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Enzymatic Synthesis of Resorcylic Acid Lactones by Cooperation of Fungal Iterative Polyketide Synthases Involved in Hypothemycin Biosynthesis

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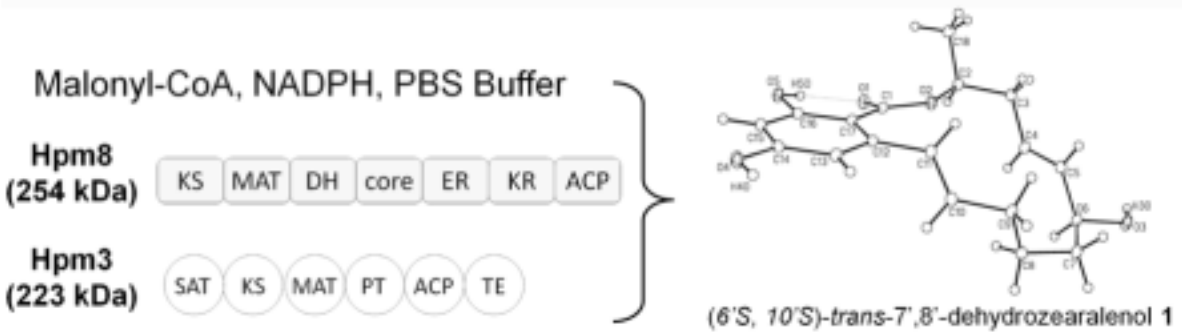
Abstract



Hypothemycin is a macrolide protein kinase inhibitor from the fungus *Hypomyces subiculosus*. During biosynthesis, its carbon framework is assembled by two iterative polyketide synthases (PKSs), Hpm8 (highly reducing) and Hpm3 (non-reducing). These were heterologously expressed in *Saccharomyces cerevisiae* BJ5464-NpgA, purified to near homogeneity and reconstituted *in vitro* to produce (*6′S, 10′S*)-*trans*-7′,8′-dehydrozearalenol (**1**) from malonyl-CoA and NADPH. The structure of **1** was determined by x-ray crystallographic analysis. In the absence of functional Hpm3, the reducing PKS Hpm8 produces and offloads truncated pyrone products instead of the expected hexaketide. The non-reducing Hpm3 is able to accept an *N*-acetylcysteamine thioester of a correctly functionalized hexaketide to form **1**, but it is unable to initiate polyketide formation from malonyl-CoA. We show that the starter unit acyltransferase (SAT) of Hpm3 is critical for crosstalk between the two enzymes and that the rate of biosynthesis of **1** is determined by the rate of hexaketide formation by Hpm8.

Polyketides of fungal origin represent an important family of natural products and display a wide range of biological activities.[1] Among them are the resorcylic acid lactones (RALs),

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Supporting Information Available: Experimental details and spectroscopic information are available free of charge via the internet at http://pubs.acs.org.

which are 12 or 14 member macrolides that contain a resorcinol carboxylate moiety, for example, zearalenone,[2] hypothemycin3 and radicicol.3–4 Hypothemycin (Figure 1) irreversibly inhibits a subset of kinases at nanomolar concentrations, including mitogen activated protein kinase (MEK) and human ERK2.5 Biosynthesis of fungal polyketides often utilizes iterative type I polyketide synthases (PKSs) that have single copies of domains that are used repeatedly in a highly programmed fashion.6 The representative hypothemycin (Hpm) PKS from *Hypomyces subiculosus* contains two iterative PKSs Hpm8 and Hpm3.[3] The highly reducing PKS Hpm8 is proposed to synthesize the reduced hexaketide (*7S, 11S, 2E, 8E*)-7,11-dihydroxy-dodeca-2,8-dienoate **2**, which is transferred to the downstream nonreducing PKS Hpm3. Hpm3 is proposed to extend **2** to a nonaketide, followed by regioselective cyclization and macrolactonization to afford 7′,8′-dehydrozearalenol **1**. Heterologous expression of both hypothemycin PKS genes (but neither gene alone) resulted in production of a product assigned as the *6′R*-epimer of **1**.[3]

The two Hpm PKSs are therefore proposed to be chemically “modular”, in which each iterative PKS specializes in generating polyketide backbones of different structural features. Crosstalk between the two PKSs transfers the correct intermediate **2** from the upstream Hpm8 to the downstream Hpm3, and is a crucial step in the biosynthesis. The protein-protein interaction between two megasynthases is similarly required in the biosynthesis of other RALs, the aflatoxin precursor norsolorinic acid,[7] and the recently discovered asperfuranone.[8] In this study we demonstrate the complete reconstitution of Hpm8-Hmp3 activities in vitro to synthesize **1**, and show that the *N*-terminal SAT domain plays a key role in facilitating acyltransfer of **2** bound to Hpm8.

Both Hpm8 (254 kDa) and Hpm3 (223 kDa) were solubly expressed with *C*-terminal hexahistidine tags in *Saccharomyces cerevisiae* BJ5464-NpgA[6b] and were purified to near homogeneity with yields of 1.5 mg/L and 2 mg/L, respectively (Figure S1). The Hpm8 acyl carrier protein (ACP) domain was mapped with Fourier transform mass spectrometry (FTMS), and phosphopantetheinylation was confirmed by Ppant ejection assays (Figures S2–S4).[9] The activities of the minimal PKS domains of Hpm8 were first probed in the presence of 2 mM malonyl-CoA without NADPH. LC-MS analysis of the organic extract showed accumulation of triketide α-pyrone **3**, which is formed through spontaneous cyclization of the unreduced triketide (Figure 2A). Addition of NADPH to Hpm8 with malonyl-CoA yielded the tetraketide and pentaketide α-pyrones **4** and **5**, respectively. The structures of **4** and **5** were confirmed by comparison to authentic standards made by chemical synthesis. Both **4** and **5** are shunt products that arise due to derailment of tailoring steps during later stages of chain extension, and are offloaded by Hpm8 via lactonization, in similar fashion as LovB.[6b] Hexaketide **2** was not detected in the reaction mixture, indicating off-loading of this product requires the partner protein Hpm3. In contrast, incubation of Hpm3 with malonyl-CoA did not produce any detectable polyketide products, suggesting the inability of this protein to initiate polyketide biosynthesis as a standalone PKS.

Upon adding 2 mM NADPH and malonyl-CoA to a reaction mixture containing both Hpm8 and Hpm3, a new peak (*m*/*z* $[M-H]^-$ 317) with UV absorbance pattern characteristic of resorcylic acid emerged in the organic extract (retention time (RT) = 23.3 min) (Figure 2B). Performing the Hpm8-Hpm3 reaction in the presence of [2-^{13}C]-malonate and the MatB-system[10] yielded a peak at the same RT and mass of *m*/*z* $[M-H]^-$ 326. The increase of 9 mass units is consistent with the expected nonaketide backbone, shown below to be **1**. In order to obtain sufficient **1** for structural characterization, the BJ5464-NpgA host was transformed with expression plasmids for both Hpm8 and Hpm3, and was cultured as described by Reeves et al.[3] The extract of the culture afforded a compound with the same RT on a chiral HPLC column, UV absorbance and mass fragmentation pattern as the in vitro synthesized compound shown in Figure 2B. Purification and NMR analysis supported the structure of **1**. It was then

crystallized, and subsequent x-ray analysis showed **1** to have *6′S,10′S* stereochemistry (Figure S5). Although the *10′S* stereochemistry was expected as the absolute stereochemistry of hypothemycin is known,[5b] the *6′S* configuration was surprising. If the KR domain were to catalyze all the keto reduction steps with the same facial stereochemistry, one would expect the final compound to be the *6′R,10′S*-diastereomer as initially proposed.[3]

To assess the kinetics of the synthesis of **1**, we quantified the product level by using [2-^{14}C]-malonyl-CoA and radioactive thin layer chromatography (TLC). When Hpm3 was fixed at 5 μM, the initial velocity of formation of **1** varied linearly with increasing concentrations of Hpm8, yielding an apparent rate constant of 0.11 min^{-1} (Figure S6A). In contrast, when Hpm8 concentration was fixed at 10, 20 or 30 μM, the initial velocities of formation of **1** at different Hpm3 concentrations were essentially constant at 1.1, 2.04 and 3.88 μM min^{-1}, respectively (Figure S6B). The overall reaction velocity is independent of Hpm3 concentration within this concentration range. Thus, synthesis of the hexaketide intermediate from malonyl-CoA catalyzed by Hpm8 is the rate limiting step. Transfer of this acyl intermediate to Hpm3 and the subsequent formation of **1** are faster, resulting in the rapid scavenging and offloading of **2**-Hpm8 by Hpm3.

The SAT domain at the *N*-terminus of Hpm3 is the most likely candidate to facilitate crosstalk between the two enzymes.[11] To probe the role of SAT, the Hpm3 point mutant Hpm3-SAT^0 was constructed by mutating the putative active site Ser121 within the GX*S*XG motif to alanine. Synthesis of **1** was completely abolished in the in vitro reaction containing Hpm3-SAT^0 and Hpm8 (Figure 2C), confirming the catalytic activity of the SAT domain is essential for acyl transfer between the proteins. To examine the specificity of SAT domain towards the partner PKS, we paired Hpm8 with the functionally equivalent PKS13 from the *Gibberella zeae* zearalenone pathway.[12] Surprisingly, they failed to function in tandem and no RAL products were detected. We then constructed a hybrid downstream PKS, PKSH1, in which the Hpm3 SAT domain was replaced with the noncognate PKS13 SAT domain. While PKSH1 was solubly expressed and all the domains were active in the presence of **2**-SNAC (see below), the hybrid enzyme was not able to communicate with Hpm8 towards the synthesis of **1** (Figure S7). These results illustrate that the SAT-mediated protein-protein interactions are highly specific.

To probe if an alternative priming pathway exists for Hpm3 in the presence of small-molecule precursors, we chemically synthesized the *N*-acetylcysteamine thioester hexaketide, **2**-SNAC (Scheme S3). LC-MS analysis revealed that **1** was produced by Hpm3 in the presence of **2**-SNAC in high yield (Figure 2D). The Hpm3-SAT^0 mutant was similarly primed with **2**-SNAC and produced **1** in comparable yield as the wild type (Figure 2E). This result shows that although the SAT domain is necessary for protein interaction, small molecule precursors can be directly captured by the Hpm3 to initiate biosynthesis of **1**. This SAT-independent pathway is probably facilitated by the direct priming of the KS domain (Figure 1). This is consistent with the SAT domains being dispensable in the precursor-directed biosynthesis of polyketide analogues by PKS13[12] and *Gibberella fujikuroi* PKS4.[13]

Synthesis and structural confirmation of **1** demonstrate that all ~30 catalytic steps in the synthesis of **1** have been reconstituted, including: i) formation of the reduced ketide acyl intermediate **2** by Hpm8; ii) communication between the two proteins and the successful transfer of the acyl intermediate; and iii) correct processing of the reduced intermediate by Hpm3. The presence of a single RAL **1** also illustrates only the completed hexaketide can be transferred to the downstream enzyme and elongated to a RAL. Despite this, free **2**-SNAC can be transformed to **1** by Hpm3.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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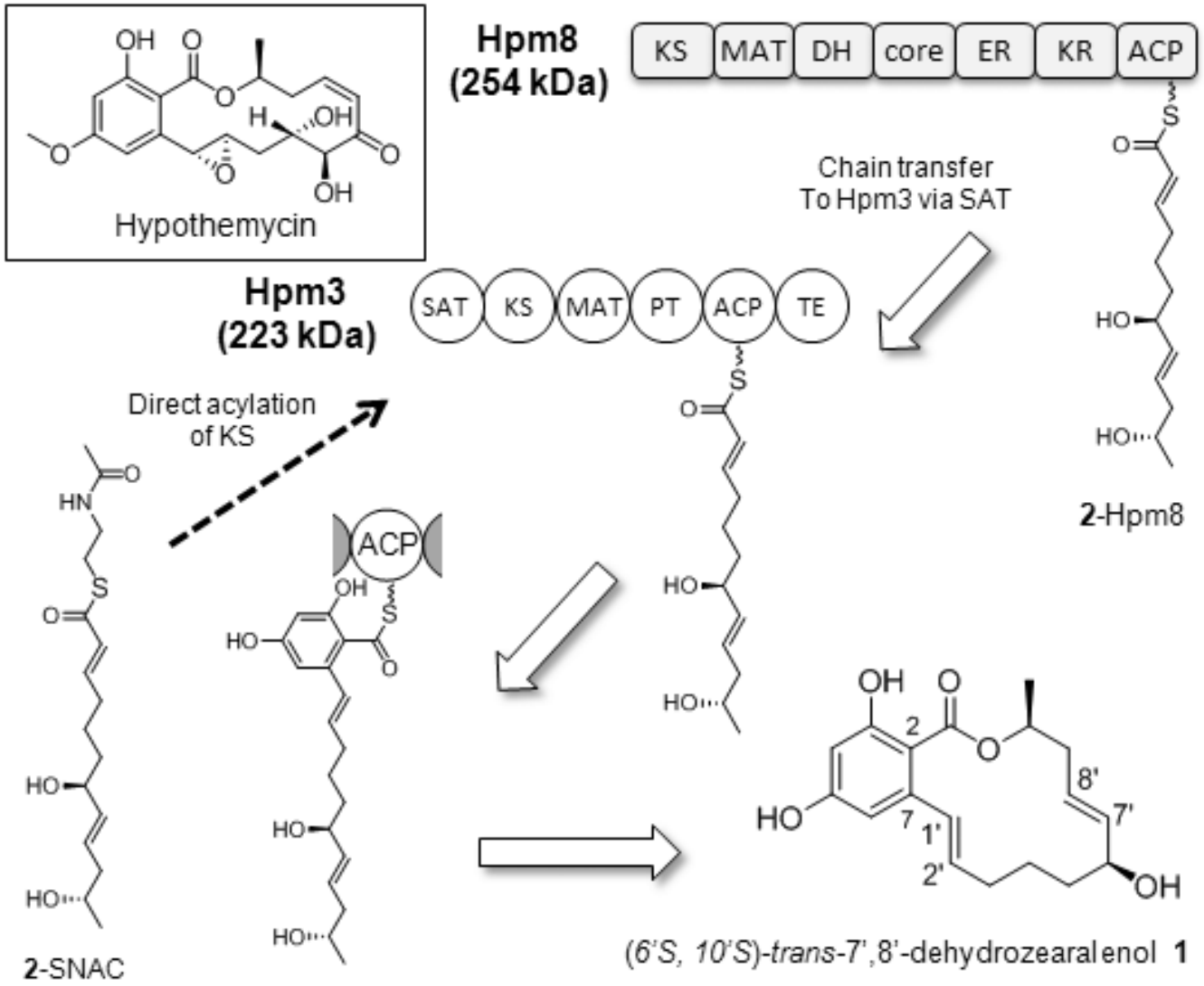

Figure 1.

Biosynthesis of (*6′S,10′S*)-7′,8′-dehydrozearalenol **1** by Hpm8 and Hpm3. Hpm8 consists of ketosynthase (KS), malonyl-CoA:ACP acyltransferase (MAT), dehydratase (DH), core, enoylreductase (ER), ketoreductase (KR), and acyl-carrier protein (ACP); Hpm3 consists of starter-unit:ACP transacylase (SAT), KS, MAT, product template (PT), ACP and thioesterase (TE). Hpm3 can accept hexaketide starter unit either from **2**-Hpm8 via SAT domain, or from **2**-SNAC directly.

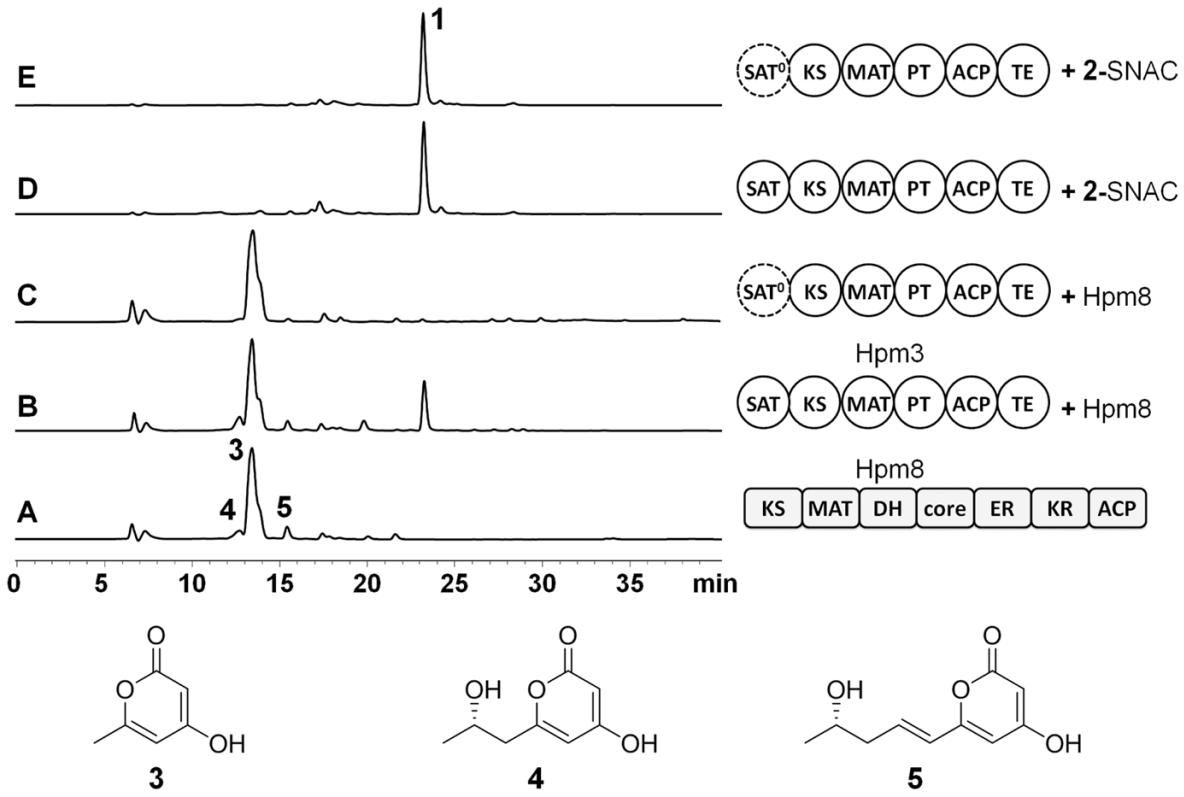

Figure 2.

Reconstitution of the Hpm iterative PKSs. HPLC analysis (300 nm) of polyketides synthesized by (**A**) Hpm8, (**B**) Hpm8 and Hpm3, (**C**) Hpm8 and Hpm3-SAT^0, (**D**) Hpm3 with 2 mM **2**-SNAC (**E**) Hpm3-SAT^0 with 2 mM **2**-SNAC. Megasynthases are added to 10 μM. In reactions **A–C**, 2 mM of NADPH and malonyl-CoA were added to PBS buffer, pH 7.4.