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Prephenate Decarboxylases: A New Prephenate-Utilizing Enzyme Family That Performs Nonaromatizing Decarboxylation en Route to Diverse Secondary Metabolites[†]

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ABSTRACT: Prephenate is the direct precursor of phenylpyruvate and 4-hydroxyphenylpyruvate in the biogenesis of phenylalanine and tyrosine by action of the decarboxylative, aromatizing enzymes prephenate dehydratase and dehydrogenase, respectively. The recent characterization of BacA in bacilysin biosynthesis as a nonaromatizing decarboxylase reveals a new route from prephenate in the biosynthesis of nonproteinogenic amino acids. This study describes two additional enzymes, AerD from *Planktothrix agardhii* and SalX from *Salinispora tropica*, that utilize the central building block prephenate for flux down distinct pathways to amino acid products, representing a new metabolic fate for prephenate and establishing a new family of nonaromatizing prephenate decarboxylases.

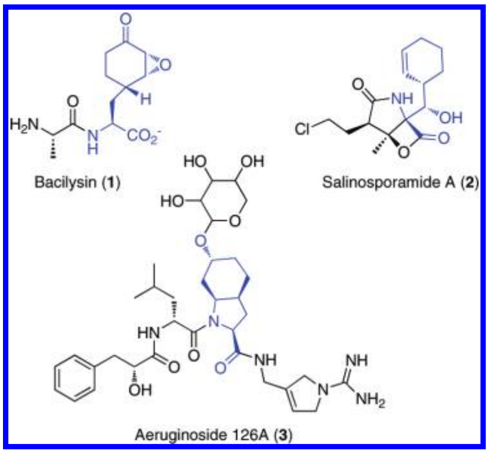

FIGURE 1: Natural products containing amino acids (blue) derived from prephenate.

Prephenate has been known for decades as the product of the celebrated 3,3-sigmatropic rearrangement of chorismate, a reaction that is classically followed by formation of the aromatic amino acids phenylalanine and tyrosine (*1*). Prephenate dehydratase and prephenate dehydrogenase are the enzymes that direct prephenate to Phe and Tyr by decarboxylation and aromatization of the cyclohexenyl ring with concomitant loss of the C-7 hydroxide or hydride, respectively. Recently, we characterized the *Bacillus subtilis* prephenate dehydratase homologue BacA, which is involved in the biosynthesis of bacilysin [**1** (Figure 1)] and catalyzes the first step of a four-enzyme pathway that transforms prephenate to a distinct nonaromatic amino acid product (*2*). Like prephenate dehydratase, BacA decarboxylates prephenate at C-4 yet uniquely protonates C-6 or C-6$'$ to yield the endocyclic diene dihydro-4-hydroxyphenylpyruvate [H_2HPP, **5** (Figure 2A)] (*2*). This diene is then subject to nonenzymatic isomerization to the exocyclic diene in a reaction that can be dramatically accelerated by BacB to yield the indicated H_2HPP regioisomer **6**. Here we identify two homologues to BacA from distinct microbial secondary metabolite pathways that catalyze the same nonaromatizing decarboxylation of prephenate, thus establishing a new family of enzymes. The first of these is AerD from the cyanobacterium *Planktothrix agardhii,* which is involved in the production of the nonribosomal glycopeptides aeruginoside 126A and B [**3** (Figure 1)] (*3*). These natural products contain the unusual amino acid residue 2-carboxy-6-hydroxyoctahydroindole (Choi, **7**) (*4*, *5*). The second enzyme is SalX in the marine actinomycete *Salinispora tropica* biosynthetic pathway to the proteasome inhibitor salinosporamide A (**2**) (*6*−*8*). Specifically, SalX is relevant to the production of the nonproteinogenic amino acid building block cyclohexenylalanine (**9**, H_4Phe) (*9*).

In a study of nonribosomal peptide synthetase gene clusters in the cyanobacterium *P. agardhii* CYA126/8, gene insertions led to the discovery and identification of novel aeruginoside glycopeptides (*3*). Aeruginosin natural products, which are potent inhibitors of serine proteases, typically contain the unusual amino acid residue Choi (*4*). Three of the genes in the aeruginosin cluster, *aerDEF*, were found to be relevant to Choi production (*3*). Ishida et al. noted the similarity of *aerDEF* to the *bacABC* genes in the bacilysin gene cluster in *B. subtilis*. However, at the time of the aeruginosin gene cluster discovery, the specific roles of the BacABC proteins were unknown, so it was unclear what catalysts the congeneric *aer* genes were encoding. For example, it was hypothesized that arogenate, not prephenate, might be the entry point onto the Choi pathway (*3*). As noted by Ishida et al., AerD is 33.8% identical to BacA, so we sought to determine whether AerD was in fact a catalytic homologue of BacA. Because the aeruginoside producer *P. agardhii* CYA126/8 and its DNA were not readily available, we chose to use a synthetic gene for AerD (DNA 2.0), which was optimized for expression in *Escherichia coli* (Figure S2 of the Supporting Information). This gene was cloned into a pET-28b vector equipped with an N-terminal His_6 tag. As shown in Figure S3 of the Supporting Information, excellent overproduction of the soluble protein ensued, allowing

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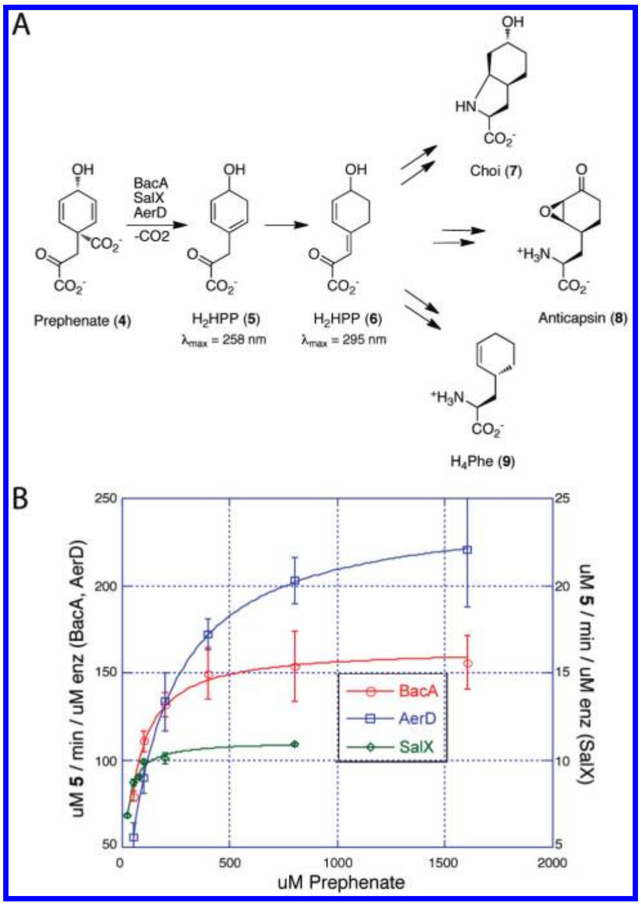

Figure 2: (A) Transformation of prephenate first to H_2HPP **6**, which is eventually incorporated into various amino acids. (B) Kinetics of BacA, AerD, and SalX.

purification of 14 mg/L of culture. We assayed AerD with prephenate as a substrate utilizing UV scanning kinetics and readily observed the conversion to an A_{258} species, indicative of endocyclic H_2HPP diene **5** (Figure 2 and Figure S4a of the Supporting Information). As we had found in studies with BacA from *B. subtilis*, H_2HPP **5** nonenzymatically isomerized to the thermodynamically favored conjugated exocyclic diene **6** (Figure 2). ^{1}H NMR analysis validated that AerD ultimately generates H_2HPP **6** (Figure S4b and Table S1 of the Supporting Information). The k_{cat} (245 ± 1 min^{-1}) and K_m (170 ± 3 μM) values for the conversion of prephenate to initial endocyclic diene **5** for AerD compare favorably with those for BacA. Thus, it is very likely prephenate is the physiological substrate for AerD in the initial step in the construction of the bicyclic amino acid Choi.

Salinosporamide A (**2**) from *S. tropica* CNB-440 has a high density of functionality built into its scaffold. The bicyclic β-lactone-γ-lactam framework is a latent warhead, and the C-2 chloroethyl side chain acts as a covalent trap for irreversible capture of the proteasome (*10*). The third element of the salinosporamide scaffold is the nonproteinogenic 3-hydroxyl-L-cyclohexenylalanyl residue [here termed β-hydroxylated H_4Phe (**9**)], which governs the binding specificity of the natural product for the proteasome (*11*). Examination of the salinosporamide biosynthetic gene cluster has indicated a set of *sal* genes that could be involved in H_4Phe formation, among them SalX (*8*). Although our initial proposal suggested SalX might function like a prephenate dehydratase for a putative dihydroprephenate substrate (*12*), we decided to test it for conversion of prephenate to **5** and then **6**, given the 35% sequence identity to BacA. Toward this end, the *salX* gene was amplified from *S. tropica* genomic DNA and ligated into an N-His_8 vector for expression in *E. coli*. As shown in Figure S3 of the Supporting Information, moderate overproduction of the soluble enzyme was obtained at 5 mg/L of culture. UV assays revealed that SalX also converts prephenate to endocyclic diene H_2HPP **5** as the initial product before spontaneous isomerization to exocyclic diene **6** (Figure 2 and Figure S4a of the Supporting Information), which was further confirmed by ^{1}H NMR (Figure S4b and Table S2 of the Supporting Information). While SalX had a 16−22-fold lower k_{cat} than AerD and BacA, it also had a lower K_m for prephenate. The SalX k_{cat}/K_m catalytic efficiency ratio of 0.73 min^{-1} μM^{-1} is only 2−4-fold lower than those of BacA and AerD, making it likely that prephenate is a physiologic substrate for SalX. To further probe whether prephenate is indeed the natural substrate for SalX and if H_2HPP **6** is an intermediate in H_4Phe production, feeding studies were preformed with a previously described *S. tropica salX*$^-$ disruption mutant that lacks salinosporamide production (*9*). H_2HPP **6** was generated and purified from an in vitro transformation of prephenate by SalX and then added aseptically to a 50 mL culture of the Δ*salX S. tropica* mutant. After 5 days, the culture was extracted and HPLC analysis indicated restoration of salinosporamide production (Figure S5 of the Supporting Information), which was confirmed by liquid chromatography−mass spectrometry analysis with authentic standards. This chemical complementation confirmed that **6** is a pathway intermediate of **2** biosynthesis in *S. tropica*.

We establish here a new family of prephenate decarboxylases that produce a nonaromatized H_2HPP product. The three new members of this family, BacA, AerD, and SalX, are capable of initiating divergent pathways that transform the primary metabolite prephenate into distinct nonproteinogenic amino acids used in natural product production. The intriguing aspect of these enzymes is that while decarboxylation occurs in a manner similar to that of prephenate dehydratases and dehydrogenases, these enzymes avoid aromatization by delivery of a proton to C-6 or C-6′ to yield a diene product. The net result is conversion of the 5,8-cyclohexadien-7-ol in prephenate to a 4,8-cyclohexadien-7-ol in H_2HPP product **5**. Isotopic labeling studies in *S. tropica* demonstrated that both halves of the cyclohexenyl ring of prephenate are distinguished during the biosynthetic transformation, suggesting a regioselectivity on the part of SalX (*12*). A similar biosynthetic observation was established in studies of the conversion of prephenate to the antibiotic 2,5-dihydrophenylalanine (*13*). However, additional detailed stereochemical analyses will be necessary to determine if all three enzymes share the same regio- and stereoselectivity with regard to protonation. Two possible mechanistic scenarios arise, one in which the reaction could involve a concerted pericyclic reaction in which the C-4 carboxylate becomes protonated and in turn donates a proton to C-6 (or C-6′) as CO_2 is evolved or a second mechanism in which a BH^+ conjugate acid in the enzymatic active site acts as the proton source. We are at present pursuing crystallization trials with BacA, AerD, and SalX to shed light on the mechanism and determine how these enzymes avoid the aromatization of prephenate that is typical of prephenate dehydratase and prephenate dehydrogenase-catalyzed transformations.

An initial BLAST search finds many other examples of proteins homologous to these enzymes, several of which are located near potential biosynthetic enzymes in host genomes such as *Nodularia spumigena*, *Clostridium celluloytican*, *Photorhabdus luminescens*, and *Erwinia amylovora*, the latter of which is known to produce 2,5-dihydrophenylalanine (DHPA) (*14*). DHPA is also produced in a variety of *Streptomyces* species, with recent data indicating production in *P. luminescens* (*15*) as well. Isotopic feeding studies in *Streptomyces arenae* demonstrated that DHPA is derived from prephenate (*13*), which suggests this enzyme family may be diverting prephenate into unique small molecule products across a broad array of microorganisms.

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SUPPORTING INFORMATION AVAILABLE

Materials and Methods, gene clusters for aeruginoside 126 and salinosporamide A, gene sequence for *aerD*, sodium dodecyl sulfate−polyacrylamide gel electrophoresis of protein expression, representative scanning kinetics for AerD and SalX, ^{1}H NMR data for the SalX and AerD products, feeding studies with *S. tropica*, alignment of BacA, AerD, and SalX with homologues from other organisms, and alignment of BacA, AerD, and SalX. This material is available free of charge via the Internet at http://pubs.acs.org.

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