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ARTICLE

Exploring the substrate promiscuity of an antibiotic inactivating enzyme

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Peptide-nucleotide conjugates have been extensively studied as scaffolds for the development of new antibiotics. However, in vivo, the efficacy of such compounds is limited by various detoxicants, such as the aminoacyl-nucleotide hydrolase MccF. MccF cleaves the amide bond between the amino acid and phosphoramine-adenylate of the aspartyl tRNA synthetase inhibitor microcin C7, providing self-immunity to the producing strains. However, MccF orthologs are also found in strains that do not produce microcin C7, suggesting a broader role in detoxification. Here, we demonstrate that MccF has no specificity for the nucleotide moiety of the antibiotic and can accept as substrates amino acids linked to any purine nucleobase. Biochemical characterization of synthetic substrate analogs, and co-crystal structure of these compounds with MccF provide a rationale for understanding this promiscuity. These findings have implications for the design of antibiotics that can avert MccF-mediated inactivation, and for understanding the function of homologs that may play roles in the metabolism of other cellular intermediates.

Introduction

Biochemical condensation of carboxyl moieties often require the activation of the carboxylate by adenylation or phosphorylation, followed by nucleophilic attack at the carbon of the anhydride with the concomitant departure of either the adenosine monophosphate (AMP) or inorganic phosphate (P_i). Phosphoadenylated carboxylic acids are ubiquitous intermediates in primary and secondary metabolic pathways, including carbohydrate metabolism, fatty acid, and protein synthesis.[1] Consequently, molecules that mimic activated carboxylic adenylates mimics with a phosphoramide or sulfamoyl linkage in place of the phosphoanhydride are inhibitors of the corresponding condensation reactions as they do not allow the second half reaction to proceed. This inhibition strategy has been utilized in the elaboration of several bioactive natural products, including the phosphoramidate-containing microcin C7,[2-3] produced by *E. coli*, as well as other strains (McC7) (**1**) (Figure 1A) and agrocin 84, which utilizes a tRNA-dependent mode of inhibition,[4-5] as well as in synthetic sulfamate compounds that inhibit non-ribosomal peptide synthetases (NRPSs),[6] cell wall biosynthesis[7] and siderophores biosynthesis.[8]

Within the target cell, the amino-terminal hexapeptide of McC7 is removed by non-specific host proteases to yield the bioactive processed McC7. Processed McC7 (**2**) mimics an aspartyl adenylate intermediate (**3**) and inhibits the charging of $tRNA^{Asp}$ by aspartyl tRNA synthetase (AspRS),[9] thus stalling protein synthesis. McC7 processing involves the proteolysis of six residues that are amino-terminal to the aspartyl adenylate. Premature processing of McC7 in the producing *Escherichia coli* strain could result in auto-toxicity, and thus necessitates one or more self-immunity mechanism(s). In addition to acetylation of **2**,[10] One immunity determinant deploys a serine carboxypeptidase, MccF,[11-12] which can hydrolyze the peptide bond that joins the amino acid and the nucleotide moieties of **2**.[13] This hydrolytic action of the enzyme generates the free amino acid aspartate, and the aminophosphorylated nucleoside, neither can bind to, nor inhibit AspRS. We have previously shown that MccF utilizes a Ser108-His311-Glu243 catalytic triad, and that substrate specificity is restricted to acidic amino acid adenylates. Although the aspartyl and glutamyl sulfamoyl adenylates (DSA (**4**) and ESA (**5**) respectively) were the only substrates accepted by the wild-type enzyme, rational engineering of the specificity determinants resulted in an enzyme with an expanded substrate scope, which accommodated and hydrolyzed adenylates with aliphatic substituents like phenylalanine sulfamoyl adenylate (FSA).[12]

Crystal structures of an inactive mutant of *E. coli* MccF in the presence of processed **2**, **4,** and **5** revealed a conserved coordination of the adenine ring by π-stacking against the side chain indole ring of Trp186. The tryptophan could also be substituted by a phenylalanine, albeit with a reduction in enzyme activity. Mutation of the tryptophan to an alanine led to a complete loss of enzymatic activity, establishing that π-stacking interactions between the indole ring of Trp186 and the adenine ring of the substrate are essential for catalysis. A subsequent crystal structure of *Bacillus anthracis* MccF complexed with AMP also revealed a similar π-stacking interaction of the adenine ring with tryptophan and phenylalanine side chains.[14] However it was not immediately clear how the favorable van der Waals interaction would offset the electrostatic repulsion between the p-orbital electrons. To explore the substrate promiscuity of MccF, particularly related

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to the nucleoside moiety, we now report the synthesis and MccF structural and activity analysis for glutamyl sulfamoyl inosine (**6**) and glutamyl sulfamoyl guanosine (**7**). As glutamyl conjugates exactly mimic the aspartyl conjugates in interactions with the nucleobase within MccF, but offer a continuous kinetic characterization of the MccF activity,[12] we chose to synthesize glutamyl conjugates as substrates in this study.

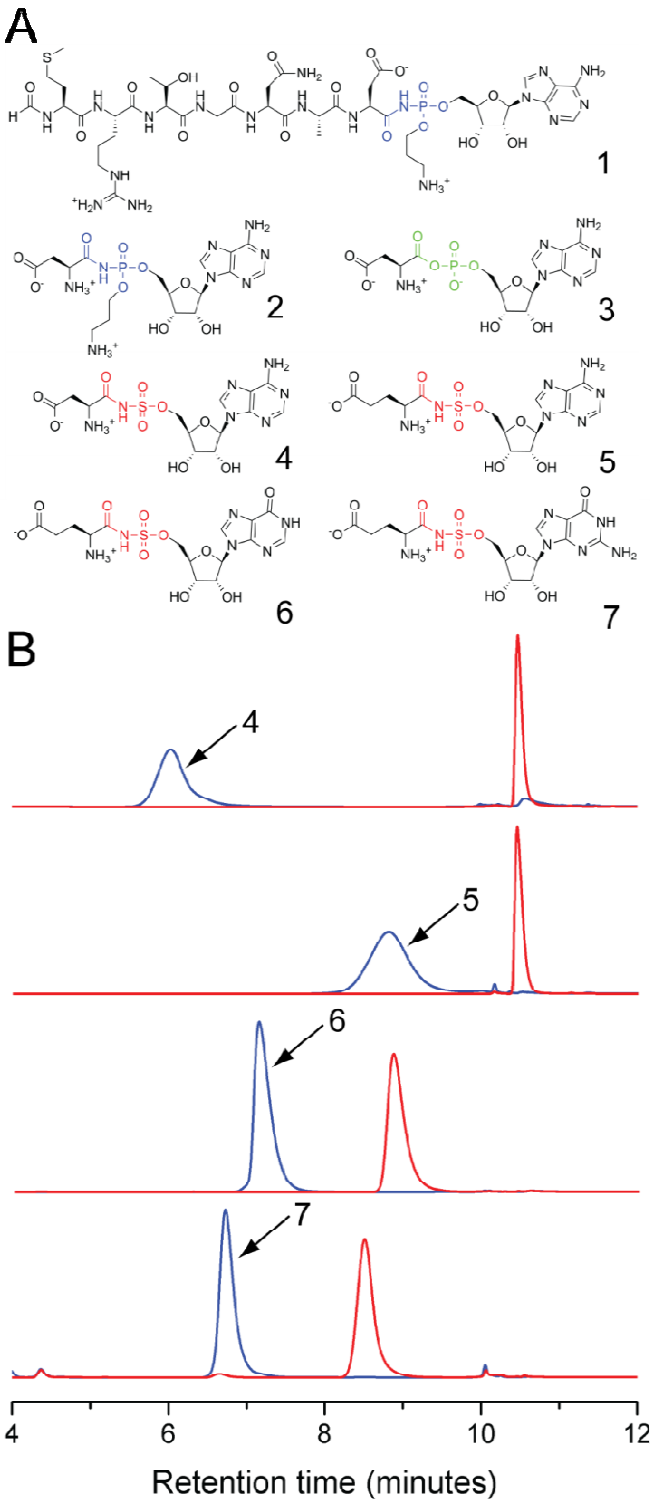

Figure 1. Chemical structures and hydrolysis of McC7 analogs by wild-type MccF enzyme. **(A)** Trojan horse antibiotic McC7 (**1**) and its processed form (**2**) which mimic the aspartate adenylate intermediate (**3**). Synthetic analogs of **2**- **4**–**7**. The phosphoramide linkage is shown in blue, phosphate ester in green and sulfamide in red. **(B)** Complete hydrolysis of **4**–**7** by wild-type MccF as determined by the reverse phase HPLC separation of substrates (blue curves) and the products- sulfamoylated nucleosides (red curves).

Results and discussion

Wild-type *E. coli* MccF could indeed accept the synthetic molecules **6** and **7** as substrates and catalyze their complete hydrolysis (Figure 1B and Figure S9-S10). Kinetic parameters for the hydrolysis of **6** and **7** were determined. Both **6** and **7** were accepted as MccF substrates at levels comparable to that of **5,** which is an analog of the physiological substrate (Table 1). Notable differences were only observed in the K_M values, with a slightly lower K_M value for **7** than for both **5** and **6**.

Table1. Kinetic parameters for the hydrolysis of **5–7** by wild-type MccF enzyme.

Substrate	k_{cat} (min^{-1})	K_M (μM)	*(k_{cat}/K_M)
ESA (**5**)	35.0 ± 4.1	51.6 ± 4.9	1
ESI (**6**)	38.1 ± 4.2	90.9 ± 10.5	0.6
ESG (**7**)	34.9 ± 3.1	33.4 ± 2.2	1.5

*(k_{cat}/K_M) value relative to **5**

In order to determine how alterations in the nucleotide could be accommodated in the enzyme active site, we determined the co-crystal structures of a catalytically deficient MccF variant (MccF-S118A) in complex with either **6** or **7**.[12] Data collection and refinement statistics are provided in Table S1. Clear electron density was observed in the MccF active site for the respective substrate molecules (Figure 2). The Glu side chain of **6** and **7** is coordinated through interactions with the side chains of Asn220 and Lys247, analogous to what is observed in the co-crystal structure with **5** (PDB id: 3TLE)[12] (Figure S11). Interactions with the nucleobases are mediated by stacking interactions with the Trp186 indole, and the hydrogen bonding of the two ribose oxygens with the side chains of Glu277 and Arg246. Also conserved are interactions that were previously postulated to stabilize the oxyanion hole and the phospho oxygen atoms.[12] For **7**, an additional hydrogen bond exists between the 2-NH_2 and Ser183 side chain hydroxyl. In complex with 6, Ser183 side chain is stabilized by interactions with Arg246 side chain guanidine group. Despite extensive efforts, dissociation constants for the MccF-S118A complexes with **5**, **6**, **7** or with AMP could not be determined by ITC due to protein instability in the titration cell.

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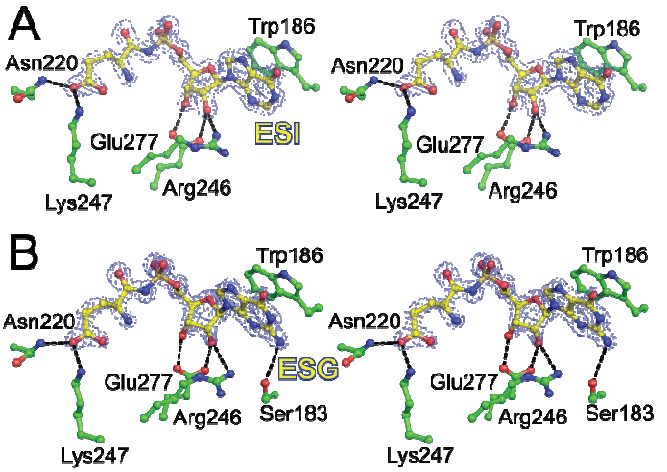
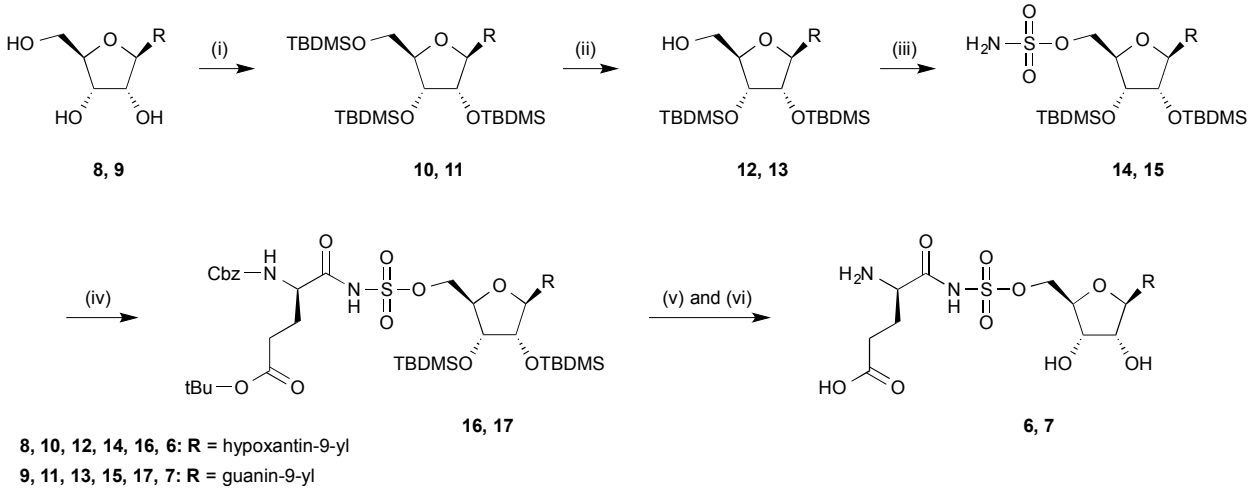


Figure 2. The MccF-S118A enzyme active site with bound **6** and **7**. Stereoview showing the active site features of the MccF-S118A in complex with **(A) 6** and **(B) 7**. The MccF carbon atoms are shown in green ball-and-stick and ligand carbon atoms are colored in yellow. Superimposed is a $|F_{obs}| - |F_{calc}|$ Fourier electron density map (contoured at 2.0σ over background in blue) calculated with phases from the final model with the coordinates of the ligands deleted prior to one round of refinement.

Scheme 1: Synthesis of glutamylated nucleoside analogs 6 and 7.



Reagents and conditions: (i) TBDMSCl, imidazole, dry DMF, rt, 3 days and then 50°C, 5h (quantitative); (ii) TFA-Water, THF, 0°C, 6h (> 95%); (iii) $ClSO_2NCO$, formic acid, DMA (**14**: 60%; **15**: 63%); (iv) Boc-Glu(OtBu)-OSu, DBU, DMF, rt, 6h, followed by (v) TFA:water (5:2), rt, 3h, and (vi) TEA.3HF, THF, rt, overnight (combined yield over 3 steps for **6**: 65% and **7**: 71%).

The modest changes in the k_{cat} values for the hydrolysis of **5**, **6**, and **7**, despite the change in identity of the nucleobase, can be attributed to the preserved glutamate side chain in these compounds. As has been demonstrated for serine proteases,[15-17] and noted in our earlier studies with MccF,[12] the k_{cat} value for substrate hydrolysis is dictated primarily by the stabilization of the departing amino acid. It should also be noted that the positions of the catalytic triad amino acid side chains and the oxyanion-hole stabilizing interactions, both of which are essential for catalysis, are identical in the crystal structures of MccF-S118A in complex with **5**, **6,** and **7**.

The similar kinetic constants for the hydrolysis of **5**, **6,** and **7** by MccF, and similar binding modes of these compounds to MccF-S118A pose an interesting dilemma in the context of electrostatic charge density localization. Calculated vacuum electrostatics for aromatic interactions have suggested various interesting modes of interactions between biological macromolecules and corresponding ligands, an example of which is the cation-π interaction observed in the structure of ligand gated ion channels.[18]

The calculated electrostatic charges for three nucleobase rings-adenine, hypoxanthine and guanine in **5**, **6,** and **7,** respectively, differ significantly only for the amine and carbonyl substituents on the six-membered rings. The fused five-membered rings are quite similar in their charge distributions (Figure 3). For the indole ring, the majority of the negative charge density is

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localized on the benzene ring, with the fused pyrrole ring being less electron rich.[19] The mode of π-stacking of substrates with Trp186 in MccF positions the six-membered ring of the nucleobase away from the negative charge bearing benzene ring of the indole, so as to minimize charge repulsion. Therefore, it is not surprising that this arrangement is strictly conserved for the interaction of MccF with the hypoxanthine and guanine rings of **6** and **7**, respectively. The double bonded oxygen atom of hypoxanthine and guanine ring of **6** and **7** establishes a negative charge in place of the positively charged primary amine in the analogous position in adenine. Nonetheless, the mode of binding of **6** and **7** positions this double bonded oxygen atom (and the positively charged primary amine of adenine) away from the indole side chain to minimize charge-charge interactions, while preserving the attractive van der Waals interaction with the nucleobase imidazole ring (Figure 2). Hence it is apparent that the MccF active site utilizes van der Waals attractive force as a primary binding determinant, while minimizing the electrostatic charge-charge interactions within the active site. This observation provides the rationale for the substrate promiscuity of MccF, as reflected in the similar K_M parameters for the hydrolysis of **5**, **6,** and **7**. The only difference apparent between the binding of adenine, guanine and hypoxanthine rings of the substrates is the additional hydrogen bond provided by the side chain of Ser183 to the primary amine of the guanine, which may result in the lower K_M value observed for **7**.

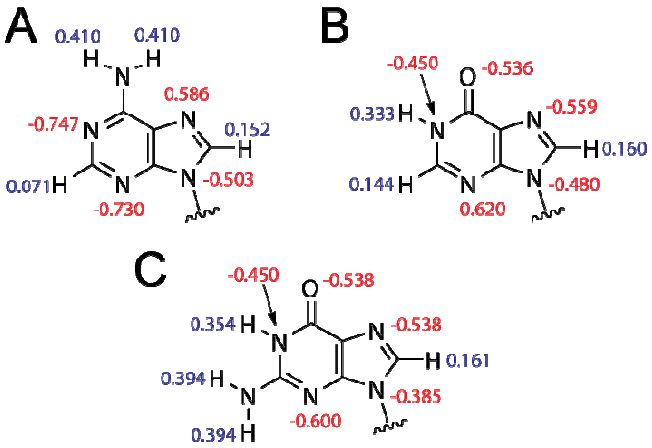

Figure 3. Calculated electrostatic charges for the peripheral atoms for the heterocyclic nucleobases: (A) adenine (B) inosine and (C) guanine. Positive charges are listed in blue, and the negative charges are listed in red. Note that the electrostatic charges for the atoms on the five membered rings are similar for all three nucleobases, while the charges on the six member rings are different.

Bioinformatics analyses reveal the existence MccF-like genes in the genomes of pathogenic bacteria that lack the biosynthetic machinery to generate microcin C7. Our studies, in conjunction with our earlier characterization of specificity determinants for recognition of the amino-acyl side chain, demonstrate MccF and homologs to be tolerant to a range of purine nucleobase-conjugated substrates. The prevalence of these MccF-like genes suggests that synthetic analogs of aminoacyl tRNA synthetase intermediates may not have the previously anticipated universal utility as antibiotics. For example, the biosynthetic cluster for agrocin 84 in *Agrobacterium radiobacter* K84 contains an *mccF*-like gene, even though agrocin 84 does not act like a simple aminoacyl tRNA synthetase intermediate mimic.[5] Consequently, the design of intermediate mimics for use as antibiotic leads must focus on the possibility of inactivation by MccF-like enzymes.

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Notes and references

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NMR characterization of synthetic molecules is described in detail in the Supporting Information. Wild type MccF enzyme and mutants were expressed, purified and crystallized in a manner described previously,[12] and described in detail in the Supporting Information. Enzyme activity assays were also conducted in a manner identical to as described previously,[12] and described in detail in the Supporting Information. Electrostatic charges were calculated using the online R.E.D. server.[20] Crystal structures of MccF-S118A in complex with **6** and **7** have been deposited in the Protein Data Bank (www.pdb.rcsb.org) with the accession codes 4IIY and 4IIX respectively.

Electronic Supplementary Information (ESI) is available online and contains experimental methods and compound characterization data.See DOI: 10.1039/b000000x/

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