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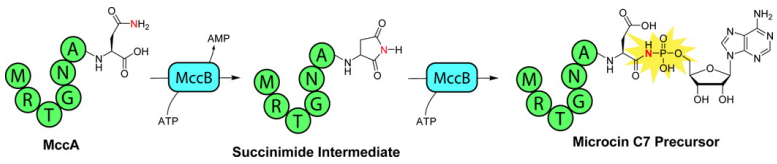

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Maturation of an *Escherichia coli* Ribosomal Peptide Antibiotic by ATP-Consuming N−P Bond Formation in Microcin C7

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Abstract: Synthetic phosphoramidate analogues of nucleosides have been used as enzyme inhibitors for decades and have therapeutic applications in the treatments of HIV and cancer, but little is known about how N−P bonds are fashioned in nature. The heptapeptide MccA undergoes post-translational processing in producer strains of *Escherichia coli* to afford microcin C7 (MccC7), a "Trojan horse" antibiotic that contains a phosphoramidate linkage to adenosine monophosphate at its C-terminus. We show that the enzyme MccB, encoded by the MccC7 gene cluster, is responsible for formation of the N−P bond in MccC7. This modification requires the consumption of two ATP molecules per MccA peptide and formation and breakdown of a peptidyl-succinimide intermediate.

Introduction

Microcins are low-molecular-weight (<10 kDa), ribosomally encoded peptide antibiotics that are secreted by some strains of Enterobacteriaceae to kill their sensitive bacterial competitors.[1,2] The post-translational maturation steps of microcins are diverse and critical for their physiological function. Examples include the heterocyclization of the microcin B17 peptide backbone, which affords a DNA gyrase inhibitor, macrocyclization of the microcin J25 peptide backbone, which creates a RNA polymerase inhibitor, and attachment of an enterobactin derivative to the microcin E492 C-terminus, which smuggles the peptide antibiotic into enterobacteria that express siderophore uptake pumps.[1,2] In this work, we focus on the post-translational tailoring of the ribosomally derived microcin peptide MccA, which results in linkage of an AMP moiety to its C-terminus through a N−P bond.[3] This modification constitutes a key step in the maturation of microcin C7 (MccC7, **1**), a "Trojan horse" antibiotic. Following export from the producing *Escherichia coli* cell, MccC7 is recognized by transporters, including *E. coli* YejABEF,[4] and imported into the target bacterial cell. The peptide is cleaved between its sixth and seventh residues by an endogenous protease, and release of Asp-NH-AMP (**1a**), a toxic adenylated aspartic acid analogue, occurs (Figure 1). This species targets the aspartyl-tRNA synthetase and inhibits protein synthesis.[5]

Microcin gene clusters include the structural gene for the nascent peptide and genes that encode proteins involved in maturation, immunity, and export. The MccC7 gene cluster contains six genes, *mccABCDEF*, five of which are necessary for MccC7 production, immunity, and export (Figure S1, Supporting Information).[6] An independently isolated antibiotic, microcin C51, is structurally identical to MccC7,[7] and its five genes have 98−100% sequence identity to *mccA*−*mccE*.[8] *mccA*, the smallest known bacterial gene with only 21 base pairs, encodes MccA ($fMet_1$-Arg_2-Thr_3-Gly_4-Asn_5-Ala_6-Asn_7), the heptapeptide precursor of MccC7.[9] *mccB*, *mccD*, and *mccE* are required for the maturation of MccC7, which results from three modifications to MccA: (i) conversion of Asn_7 to a derivatized $isoAsn_7$, (ii) formation of a N−P bond between the amide group of $isoAsn_7$ and P_α of ATP, affording the peptidyl-AMP linkage, and (iii) aminopropylation of the phosphoramidate (Figure 1). MccB (351 aa, 39.2 kDa) is homologous to adenylating

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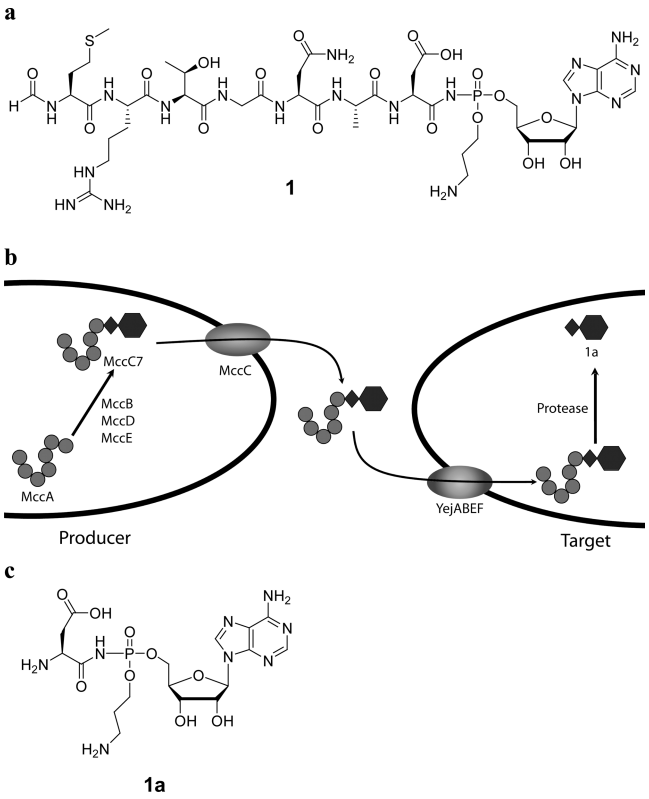

Figure 1. Biological activity of MccC7 (**1**). (a) Structure of microcin C7.[3] (b) Production of the toxic metabolite. (c) Structure of the toxic adenylated aspartic acid analogue (**1a**).

enzymes, including eukaryotic E1 ubiquitin ligases[10,11] and bacterial ThiF from thiamin pyrophosphate biosynthesis,[12] and is therefore the candidate for catalysis of N−P bond formation and the focus of the present work.

Results and Discussion

Preparation of MccB and Substrates. To elucidate the role of MccB in MccC7 biosynthesis and, specifically, in N−P bond formation, *mccB* (1053 bp) was subcloned from pMM550[3,13] and the protein product overexpressed in *E. coli* as a N-terminal His_6 fusion (Figure S2) in yields of 5 mg/L culture. The MccA heptapeptide was synthesized in the *N*-acetyl, *N*-formyl, and free amine forms by standard solid-phase methodology. Because each peptide gave equivalent activity in initial assays with MccB (data not shown), free amine **2** (MRTGNAN, Scheme 1) was used in all subsequent studies.

In Vitro Characterization of MccB. HPLC analysis of incubations of MccB with **2** revealed that three products formed in an ATP- and Mg^{2+}-dependent fashion (Figure 2a). Two of the products absorbed at 260 nm (Figure 2b), consistent with the presence of an adenosine moiety. One of these peaks was identified as AMP following isolation, ion-exchange chromatography, and electrospray ionization mass spectrometry (ESI-MS) (Figure 2c). ESI-MS analysis of substrate **2** and product **3** revealed a mass increase of 329 mu (Table 1), consistent with the addition of AMP to the heptapeptide (Scheme 1**)**. The k_{cat} value was 6−12 s^{-1}, indicating robust catalysis of ATP cleavage (Table 2). The third product, **4**, showed no adenosine absorption (260 nm) and accounted for ≤6% of the reaction components. ESI-MS analysis of **4** indicated a loss of 18 mu relative to **2**, which suggested dehydration, potentially resulting from cyclization of Asn_7, and formation of a succinimide (Figure 3a).

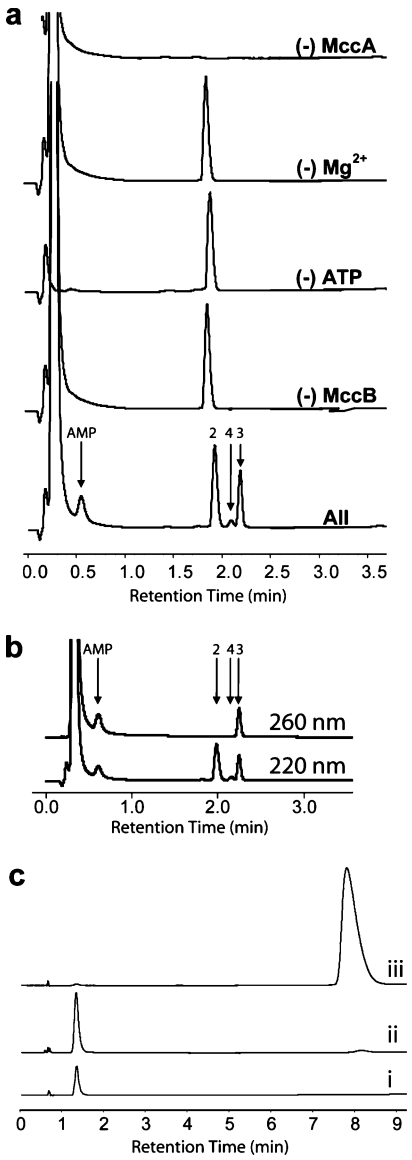

Figure 2. Characterization of MccB activity. (a) HPLC traces (220 nm) showing the products resulting from incubation of MccB (5 μM) with **2** (250 μM) in reaction buffer at pH 8 or in buffer lacking the indicated component. (b) HPLC traces of samples containing all reaction components with absorbance at 220 and 260 nm. (c) Identification of the ∼0.6-min peak in (a) and (b) as AMP by ion-exchange chromatography: (i) AMP standard, (ii) the isolated and purified ∼0.6-min peak, and (iii) ADP standard.

To establish the structure of **3**, multimilligram quantities of the reaction product were obtained from preparative-scale reactions and HPLC purification. The ^1H NMR spectrum of **3** was consistent with the published spectrum of MccC7, but lacked the propylamine resonances (Figure S3 and Table S1). Existence of the N−P bond was established by 1D ^{15}N NMR, 1D ^{31}P NMR, [$^{15}N^1H$]-HSQC, and [$^{31}P^1H$]-HMBC NMR analysis of the ^{15}N-labeled form of product **3** (Figure 4). The one-bond $^{15}N-^{31}P$ coupling constant measured in the four different spectra varied from 27.6 to 28.2 Hz. The connectivity

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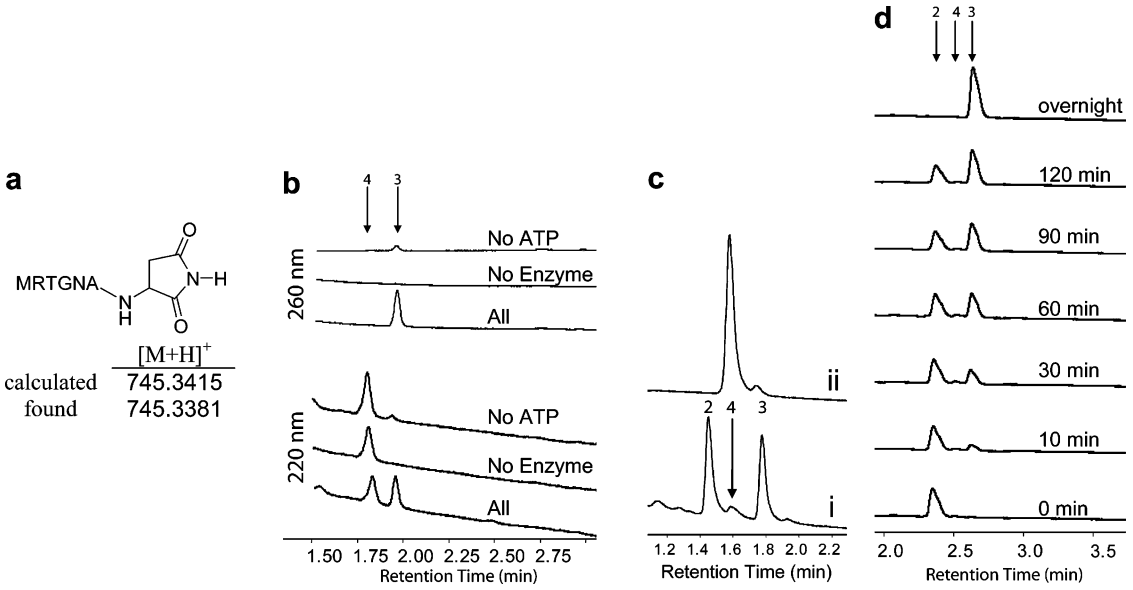
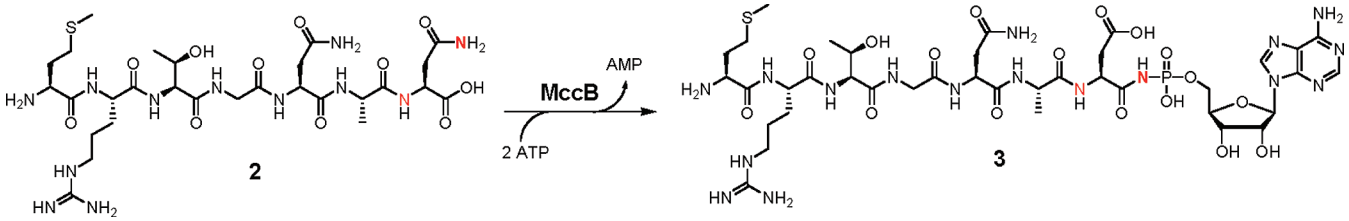


Figure 3. Characterization of the MccB intermediate **4**. (a) Structure and mass spectral analysis of the succinimide intermediate, **4**. (b) MccB converts the succinimide intermediate **4** to product **3**. MccB assayed with purified **4**, obtained by preparative RP-HPLC, and ATP in reaction buffer at pH 8 or in the absence of the indicated component for 20 min at room temperature. (c) The synthetic succinimide standard (ii) coelutes with intermediate **4** from incubation of **2** and ATP with MccB (i). (d) Time course (220 nm) for the MccB-mediated adenylation of **2** to form **3.**

Scheme 1. Reaction Scheme for C-Terminal Adenylation of **2** by MccB[a]



[a] The ^{15}N-labeled nitrogens in the labeled versions of **2** and **3** are highlighted in red.

Table 1. High-Resolution Mass Spectral Analysis of the MccB Reaction Components[a]

	unlabeled		^{15}N labeled	
	calcd $[M+H]^+$	found $[M+H]^+$	calcd $[M+H]^+$	found $[M+H]^+$
MccA	763.3521	763.3533	765.3521	765.3432
MccB product (**3**)	1092.4046	1092.4007	1094.4046	1094.3961
difference	329.0525	329.0474	329.0525	329.0529

[a] Compound **3** was purified from a large-scale incubation of MccB with ATP and either unlabeled **2** ($C_{28}H_{50}{}^{14}N_{12}O_{11}S$) or **2** containing a ^{15}N-labeled C-terminal asparagine ($C_{28}H_{50}{}^{14}N_{10}{}^{15}N_2O_{11}S$).

Table 2. Kinetic Parameters for MccB

substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\,\mu M^{-1}$)
MccA (**2**)	61 ± 16	6.4 ± 1.8	0.10
ATP	70 ± 30	12.5 ± 2.4	0.18
succinimide (**4**)	130 ± 40	29.6 ± 4.8	0.23

of the N-AMP to C_α of $IsoAsn_7$ was verified by [$^{15}N^1H$]-TROSY-H(N)CA NMR analysis and through comparison of the direct observe ^{13}C NMR spectra of the ^{15}N-labeled form of **3** obtained with and without ^{15}N decoupling (Figures S4 and S5).

As MccA is converted to MccC7, there is an asparagine-to-isoasparaginyl moiety transformation in the MccB-catalyzed tailoring of the heptapeptide C-terminus. We therefore employed a heptapeptide with ^{15}N-labeled Asn_7 in an activity assay to determine whether the Asn_7 carboxamido nitrogen atom is retained in **3** (red N's; Scheme 1). ESI-MS analysis indicated that the conversion of ^{15}N-labeled **2** to product **3** resulted in an increase of 329 mu, identical to the mass difference of the unlabeled reaction (Table 1), revealing that both nitrogen atoms of Asn_7 are retained.

Compound **4** was isolated from an incubation with MccB and was converted to product **3** in a MccB- and ATP-dependent manner (Figure 3b), indicating that it was a chemically competent intermediate. To confirm the identity of **4**, the putative peptidyl-succinimide was synthesized[14,15] for use as a substrate and standard. HPLC analysis indicated that the synthetic succinimide coeluted with intermediate **4** (Figure 3c) and was also accepted by MccB and converted to **3** (data not shown). This intermediate was kinetically competent with a k_{cat} value ~4-fold greater than that of **2** (Table 2). Similar cyclization reactions and succinimide intermediates are evoked as intermediates in spontaneous peptide degradation,[16] in protein splicing,[17] and in side reactions during solid-phase peptide synthesis.[18] In the proposed biosynthetic pathway of agrocin 84, a naturally occurring antibiotic with structural similarities to MccC7, an aspartyl-tRNA synthetase homologue is hypothesized to form a phosphoramidate linkage

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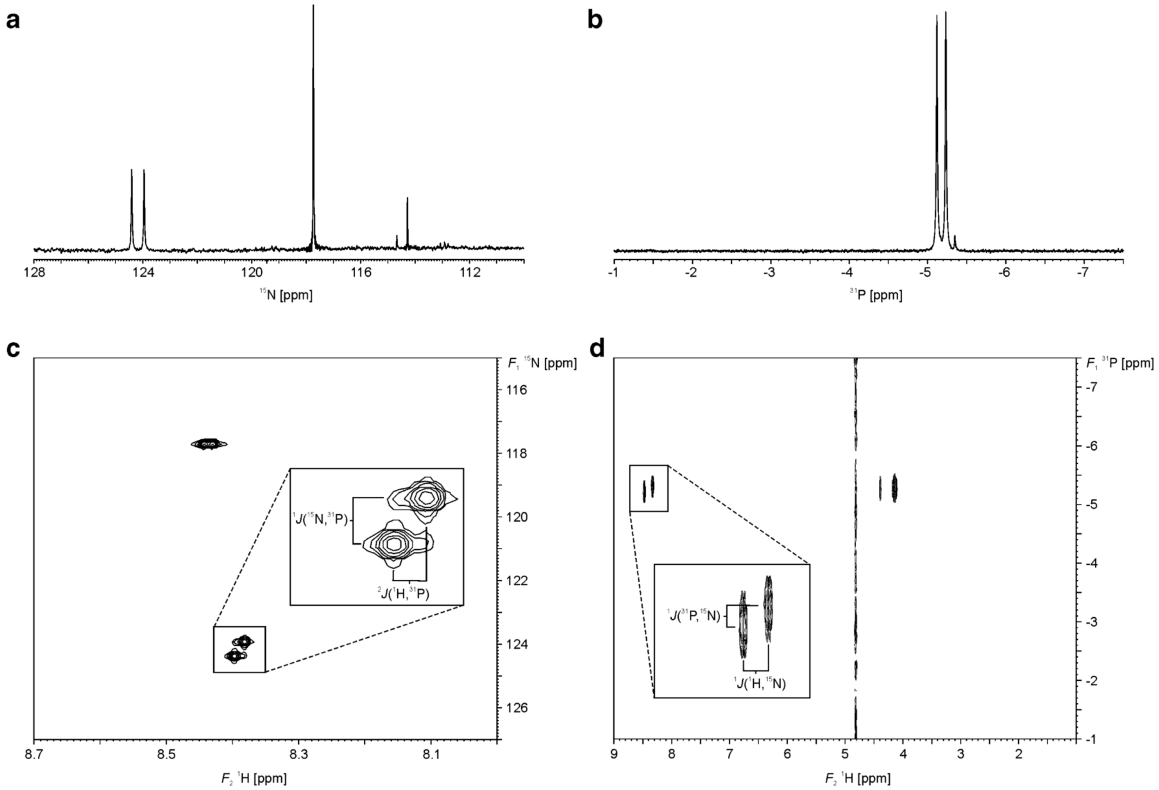

Figure 4. Characterization of the N−P bond in **3**. (a) 1D ^{15}N NMR, (b) 1D ^{31}P NMR, (c) [^{15}N^{1}H]-HSQC, and (d) [^{31}P^{1}H]-HMBC analysis of ^{15}N-labeled **3**. Insets: The cross-peaks in the [^{15}N^{1}H]-HSQC and [^{31}P^{1}H]-HMBC spectra relevant to the nitrogen−phosphorus linkage reveal E.COSY-like multiplet structures resulting from scalar couplings involving the passive ^{31}P and ^{15}N nuclei, respectively.

between an amino acid and AMP through a succinimide intermediate.[19]

Formation of phosphoramidate **3** from succinimide **4** could occur by two possible routes, both of which require ring-opening of the succinimide and addition of the AMP moiety of ATP. To determine if ring-opening occurs before nucleophilic attack on ATP, MccA-N7isoAsn was evaluated as a substrate. It was not accepted by MccB, and it inhibited the MccB-catalyzed modification of **2**, demonstrating that it bound in the active site (Figure S6). This result suggested that succinimide **4** is not ring-opened to the isoasparaginyl-α-carboxamide before enzymatic N−P bond formation.

Peptides **2** and **4** were both consumed to yield **3** in overnight incubations with MccB (Figure 3d). The observations that (i) AMP was a product of the MccB-catalyzed modification of **2** and (ii) formation of **3** and **4** both require ATP suggested that 2 equiv of ATP are required per equiv of substrate **2** to generate **3**. In accord with this notion, complete conversion of **2** to **3** was only observed when MccA was incubated with MccB and ≥2 equiv of ATP (Figure 5).

In vivo studies revealed that mutations to the seventh residue of MccA abolished MccC7 production.[20] To provide further insight into the MccB mechanism and in an effort to trap the adenylated intermediate **5** (Scheme 2) by removing the amide nitrogen, MccA-N7D was prepared. This peptide was neither a substrate nor an inhibitor of MccB (Figure S7), indicating that

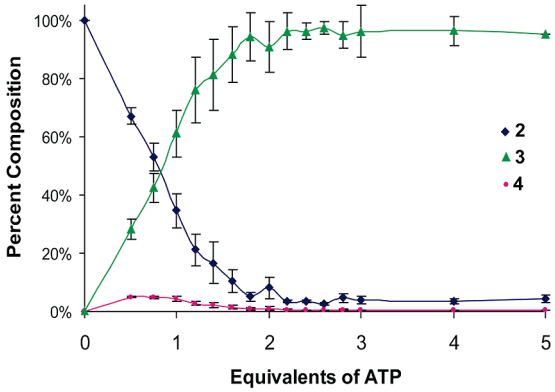

Figure 5. MccB consumes 2 equiv of ATP to form **3** from **2**. Reactions with MccB (5 μM), **2** (250 μM), and varying concentrations of ATP (0−1250 μM) were incubated overnight at room temperature in reaction buffer at pH 8 and analyzed by HPLC. Green triangles, MccB product **3**; blue diamonds, MccA **2**; pink circles, intermediate **4**. Percent composition was plotted against the equivalents of ATP ([ATP]/[MccA]) with standard error from four independent trials.

the C-terminal carboxamido nitrogen atom is crucial for catalysis and initial structural recognition.

Mechanism for the MccB-Catalyzed C-Terminal Modification of MccA. On the basis of these observations, we propose a two-stage reaction scheme for the MccB-catalyzed post-translational modification of MccA that accounts for N−P bond formation, conversion of Asn_7 to an $isoAsn_7$ moiety, and consumption of 2 equiv of ATP (Scheme 2). In the first stage, the α-carboxylate of Asn_7 attacks P_α of the first ATP equivalent, yielding the hydrolytically labile heptapeptidyl-CO-AMP, **5**. The MccB homologues E1 and ThiF carry out similar C-terminal

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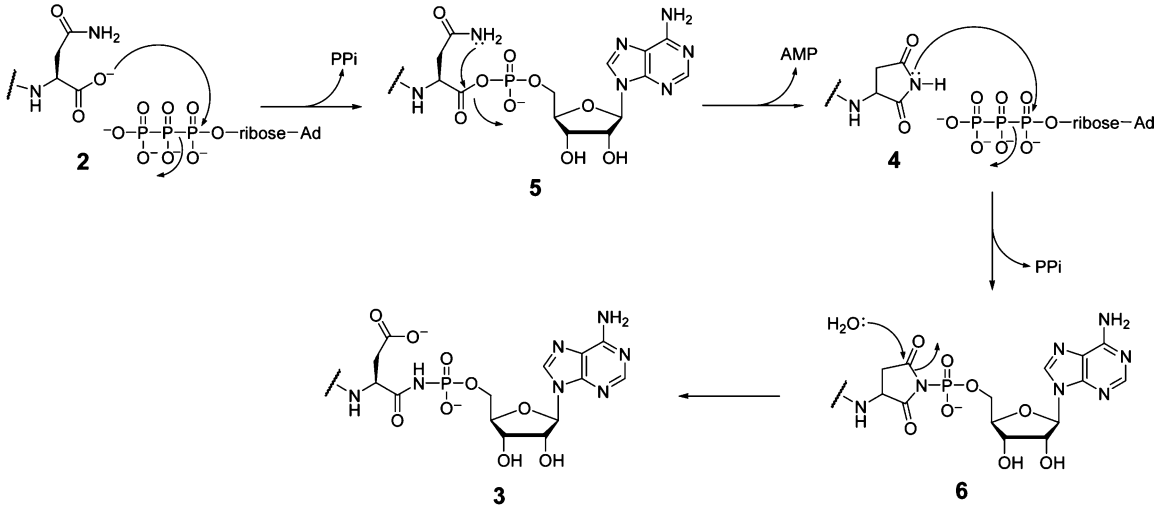
Scheme 2. Proposed MccB Mechanism[a]



[a] The six N-terminal residues (MRTGNA) of MccA are removed for clarity.

carboxyl activation of polypeptide substrates during ubiquination and thiamin biosynthesis, respectively.[10−12] The β-carboxamido nitrogen atom of Asn_7 then captures the C-terminal acyl-AMP anhydride **5** intramolecularly, yielding the tetrahedral adduct, from which subsequent expulsion of AMP affords succinimide **4**. Although carboxamido nitrogens are poor nucleophiles, there are many examples in biology, including the modification of Asn side chains in protein N-glycosylation, where weak nitrogen nucleophiles are specifically employed.[21] In the second stage, the succinimidyl nitrogen atom in **4** is the nucleophile that attacks P_α of a second ATP molecule, forming the N−P bond and yielding **6**. The succinimidyl NH is likely more easily activated for nucleophilic attack via a general base catalyst than the starting Asn_7 carboxamide NH_2 because of its greater acidity. Subsequent addition of water to the carbonyl is followed by directed ring-opening to generate the C-terminal isoasparaginyl moiety in **3**.

Concluding Remarks

Enzymatic installation of the stable N−P bond in MccC7 follows reasonable chemical logic. Creation of this stable mimic of an acyl-AMP mixed anhydride provides the producing bacteria with a "Trojan horse" toxin and hence a tool for survival and successful colonization in the host. Elucidating the enzymatic machinery and mechanisms for the formation of N−P bonds in other naturally occurring nucleoside analogues will provide evolutionary insight into the adaptation of adenylating enzymes. Relevant examples include the secondary metabolites phosmidosine and agrocin 84, amino acid-N-AMP conjugates with antibiotic properties.[22−24] If the aspartyl-tRNA synthetase homologue in the agrocin 84 biosynthetic pathway is responsible for the formation of a N−P bond,[19] convergent evolution of adenylating enzymes to form phosphoramidates is suggested. From a practical standpoint, synthetic nucleoside analogues containing N−P bonds have established applications as enzyme inhibitors and as anti-HIV and anticancer therapies.[25] Deciphering if MccB-catalyzed N−P bond formation is portable to other peptide substrates may provide a new route for the design of antibiotic drugs based on AMP phosphoramidate enzyme ligation chemistry.

Experimental Section

Bacterial Strains, Plasmids, Materials, and Instrumentation. All chemicals were purchased from Sigma-Aldrich. *E. coli* TOP10 and BL21(DE3) cells were purchased from Invitrogen. The pET-28b plasmid was obtained from Novagen. Restriction enzymes were purchased from New England Biolabs. Nickel nitrilotriacetic acid agarose (Ni-NTA) gel was purchased from Qiagen. Isotopically labeled amino acids and NMR solvents were purchased from Cambridge Isotopes. ^{1}H NMR spectra were recorded on a Varian 600 MHz Fourier transform NMR spectrometer and referenced to internal solvent peaks. Low-resolution electrospray ionization mass spectra were collected by liquid chromatography−mass spectrometry (LC−MS) using a Shimadzu LC−MS QP8000α operating in positive ion mode. High-performance liquid chromatography (HPLC) data was collected on a Beckman Coulter System Gold (126 solvent module, 168 detector). A Clipeus C18 (5 cm × 4.6 mm) column was used for analytical HPLC. A Phenomenex Luna C18 (250 × 21.2 mm) column was used for preparative HPLC. Absorption at 220 and/or 260 nm was monitored for all liquid chromatography. DNA sequencing was performed by staff at the Biopolymers Facility, Department of Genetics, Harvard Medical School, Boston, MA. High-resolution mass spectra were obtained by staff at the Massachusetts Institute of Technology Department of Chemistry Instrumentation Facility, Cambridge, MA. The microcin C7 gene cluster, pMM550,[3,13] was provided by Dr. Felipe Moreno at Unidad de Genética Molecular, Hospital Ramón y Cajal, Madrid, Spain.

Cloning and Expression of MccB. The *mccB* gene was obtained by PCR amplification from the plasmid pMM550.[3,13] Primers for *mccB* (5′-*ggaattccat*atg*gattatatattgggtcgctatg*-3′ *and* 5′-*gatcctc*gag**tca**cattc-tatttccacacacagag-3′: italicized, modified sequences; underlined, restriction site; bold, stop codon) contained *Nde*I and *Xho*I restriction sites, respectively, and the amplified gene was ligated into the corresponding sites of pET-28b using T4 DNA ligase. The construct was transformed into *E. coli* TOP10 cells, and the plasmids were obtained with a Qiagen miniprep kit. The identity of the *mccB* insert was confirmed by DNA

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sequencing and differed from the published sequence[6] with a T-C conversion at base pair 98, resulting in a valine-alanine substitution at residue 33.

E. coli BL21(DE3) cells were transformed with the pET-28b-*mccB* and were grown in Luria broth medium supplemented with kanamycin (50 μg mL^{-1}) at 37 °C. The cells were grown at 37 °C until $OD_{600} \approx 0.6$, and expression of MccB was induced by addition of 400 μM isopropyl β-D-thiogalactoside (IPTG). The temperature was reduced to 15 °C, and the cells were grown for 24 h. Cells were harvested by centrifugation (10 min, 3500 rpm) and resuspended in lysis buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl and 10 mM $MgCl_2$). The resuspended cells were lysed by two passes at 10 000−15 000 psi in an Avestin EmulsiFlex-C5 high-pressure homogenizer, and the cell debris was removed by ultracentrifugation (35 min, 35000 rpm).

N-His_6-tagged MccB was purified by Ni-NTA affinity chromatography. Fractions were eluted from the column in lysis buffer with a gradient of 25−200 mM imidazole. Fractions collected from 75 to 150 mM imidazole were pooled and dialyzed in buffer (25 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM DTT, 10% glycerol) overnight. The final yield of MccB was 5 mg/L of culture. MccB was flash frozen in liquid nitrogen and stored at −80 °C. The protein concentration was determined by a Bradford Assay.[26]

Syntheses of Peptide Substrates. All peptides were synthesized via solid-phase peptide synthesis by staff at the Biopolymers Facility, Department of Biochemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA. Their purity and identities were confirmed by HPLC/LC−MS. The *N*-formyl, *N*-acetyl, and free amine (**2**) forms of MccA contained the sequence MRTGNAN. MccA-N7D and MccA-N7isoAsn have the sequences MRTGNAD and MRTGNAisoN, respectively. The ^{15}N-labeled version of **2** was synthesized using an ^{15}N-labeled asparagine, where both nitrogen atoms were labeled, as the C-terminal residue. Asn_5 was unlabeled.

Synthesis of Succinimide 4. The hexapeptidyl succinimide **4** was prepared by adapting established protocols.[14,15] The peptide MRTGNA-isoAsn(OBzl) (21.2 mg, 24.9 μmol) was dissolved in 1.4 mL of dimethylformamide, and 11 equiv of triethylamine were added. The reaction was stirred at room temperature overnight, and reaction progress was monitored by LC−MS and analytical HPLC. Product **4** was purified by preparative HPLC using a gradient of 0−20% acetonitrile in 0.1% TFA/H_2O in 40 min and lyophilized to dryness (5.3 mg, 7.1 μmol, 29%). It eluted at 24.5 min, and its identify was confirmed by LC−MS ($[M + H]^+$ calcd, 745.3; found, 745.6).

Preparation of 3 and 4 from Large-Scale MccB Incubations. MccB (10 μM) was incubated with **2** (1 mM) and ATP (10 mM) at pH 8.0 (75 mM Tris-HCl, 5 mM $MgCl_2$, 5 mM TCEP) in a volume of 20 mL for 24−48 h at room temperature. The reaction was quenched with 17 mL of 0.6% TFA in water and syringe filtered to remove precipitated protein. The reaction products were separated and purified by using preparative HPLC at 10 mL/min and a solvent gradient of 0−40% B in 40 min (solvent A: 0.1% TFA in H_2O; solvent B: acetonitrile). Compounds **2**, **4**, and **3** eluted at 16.75, 18.3, and 18.8 min, respectively. Samples were lyophilized, and their purity was confirmed using analytical HPLC. MccA (**2**; $[M + H]^+$ calcd, 763.3521; found, 763.3533); product **3** ($[M + H]^+$ calcd, 1092.4007; found, 1092.4007); succinimide intermediate **4** ($[M + H]^+$ calcd, 745.3415; found, 745.3381).

NMR Characterization of the N−P Bond in 3. ^{15}N and ^{31}P NMR spectra were recorded at 20 °C on a Bruker Av600 spectrometer equipped with ^{1}H/^{13}C/^{15}N or ^{1}H/^{13}C/^{31}P triple resonance cryoprobes optimized for ^{1}H and ^{13}C detection with samples containing 5% D_2O for field locking and 0.15 mM DSS for internal chemical shift referencing. The 1D ^{15}N spectrum resulted from addition of 2304 scans using a spectral width of 250 ppm and an acquisition time of 537 ms. Power-gated ^{1}H decoupling was applied during relaxation and acquisition periods. The inverse-detected proton-nitrogen correlation employed a gradient-sensitivity enhanced HSQC pulse sequence[27] supplemented with water-flip back.[28] Spectral widths were 10 and 120 ppm along the ^{1}H and ^{15}N dimensions, respectively. Eight scans per FID and 1536 × 1024 complex data points were recorded, corresponding to acquisition times of 255 (^{1}H) and 140 ms (^{15}N). In the 1D ^{31}P experiment, proton decoupling was applied during the 1.68-s acquisition period only. Sixty-four scans were accumulated using a spectral width of 80 ppm. The 2D proton-phosphorus correlation was obtained with eight scans/FID using a gradient-coherence selected HMBC sequence.[29] The *J*-evolution delay was adjusted to 50 ms. Spectral widths were 10 ppm in both dimensions, while acquisition times were 340 ms (2048 complex data points) and 26 ms (64 t_1 increments) in the ^{1}H and ^{31}P dimensions, respectively. Spectra processing was carried out using Bruker TopSpin 2.0 software. Exponential broadening of 0.5 Hz was applied before Fourier transformation of 1D spectra. Cosine-squared window functions were applied in both dimensions of the [^{15}N^{1}H]-HSQC. The [^{31}P^{1}H]-HMBC spectrum is shown in the mixed-mode representation (pure absorption along F_1, magnitude mode along F_2). Apodization involved multiplication with an unshifted sine-bell function along the ^{1}H dimension and a cosine-squared function along the ^{31}P dimension.

MccB Activity Assays. MccB (5 μM) was incubated with MccA (**2**, 250 μM) and ATP (5 mM) at pH 8.0 in the reaction buffer (75 mM Tris-HCl, 5 mM $MgCl_2$, 2.5 mM TCEP). Product **3** formation was monitored by analytical HPLC using a gradient of 0−40% B in 8 min with a flow rate of 4 mL/min (solvent A: 0.1% TFA in H_2O; solvent B: acetonitrile). This general protocol was used for assays with modified peptides.

Kinetic Characterization of the MccB-Catalyzed Reaction. All kinetic investigations were conducted at pH 8 in the reaction buffer, and product formation was monitored by analytical HPLC. To determine k_{cat} and K_m of MccB for its accepter substrate, MccA, the concentration of ATP was held constant at 5 mM and the concentration of MccA was varied from 10 to 300 μM ([MccB] = 1 μM) in 100-μL reactions. The reactions were incubated at room temperature and quenched at t = 20 min by addition of an equal volume of 0.6% TFA/H_2O. To determine k_{cat} and K_m of MccB for its donor substrate, ATP, the concentration of MccA was 500 μM and the ATP concentration was varied from 10 to 750 μM ([MccB] = 1 μM). The 100-μL reactions were incubated for 10 min at room temperature and quenched with an equal volume of 0.6% TFA/H_2O. The k_{cat} and K_m values of MccB for the succinimide intermediate **4** were determined as described for MccA except that the MccB concentration was 100 nM, the concentration of **4** was varied from 0.04 to 1.5 mM, and the reactions were quenched after 5-min incubations at room temperature. The product peaks (260 nm) were integrated, and the resulting values were converted to molecules formed by using an ATP standard curve (62.5−500 μM ATP). The data were fit to the Michaelis−Menten equation, and all reported values are the mean ± standard deviation of at least three independent trials.

Identification of AMP as a Product. Ion-exchange chromatography was performed on a Vydac 302IC4.610 4.6 × 100 mm column at 2 mL/min and a solvent gradient of 0% B for 2 min followed by 1−100% B in 17 min (solvent A: 25 mM total of 1:1 NaH_2PO_4/Na_2HPO_4 at pH 2.8; solvent B: 125 mM total 1:1 NaH_2PO_4/Na_2HPO_4 at pH 2.9). The absorbance at 260 nm was monitored. The product of the MccB reaction that eluted at 0.5 min from reverse-phase HPLC was isolated and purified and subsequentially injected onto the ion-exchange column. Its retention time was compared to those of authentic ATP, ADP, and AMP standards. The ATP, ADP, and AMP standards eluted at 1.5, 8.0, and 16.5 min, respectively. The product was confirmed as AMP by LC−MS ($[M + H]^+$ calcd, 348.1; found, 348.1).

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Supporting Information Available: Figures S1−S5 and Table S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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