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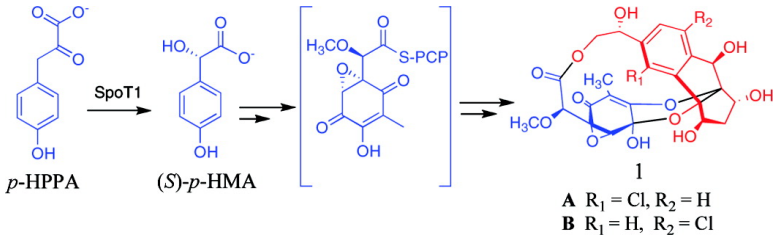

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Unraveling the Biosynthesis of the Sporolide Cyclohexenone Building Block

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Sporolides A and B (**1**) are novel polycyclic macrolides from the marine bacterium *Salinispora tropica* that are composed of a chlorinated cyclopenta[*a*]indene ring and a cyclohexenone moiety (Figure 1A).[1] The former is hypothesized to be nonenzymatically derived from an unstable nine-membered enediyne precursor by the nucleophilic addition of chloride.[2] Experimental data verifying this suggestion were lacking until sequence analysis of the 5 183 331 bp *S. tropica* CNB-440 genome led to the identification of two biosynthetic loci encoding enediyne polyketide synthases (PKSEs).[3] Analysis of the respective gene sequences strongly suggests one PKSE to catalyze the biosynthesis of a 9-membered enediyne (*spo*), which might be the nonaromatized predecessor of the sporolides, whereas the second PKSE (*pks1*) is associated with a 10-membered enediyne of unknown molecular architecture. The biosynthetic origin of the cyclohexenone building block of **1**, however, was unclear. Herein, we report the in vitro characterization of the gene *spoT1*, which is located within the putative sporolide cluster (Figure 2) and whose product catalyzes the initial biochemical reaction in the pathway to the cyclohexenone epoxide moiety of **1**.

Naturally occurring cyclohexane epoxide structural units are derived from a plethora of biosynthetic pathways.[4] We envisioned a multitude of biosynthetic scenarios via polyketide and shikimate origins to yield the highly functionalized cyclohexenone unit of **1**, via the hypothetical precursor **2**, which we confirmed in vivo originates from L-tyrosine and two L-methionine-derived methyl groups. MS analyses revealed incorporation rates of ~30% after assimilating the corresponding ^{13}C-labeled amino acids into **1** (see Supporting Information). Of particular interest was the finding that administration of tyrosine led to ~10-fold increase in sporolide production, identifying this amino acid as a limiting nutrient. Even though sporolide titers obtained from *S. tropica* CNB-440 are low (~100 μg/L), the feeding experiment underlines the opportunity to reach higher production levels by optimization of fermentation conditions.

To decipher the biosynthetic pathway for **2** in more detail, we focused on the gene *spoT1* that had been annotated to encode an Fe(II)-dioxygenase. Enzymes in this family are known to play a pivotal role in bacterial tyrosine degradation converting *p*-hydroxyphenylpyruvate (*p*-HPPA) to either homogentisate (HGA) or *p*-hydroxymandelate (*p*-HMA).[5] The gene product of *spoT1* may thus function as hydroxyphenylpyruvic acid dioxygenase (HPPD) or as hydroxymandelic acid synthase (HMAS), respectively. Both HGA and *p*-HMA were envisaged as probable intermediates during the biosynthesis of **2** (Figure 1A). A third plausible scenario involving the trapping of the epoxide intermediate of HGA formed during the oxidation step of HPPD reaction was also postulated.

In order to differentiate between these biosynthetic scenarios, we overexpressed and purified SpoT1 from *Escherichia coli* BL21(DE3) as an octahistidyl-tagged recombinant protein at ~100 mg/L via the pET28a(+)-based expression plasmid pHIS8.[6] Biochemical characterization with *p*-HPPA in the presence of Fe^{2+}, ascorbate, and catalase identified SpoT1 as a HMAS (Figure 1B), thereby revealing the initial biochemical step in the pathway to the cyclohexenone moiety.

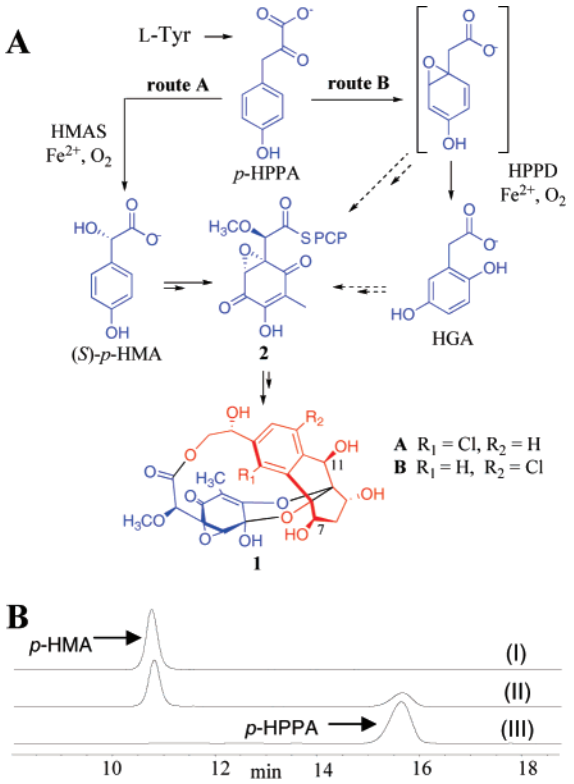

Figure 1. (A) Proposed enzymatic routes during the biosynthesis of the cyclohexenone moiety **2**. Experimental results described in the text are consistent with route A. (B) HPLC analysis of (I) *p*-HMA, (II) enzymatic reaction, and (III) *p*-HPPA without enzyme. HGA, which has a retention time of 14.6 min, was not an enzymatic product.

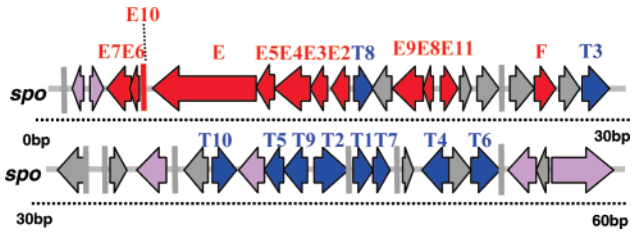

Figure 2. Organization of the 60 kb *spo* biosynthetic gene cluster. Putative assignments include *spoE*, *E2-E11*, and *spoF* in the biosynthesis of the enediyne core (red), *spoT1*−*T10* in the construction and attachment of **2** (blue), six genes involved in regulation, transport, and resistance (lilac), and 19 of unknown or unassigned function (gray).

p-HPPD was preferentially recognized by SpoT1 when testing a variety of candidate substrates (see Supporting Information). The absolute stereochemistry of *p*-HMA produced from a large-scale enzymatic reaction was established to be *S* by comparing its optical rotation ($[\alpha]_D$ +20.1, c = 0.17, H_2O) with that of synthetic (*S*)-*p*-HMA ($[\alpha]_D$ +26.3, c = 0.16, H_2O).[7] Since the corresponding

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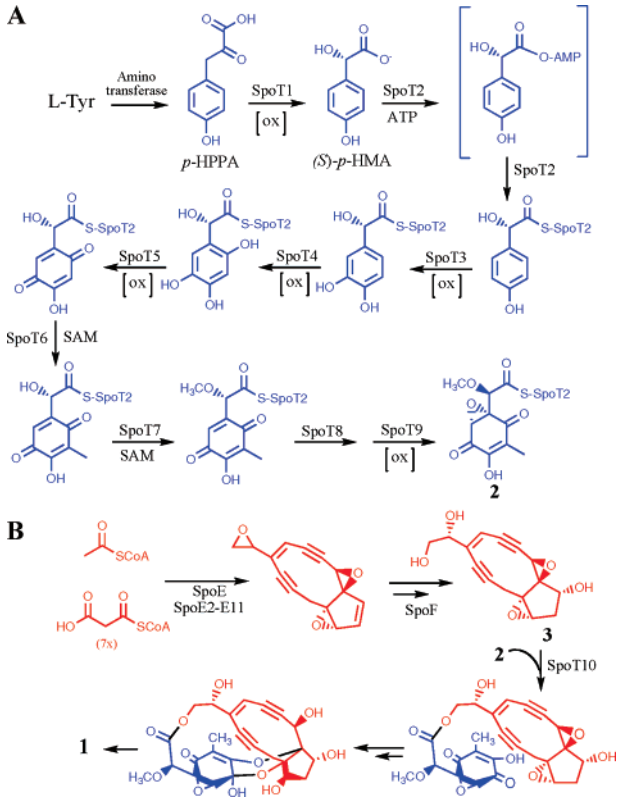
Scheme 1. Proposed Biosynthesis of the Sporolides A and B (**1**) Enediyne Precursor via Putative Precursors **2** (pathway A) and **3** (pathway B)



carbon in the natural product **1** is in the *R* configuration, epimerization of this center must occur during sporolide biosynthesis. Close inspection of the sporolide cluster revealed several candidate genes with a putative epimerase function, including *spoT8*.

The in vitro characterization of SpoT1 illuminated the initial biochemical reaction to the novel cyclohexenone epoxide unit of **1** and allowed us to postulate a pathway involving eight additional genes *spoT2*−*spoT9* to the proposed intermediate **2** (Scheme 1A). SpoT2 is a nonribosomal peptide synthetase (NRPS) adenylation−peptidyl carrier protein didomain predicted to activate hydroxyphenylglycine,[8] which is structurally analogous to *p*-HMA, suggesting that intermediates to **2** are enzyme bound. Other gene products include a phenol hydroxylase (SpoT3), a monooxygenase (SpoT4), an alcohol dehydrogenase (SpoT5), a *C*-methyltransferase (SpoT6), an *O*-methyltransferase (SpoT7), and a CYP450 monooxygenase (SpoT9). While the precise timing of these reactions remains to be determined, it is reasonable to assume that covalent tethering of the modified tyrosine residue occurs early in the pathway to divert the amino acid into secondary metabolism.[9]

The genes involved in the biosynthesis of the enediyne core of **1** share strong homology with those from other nine-membered enediyne PKS gene clusters, including C-1027, neocarzinostatin, and maduropeptin (Scheme 1B).[10] A strategy for assembly of the sporolide chromophore from the two building blocks likely takes place initially via the ester linkage, which is catalyzed by the type II NRPS condensation enzyme (SpoT10) in a related fashion to that in C-1027 biosynthesis.[9] The enzymes involved in the final ring closure via the two ether bridges are unknown and are most curious as this may facilitate the Bergmann cyclization reaction in the presporolide enediyne much like in calicheamicin[11] with its allylic trisulfide trigger for warhead cycloaromatization. The observed configuration of the OH-7 and OH-11 sporolide hydroxyl groups strongly suggests that the ether formation involves S_N2 epoxide ring openings as proposed in the biosynthesis of the polyether ionophore monensin.[12]

Inspection of the genome failed to clearly identify an apoprotein gene homologous to other nine-membered enediyne chromoprotein complexes.[10] The absence of such a protein may reflect the molecular basis for the putative nonenzymatic mechanism for the direct incorporation of a halogen atom in the enediyne products sporolides A and B.[2]

In summary, we have identified a gene cluster responsible for sporolide biosynthesis in *S. tropica* CNB-440. In vitro characterization of SpoT1 as HMAS which converts *p*-HPPA to (*S*)-*p*-HMA has shed light on how the cyclohexenone moiety is biosynthesized starting from L-tyrosine. This unusual pathway has shown a further role for HMAS, which until now has only been associated with the biosynthesis of (*S*)-hydroxyphenylglycine,[5] a non-proteinogenic amino acid incorporated into several biologically active secondary metabolites.

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Supporting Information Available: Methods section, a table of genes and their functions, and a purification gel figure of SpoT1. This material is available free of charge via the Internet at http://pubs.acs.org.

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