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Nanomolar inhibition of the enterobactin biosynthesis enzyme, EntE: Synthesis, substituent effects, and additivity

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Abstract—2,3-Dihydroxybenzohydroxamoyl adenylate (**I**) was prepared as a potential product analog inhibitor of EntE (EC# 2.7.7.58), a 2,3-dihydroxybenzoate AMP ligase from *Escherichia coli* that is required for the biosynthesis of enterobactin. This compound, obtained by the aqueous reaction of imidazole-activated adenosine 5′-phosphate and 2,3-dihydroxybenzohydroxamic acid, is a competitive inhibitor with a K_i value of 4.5×10^{-9} M. Deletion of the catecholic 3-OH group of (**I**), in compound (**II**), reduced inhibitory activity by a factor of 3.5, whereas, removal of both the 3-OH and 2-OH groups, in (**III**), reduced inhibitory activity by a factor of ~2000. Acetohydroxamoyl adenylate (**IV**), in which the entire catechol moiety of (**I**) is replaced by a hydrogen atom, gave $\leqslant$10% inhibition at 6×10^{-4} M, indicating a reduction in affinity by more than 10^5. The binding free energy of (**I**) is nearly equivalent to the sum of the corresponding values for adenosine 5′-phosphate and 2,3-dihydroxybenzoate.

Siderophores are low molecular weight chelating agents secreted by certain strains of infectious bacteria, including *Mycobacterium tuberculosis*[1] and *Yersinia pestis*[2] (cause of plague), that require iron for virulence. Their equilibrium constants (K_a) for association with free Fe^{III} are sufficiently high as to render chelation essentially irreversible,[3] and liberation of bound Fe^{III} for cellular use requires pathogen hydrolases that dismantle the ferric siderophore itself.[4] Because no equivalent systems seem to be present in mammals, the enzymes involved in siderophore biosynthesis[5] represent logical targets for the development of bactericidal agents.[6–10]

Here, we describe a new class of aryl adenylate analogs with potent inhibitory activity against EntE, an enzyme from *Escherichia coli* that catalyzes the formation of 3 equivalents of 2,3-dihydroxybenzoyl adenylate (DHB–AMP) during the early stages of enterobactin biosynthesis.[11] Using the same number of serine residues, downstream enzymes tether the DHB moieties into a ring, allowing each of the six catecholic hydroxyl groups to coordinate Fe^{III} (Scheme 1). Recent evidence suggests that production of this siderophore by *E. coli* may be correlated with virulence in urinary tract infections,[12] and similar associations have been found for catecholate-type siderophores produced by the pathogens, *Brucella abortus* and *Vibrio anguillarum*, which require the action of EntE homologues (Table 1).

Like many adenylate forming enzymes, EntE binds its product with high affinity, perhaps to protect DHB–AMP from being exposed to attack by adventitious nucleophiles in the cell and ensure that the labile acyl group is available to holo EntB for the next enzymatic transformation. By measuring the catalytic turnover of pyrophosphate released from EntE, a rate constant of $0.4\ min^{-1}$ has been determined for dissociation of the EntE–DHB–AMP complex,[11] much lower than dissociation rate constants of typical E–P complexes.[13]

We attempted to exploit the high affinity of the product binding site by designing a stable mimic of DHB–AMP as a potential inhibitor. This analog contains an *N*-acyl hydroxamoyl phosphate group in place of the labile carboxylic–phosphoric anhydride linkage, a modification that increases the distance between the AMP and aryl moieties by 2 Å and renders the molecule unreactive. The choice of a phosphoryl group, rather than an

Abbreviations: DHB, 2,3-dihydroxybenzoic acid; DHB–AMP, 2,3-dihydroxybenzoyl adenylate; AMP, adenosine 5′-monophosphate; Me-DHB, methyl 2,3-dihydroxybenzoic acid; DHB-Hx, 2,3-dihydroxybenzohydroxamic acid.

Keywords: Adenylate analog; Inhibitor; Additivity; EntE; Enterobactin; Siderophore; Adenylate-forming; Bisubstrate; Iron; Inhibition; Binding energy.

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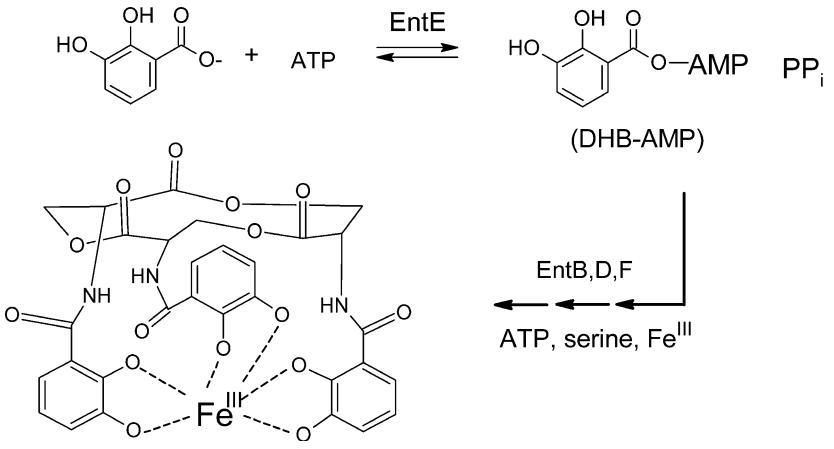

Scheme 1.

Table 1. DHB–AMP forming enzymes in microbial siderophore biosynthesis

EntE homologue	Catecholate siderophore	Microbe
DhbE[a]	Bacillibactin	*Bacillus subtilis*
AngE[b]	Anguibactin	*Vibrio anguillarum* (fish pathogen)
VibE[c]	Vibriobactin	*Vibrio cholerae* (cause of cholera)
DhbE[d]	Brucebactin	*Brucella abortus* (ruminant pathogen)
AgbE[e]	Agrobactin	*Agrobacterium tumefaciens* (plant pathogen)

[a] May, J. J.; Wendrich, T. M.; Marahiel, M. A. *J. Biol. Chem.* **2001**, 276, 7209.
[b] Lorenzo, M. D.; Stork, M.; Tolmasky, M. E.; Actis, L. A.; Farrell, D.; Welch, T. J.; Crosa, L. M.; Wertheimer, A. M.; Chen, Q.; Salinas, P.; Waldbeser, L.; Crosa, J. H. *J. Bacteriol.* **2003**, 185, 5822.
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[d] Bellaire, B. H.; Elzer, P. H.; Hagius, S.; Walker, J.; Baldwin, C. L.; Roop, R. M. 2nd. *Infect. Immun.* **2003**, 71, 1794.
[e] Sonoda, H.; Suzuki, K.; Yoshida, K. *Genes Genet. Syst*. **2002**, 77, 137.

uncharged surrogate, was based on the X-ray structure of the homologous enzyme from *Bacillus subtilis*, DhbE (47% identical based on its amino acid sequence), which shows a potentially favorable interaction between the side chain of a conserved active site histidine residue (H234) and the 5′-phosphoryl group of bound DHB–AMP.[14]

The preparation of hydroxamate-linked adenylate analogs was conceptually similar to the syntheses of sulfamoyl-[7] or tetrazolide-[9]linked analogs, but did not require catalysts, protecting groups, or anhydrous solvent conditions. Compound **I** was obtained by combining imidazole-activated adenosine 5′-phosphate (AMP-Im)[15] (0.2 mmol) with 2,3-dihydroxybenzohydroxamic acid (DHB-Hx) (0.4 mmol) in 6 ml H_2O at 25 °C, pH 7.0. After 1 h, ^{31}P NMR[16] showed 76% conversion to **I**. The solution was passed over a Q-cellulose column (1.25 × 35 ml) at a flow rate of 1.1 ml/min of potassium phosphate buffer (0.025 M, pH 6.2) at 4 °C, with the results shown in Figure S1. Fractions containing **I** were pooled, concentrated in vacuo, and desalted using a Sephadex G-10 column (38 × 1 cm). Results of high resolution ESI/MS and ^{1}H NMR were consistent with the assigned structure.[17] Stock solutions were stored at −80 °C.

Compounds **II**–**IV** were prepared in a similar manner except that the pH of the reaction was adjusted to a value roughly midway between the pK_a values of the imidazolium group of AMP-Im[18] and the respective hydroxamic acid.[19] For **III** and **IV**, reaction times were extended to 24 h and the temperature lowered to 4 °C. Yields for each of these analogs were >50%.[20]

Competitive inhibition experiments indicate that compound **I** was bound by EntE with a $K_{i(app)}$ value of 9 nM. That affinity is 10^2 more favorable than that of DHB (K_m, 2.7 μM) and 10^5 more favorable than that of ATP (K_m, 1120 μM),[11] suggesting that **I** may interact with both substrate binding sites at once.

Binding of compound **I** is competitive with respect to substrate DHB. Figure S3 shows that increasing the concentration of **I** results in an equivalent increase in the apparent K_m value of DHB. The K_i value of **I**, corrected for substrate competition, is 4.5 nM. The strength of this interaction appears to be substantially greater than that reported for an adenylate analog inhibitor of DhbE in which a sulfamoyl group replaces the native phosphoryl group of DHB–AMP (K_i, 85 nM, 37 °C).[10] A second sulfamoyl-linked analog has been reported to inhibit the salicyl activating enzyme, YbtE, from *M. tuberculosis*, but binding was noncompetitive with respect to salicylate.[7] Although trivial explanations for different inhibitor modalities are possible, a likely explanation is that, despite their sequence similarity (42% identical based on amino acid sequence), EntE and YbtE may follow different kinetic mechanisms (random vs ordered, respectively).

To investigate the basis for the strong interaction between EntE and **I**, compounds **II**–**IV** were prepared and tested as potential inhibitors, with the results summarized in Table 2. Deletion of the catecholic 3-OH group of **I**, in compound **II**, resulted in only a minor (3.5-fold) reduction in the observed inhibitory activity. For compound **III**, in which both hydroxyl groups are removed, enzyme affinity was reduced by a factor of 2000. This result is in reasonable agreement with the substrate specificity of EntE, described by Rusnak et al.[11] Thus, 2-hydroxybenzoate is activated by EntE 10-fold less efficiently (k_{cat}/K_m) than DHB, whereas 3-hydroxybenzoate and benzoate are not acted upon by EntE to any measurable extent. Together, these results indicate the importance of binding interactions between EntE and the catecholic 2-OH group. The superior inhibition displayed by **III**, compared with **IV** ($\leqslant$10% inhibition at 5.9×10^{-4} M), suggests that noncovalent forces of attraction are also at work between the unsubstituted benzene ring and the EntE active site (Table 2).

If compound **I** is represented as A–B, and its components, AMP and DHB, are represented as A and B, it would be of interest to know whether the free energy of binding of A–B is equivalent to the sum of the free energies of binding of A and B individually:

$$\Delta G^{A-B}_{bind} = \Delta G^{A}_{bind} + \Delta G^{B}_{bind}$$

Such a comparison, evaluating the additivity of inhibitor binding interactions, might be expected to be sensitive to differences in entropy, strain, or solvation, associated with binding of A–B relative to A or B.[21] As shown in Figure 1, however, the binding of compounds **I** and **II** appears to be purely additive.[22] In future experiments, it would be of interest to explore the possibility that adjustments in the length and flexibility of the linker between the aryl and AMP moieties of compound **I** might disturb that relationship, and permit its two fragments to be bound synergistically.

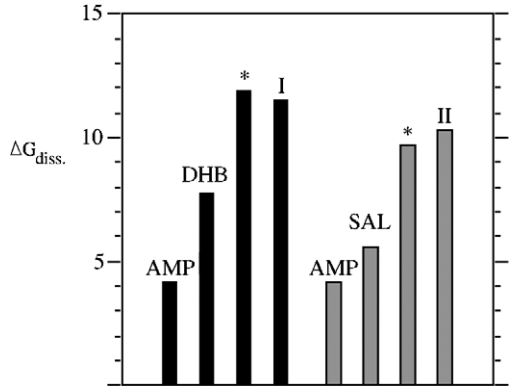
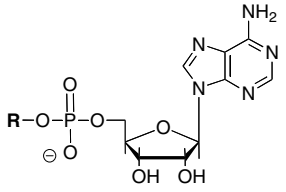
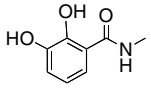
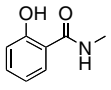
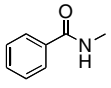
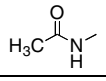


Figure 1. Estimated free energy of dissociation of EntE with substrates and inhibitors [$\Delta G_{diss} = -RT\ln(K_{diss})$]. Asterisks indicate the algebraic sum of the free energies of dissociation of the substrate, using K_m values reported by Rusnak et al., and AMP using the $K_{i(app)}$ value of 9×10^{-4} M determined in this work. SAL: 2-hydroxybenzoate.

Table 2. $K_{i(app)}$ values with EntE (uncorrected for substrate competition)



Inhibitor	R	$K_{i(app)}$ (M)
I		9×10^{-9}
II		3.7×10^{-8}
III		1.3×10^{-5}
IV		$\geqslant 1 \times 10^{-3}$

Compound **I** is a powerful inhibitor of EntE, suggesting that the *N*-acyl hydroxamoyl phosphate group effectively mimics the acyl phosphate group present in DHB–AMP. Inhibitor synthesis is carried out in water, gives high yields, and uses reactants that are storable and in some cases commercially available. Comparisons with the inhibitory activity of derivatives **II** and **III** indicate that the catecholic 2-OH group is critical for tight binding. Tight binding of the EntE–**I** complex also appears to be a consequence of the near additivity of the free energies of binding of its AMP and DHB moieties.

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Supplementary data

Experimental details for all the syntheses and the inhibition results are available in Supplementary data. Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bmcl.2006.04.024.

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17. ^{1}H NMR (D_2O) δ 8.23 (s, 1H), 8.06 (s, 1H), 6.79 (d, 1H, J = 8 Hz), 6.72 (d, 1H, J = 8 Hz), 6.48 (t, 1H, J = 8 Hz), 5.96 (d, 1H, J = 5.5 Hz), 4.65 (t, 1H), 4.49 (m, 1H), 4.38 (ddd, 1H, J = 11, 5, 2 Hz), 4.33 (m, 1H), 4.14 (dt, 1H, J = 11, 3.7 Hz).
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