smegmatis*.⁴⁷ Unfortunately, this α -hydroxy modification also leads to a >2 – 3 order-of-magnitude decrease in inhibitory potency against the cysteine adenylation domain of HMWP2, when compared to the corresponding α -amino derivatives. While some loss in binding affinity is acceptable if it provides cell permeability, further studies will be required to develop inhibitors that retain sufficient binding affinity to exhibit cellular activity. In the context of the cysteine adenylation domain of HMWP2, inclusion of the β -thiol side chain in cysteyl-AMS (**4c**) and α -hydroxycysteyl-AMS (**4d**) increases inhibitory potency by $\approx$ 30–60-fold compared to the corresponding alanine-based inhibitors alanyl-

AMS (**4a**) and lactyl-AMS (**4b**). This brings the IC₅₀ of α -hydroxycysteyl-AMS (**4d**) to within 15-fold of alanyl-AMS (**4a**).

Thus, in the macrocycle series, it may similarly be possible to recapture much of the binding affinity lost due to the α -amino-to- α -hydroxy modification by installing this β -thiol side chain (*e.g.*, *cyclo*-hydroxycysteyl-AMS). This will require development of an efficient, diastereoselective synthetic route to access these significantly more challenging molecules, and determination of the appropriate stereochemical configuration at the β -substituent, which is unclear from inspection of the GrsA phenylalanine adenylation domain structure.⁷⁴ Other amino acid adenylation domains could then similarly be targeted specifically by incorporating the corresponding β -substituent. If these α -hydroxyacyl-AMS macrocycles also exhibit improved bacterial cell penetration, by analogy to the trends observed in the linear series,⁴⁷ then this would provide potent, selective, and cell-penetrant inhibitors of NRPS amino acid adenylation domains. Such inhibitors would then be powerful probes to study the biological functions of the NRPS-derived natural products in bacterial processes, including virulence. They could also be used to evaluate the therapeutic potential of targeting natural-product virulence factor biosynthesis, and in particular could be used to assess pharmacologically the propensity for bacteria to develop resistance to such antivirulence factor drugs. Efforts to synthesize and evaluate such fully elaborated inhibitors are underway and will be reported in due course.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Amino acid adenylation reaction and inhibitors

(a) Both NRPS amino acid adenylation domains and aminoacyl-tRNA synthetases use a two-step mechanism to activate and couple amino acid substrates via a tightly-bound aminoacyl-AMP intermediate (**2**). (b) Structures of linear (**4**) and macrocyclic (**5**) amino acid adenylation domain inhibitors designed to target the cysteine adenylation domain of *Y. pestis* HMWP2. AMS = adenosine 5'-O-monosulfamate; PCP = peptidyl carrier protein with phosphopantetheine thiol nucleophile; tRNA = transfer RNA with 2'- or 3'-hydroxyl nucleophile.

Figure 2. Targeting the unique cisoid binding pharmacophore of NRPS amino acid adenylation domains

(a) Extended conformation of Phe-AMP bound to Phe-tRNA synthetase from *Thermus thermophilus* (PDB ID: 1B7Y).⁷³ (b) Cisoid conformation of Phe and AMP bound to the Phe adenylation domain of *Bacillus brevis* gramicidin S synthetase 1 (PDB ID: 1AMU),⁷⁴ with a 4.1 Å unobstructed space between C8 of the adenine ring and the β -carbon of the amino acid. (c) Structure and non-selective inhibitory activity of linear alanyl-AMS (**4a**) against both the Cys adenylation domain of *Y. pestis* HMWP2 and aminoacyl-tRNA synthetases (IVT). (d) Structure and selective inhibitory activity of macrocyclic *cyclo*-alanyl-AMS (**5a**) against the Cys adenylation domain of HMWP2. HMWP2 = high molecular-weight protein 2 from *Y. pestis* yersiniabactin synthetase. IVT = *in vitro* translation assay with rabbit reticulocyte lysates and luciferase readout.

amino acid	X	Y		HMWP2 ^a IC ₅₀ (μM)	IVT IC ₅₀ (μM)		HMWP2 ^a IC ₅₀ (μM)	IVT IC ₅₀ (μM)
Ala	NH ₃ ⁺	H	4a	1.15 ± 0.17	0.118 ± 0.002	5a	0.34 ± 0.05	>250
Lac	OH	H	4b	>1,000	166.4 ± 24.7	5b	>1,000	>250
Cys	NH ₃ ⁺	SH	4c	0.04 ± 0.01	3.27 ± 0.13			
HO-Cys	OH	SH	4d	16.53 ± 0.50	221.5 ± 10.8			

Figure 3. Structure–activity relationships at α- and β-positions of acyl-AMS inhibitors
 HMWP2 assay conditions: 30 nM HMWP2^(1–1491), 0.04 U inorganic pyrophosphatase, 0.1 U purine nucleoside phosphorylase, 300 mM NH₂OH, 0.2 mM MesG, 5 mM cysteine, 2.5 mM ATP, 5 mM MgCl₂, 1.5 mM DTT, 50 mM Tris pH 8.0. The *in vitro* translation (IVT) assay was performed using the Promega TNT T7 Coupled Reticulocyte Lysate System with the luciferase T7 control DNA and luciferin reagent. Values shown for average of three independent experiments, with standard error of the mean. See Supplementary Data for complete details. ^a K_i^{app} (ATP-³²PP_i isotope exchange assay⁴⁶): **4a** = 2.5 ± 0.2 μM, **4c** = 0.24 ± 0.02 μM, **5a** = 1.7 ± 0.1 μM.

Figure 4. Electrostatic interaction of α -amino group with conserved active site aspartate
Structure of phenylalanine (Phe) and AMP, the hydrolysis products of the Phe-AMP reaction intermediate, bound in the active site of the *B. brevis* gramicidin S synthetase 1 phenylalanine adenylation domain (PDB: 1AMU),⁷⁴ with key interaction with Asp-235.

4d (HO-Cys-AMS)

Scheme 1. Synthesis of linear α-hydroxycysteyl-AMS analogue 4d

Reagents and conditions: a) TrtSH (1.5 equiv), Et₃N (1.0 equiv), MeOH, 65 °C, 24 h, 69%; b) TBSCl (4.3 equiv), imidazole (3.5 equiv), CH₂Cl₂, 0 → 23 °C, 42 h, 100%; c) LiOH (0.4 M aq, 5.0 equiv), THF, 0 → 23 °C, 24 h, then aq HCl (1.0 N) to pH 4, 0 °C, 95%; d) NHS (1.0 equiv), DCC (1.1 equiv), THF, 23 °C, 20.5 h, 76%; e) **8** (1.5 equiv), **9** (1.0 equiv), DBU (1.5 equiv), CH₃CN, 23 °C, 2 h, 81%; f) 5:1 TFA/H₂O, 0 → 23 °C, 3 h, then MeOH, 0 °C, 79%. DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene; DCC = *N,N'*-dicyclohexylcarbodiimide; NHS = *N*-hydroxysuccinimide; TBS = *t*-butyldimethylsilyl, TFA = trifluoroacetic acid; THF = tetrahydrofuran; Trt = trityl.

Scheme 2. Retrosynthetic analysis (a) and synthesis (b) of macrocyclic *cyclo*-lactyl-AMS analogue **5b**

Reagents and conditions: a) ethynyltrimethylsilane (1.5 equiv), *n*-BuLi (1.7 M in hexanes, 1.5 equiv), toluene, 0 °C, 15 min, then Et₂AlCl (1.5 equiv), 0 °C, 45 min, then **6**, 0 $\rightarrow$ 23 °C, 90 min, 94%; b) TBAF (1.0 M in THF, 1.0 equiv), THF, 23 °C, 2 h, 92%; c) TBSCl (4.3 equiv), imidazole (3.5 equiv), CH₂Cl₂, 0 $\rightarrow$ 23 °C, 42 h, 86%; d) **11** (1.5 equiv), **12** (1.0 equiv), *i*-PrNEt₂ (2.0 equiv), CuI (0.70 equiv), Pd(PPh₃)₄ (0.35 equiv), DMF, 23 °C, 23 h, 94%; e) sulfamoyl chloride (5.6 equiv), DMA, 0 °C, 1 h, 76%; f) 30% Pd/C (2.5 equiv),

H₂ (balloon), MeOH, 23 °C, 6 h, 100%; g) LiOH (0.4 M aq, 1.5 equiv), THF, MeOH, 0 → 23 °C, 15 h; h) PyBOP (1.8 equiv), HOBt (2.8 equiv), DBU (1.5 equiv), CH₃CN, 23 °C, 4.5 h, 48% over 2 steps; i) 5:1 TFA/H₂O, 0 → 23 °C, 3 h, then MeOH, 0 °C, 37%. DMA = *N,N*-dimethylacetamide; DMF = *N,N*-dimethylformamide; HOBt = hydroxybenzotriazole; Lac = lactyl; PyBOP = benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate; TBAF = tetrabutylammonium fluoride.