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A Novel 4-Methylideneimidazole-5-one-Containing Tyrosine Aminomutase in Enediyne Antitumor Antibiotic C-1027 Biosynthesis

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C-1027, a chromoprotein natural product produced by *Streptomyces globisporus*, belongs to the enediyne family of antitumor antibiotics.[1,2] The C-1027 chromophore (**1**) is characterized by an enediyne core, a deoxy aminosugar, a benzoxazolinate, and an (*S*)-3-chloro-4,5-dihydroxy-β-phenylalanine moiety (**2**). We recently cloned and characterized the C-1027 biosynthetic gene cluster from *S. globisporus* and proposed that **2** is derived from L-tyrosine (**3**), implicating the involvement of an aminomutase in converting the α-amino acid precursor into the β-amino acid intermediate (Figure 1).[3] We now report that this reaction is catalyzed by SgcC4, a novel tyrosine aminomutase that converts **3** into (*S*)-β-tyrosine (**4**) during the biosynthesis of **1**. We also demonstrate that it employs the 4-methylideneimidazole-5-one (MIO) cofactor, of which dehydroalanine is a component.

Aminomutases characterized to date fall into four groups: radical-based enzymes that use either cobalamin or *S*-adenosylmethionine plus an iron−sulfur cluster,[4] pyridoxamine phosphate-dependent enzymes,[5] and ATP-dependent enzymes.[6] An aminomutase exhibiting characteristics different from the above groups has also been reported recently but was only in the form of cell-free extracts;[7] the exact nature of its catalytic mechanism remains to be determined.

None of the genes identified within the C-1027 gene cluster encode proteins with significant homology to known aminomutases. Instead, we identified *sgcC4*, whose deduced product shows high homology, including the signature Ala-Ser-Gly motif, to a family of ammonia lyases. These include the histidine ammonia lyase from *Streptomyces griseus*[8] (39% identity and 56% similarity) and the phenylalanine ammonia lyase from *Streptomyces maritimus*[9] (38% identity and 56% similarity). We inactivated *sgcC4* by replacing it with a mutant copy in which *sgcC4* was disrupted by *aac(3)IV* according to published procedures.[3] The resultant *S. globisporus* SB1007 mutant lost its ability to produce **1**, confirming that *sgcC4* is essential for the biosynthesis of **1**. On the basis of these results, we proposed that SgcC4 is a novel tyrosine aminomutase that acts via α,β-elimination of **3** to yield enzyme-bound ammonia and *p*-hydroxycinnamate (**5**), with subsequent Michael addition between ammonia and **5** to afford **4**. This represents an unprecedented mechanism for an aminomutase (Figure 2).

To validate this hypothesis, the *sgcC4* gene was amplified from pBS1005[3b] by PCR, and the resultant product was cloned as an *Nde*I-*Hin*dIII fragment into the same sites of pET28a and sequenced to confirm PCR fidelity, yielding the expression construct pBS1022.[10] Introduction of pBS1022 into *E. coli* BL-21(DE-3) resulted in the overproduction of SgcC4 as a His_6-tagged fusion protein, which was purified by affinity chromatography on Ni-NTA resin.[10] The purified SgcC4 (0.5 mg/mL) was incubated with **3** (0.5 mM) in

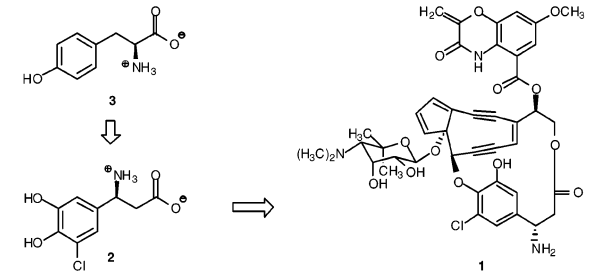

Figure 1. Structure of the C-1027 chromophore (**1**) and proposed biosynthetic origin of the (*S*)-3-chloro-4,5-dihydroxy-β-phenylalanine moiety (**2**) from L-tyrosine (**3**).

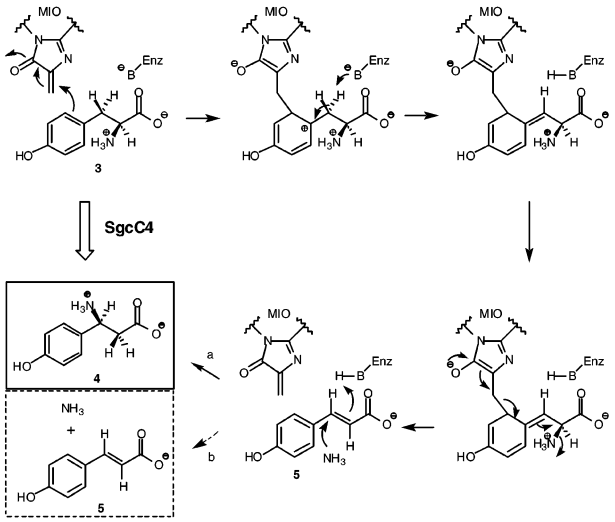

Figure 2. Proposed mechanism of the SgcC4 tyrosine aminomutase that catalyzes **3**-to-**4** conversion and employs MIO at its active site.

100 mM Tris-HCl, pH 8.8, at 25 °C to test directly the aminomutase activity in vitro. The reaction was terminated by addition of HCl to pH < 2. The quenched mixture was derivatized with *o*-phthaldialdehyde[11] and subjected to HPLC analysis.[12] Baseline separation for **3** and **4** as *o*-phthaldialdehyde derivatives and **5** was achieved (Figure 3, inset). As shown in Figure 3, >80% of **3** was converted into **4** with **5** as a minor product (<10%) after 180 min of incubation. The identities of **4** and **5** were verified by cochromatography with authentic standards and by characteristic UV absorption patterns. HPLC analysis using a chiral column[13] with both authentic (*S*)- and (*R*)-**4** as standards confirmed the enzymatic product to be (*S*)-**4**, as would be predicted from the stereochemistry of **1**. These results unambiguously establish SgcC4 as a tyrosine aminomutase that catalyzes the conversion of **3** to **4**. The fact that **5** was detected as a minor product provides direct evidence supporting the hypothesis that the SgcC4 catalyzed **3**-to-**4** conversion proceeds via **5** as an intermediate.

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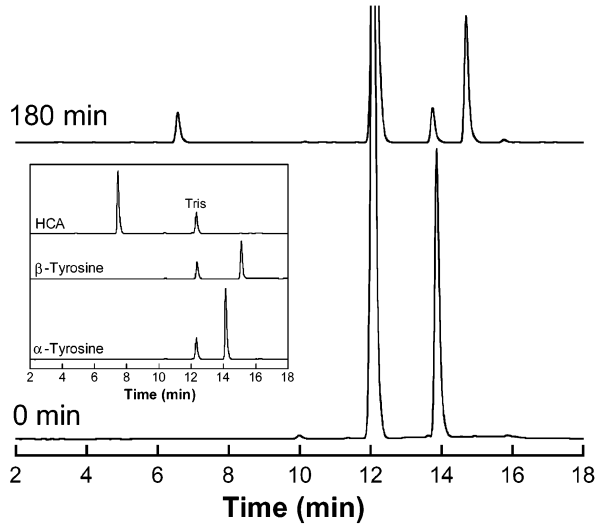

Figure 3. HPLC analysis of in vitro synthesis of (*S*)-*β*-tyrosine (**4**) from L-tyrosine (**3**) catalyzed by SgcC4. Traces for samples taken after 0 and 180 min of reaction are shown. Authentic standards are shown in the inset. The amino acids were analyzed as *o*-phthaldialdehyde derivatives and *p*-hydroxycinnamate (HCA) (**5**) in its underivatized form.

The active site of ammonia lyases was historically considered to contain a simple dehydroalanine residue until recent X-ray crystallographic studies provided very strong support in favor of MIO that is formed autocatalytically from the conserved Ala-Ser-Gly motif.[14] The MIO group is known to be inactivated by borohydride and cyanide.[15] To ascertain that the ScgC4 aminomutase utilizes MIO at its active site, we pretreated SgcC4 with either 10 mM $NaBH_4$ or 2 mM KCN. After removal of the excess reagents by filtration, the treated enzyme was assayed for tyrosine aminomutase activity. Consistent with the presence of MIO, pretreatment of SgcC4 with $NaBH_4$ rendered the enzyme totally inactive, and KCN treatment of SgcC4 resulted in >98% loss of its aminomutase activity. Inclusion of **3** (1 mM) during KCN pretreatment largely prevented the activity loss. In contrast, phenylalanine did not provide any protection against KCN inhibition, nor did it serve as a substrate for SgcC4. This implicates **3** as the precursor for **2** in the biosynthesis of **1**.

Finally, to confirm that Ser153 from the conserved Ala-Ser-Gly motif of SgcC4 plays a critical role in the MIO-mediated 2,3-aminomutase activity, we replaced Ser153 with Ala by site-directed mutagenesis.[16] The S153A mutation resulted in a 340-fold decrease in k_{cat}/K_M for SgcC4 as a tyrosine aminomutase. This compares well with the 430-fold decrease in k_{cat}/K_M for phenylalanine ammonia lyase[17] and the 1170-fold decrease in k_{cat}/K_M for histidine ammonia lyase[18] engendered by the analogous mutation. The difference UV spectrum of SgcC4 and its S153A mutant exhibited a discrete absorption maximum around 308 nm, characteristic for MIO.[19] Taken together, these results strongly support the hypothesis that SgcC4 is an unprecedented aminomutase that requires MIO for activity.

Our finding that SgcC4 is a tyrosine aminomutase extends known MIO-dependent chemistry from simple ammonia lyases to that of aminomutases. On the basis of the MIO-dependent ammonia lyase paradigm,[14] we envision that SgcC4 catalyzes the α,β-elimination (i.e., ammonia lyase reaction) of **3** to yield ammonia and **5** as enzyme bound intermediates. Instead of releasing ammonia and **5** as the end products, as do ammonia lyases (Figure 2, path b), SgcC4 subsequently catalyzes a Michael addition of ammonia to the *β*-position of **5** (Figure 2, path a), acting overall as an aminomutase catalyzing the net 2,3-ammonia migration (Figure 2). The detection of **5** as a minor product apparently results from a competition between the Michael addition of ammonia to **5** and the release of the intermediates from the enzyme complex. It remains to be determined if this phenomenon is biologically relevant in vivo. Given the high sequence homology between SgcC4 and MIO-dependent ammonia lyases, one wonders what the evolutionary relationship between SgcC4 and ammonia lyases is and whether the aminomutase activity could be engineered into ammonia lyases (and vice versa). One also wonders how widely MIO-dependent aminomutases are distributed in other organisms[7] and what the metabolic advantages of having yet another type of aminomutase are.[4−6]

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(13) Filtered SgcC4 reaction samples were loaded onto a Chirobiotic-T column (5 μm, 250 × 4.6 mm, Astec Inc., Whippany, NJ) and eluted isocratically with $CH_3OH/AcOH/Et_3N$ (100/0.1/0.1, vol/vol), at a flow rate of 1.0 mL/min with UV detection at 280 nm.
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