JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Communication

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Investigation of Fungal Iterative Polyketide Synthase Functions Using Partially Assembled Intermediates

Zhizeng Gao,[†,∥] Jingjing Wang,[‡,∥] Amy K. Norquay,[†] Kangjian Qiao,[‡] Yi Tang,*[,‡,§] and John C. Vederas*[,†]

[†]Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2G2

[‡]Department of Chemical and Biomolecular Engineering and [§]Department of Chemistry and Biochemistry, University of California, Los Angeles, California 90095, United States

Supporting Information

ABSTRACT: Iterative polyketide synthases (PKSs) are large, multifunctional enzymes that resemble eukaryotic fatty acid synthases but can make highly functionalized secondary metabolites using complex and unresolved programming rules. During biosynthesis of the kinase inhibitor hypothemycin by *Hypomyces subiculosus*, a highly reducing iterative PKS, Hpm8, cooperates with a non-reducing iterative PKS, Hpm3, to construct the advanced intermediate dehydrozearalenol (DHZ). The identity of putative intermediates in the formation of the highly reduced hexaketide portion of DHZ were confirmed by incorporation of ^{13}C-labeled *N*-acetylcysteamine (SNAC) thioesters using the purified enzymes. The results show that Hpm8 can accept SNAC thioesters of intermediates that are ready for transfer from its acyl carrier protein domain to its ketosynthase domain and assemble them into DHZ in cooperation with Hpm3. Addition of certain structurally modified analogues of intermediates to Hpm8 and Hpm3 can produce DHZ derivatives.

Polyketides display a wide spectrum of biological activities that make them an important resource for drug discovery.[1−4] Iterative polyketide synthases (PKSs), which often occur in fungi, have only a single copy of each domain (e.g., keto reduction, dehydration, and enoyl reduction) that can be utilized repeatedly for multiple cycles of chain elongation and tailoring of functionality (see Figure 1).[2,5−7] The control of product functionality by an iterative PKS depends on the structure of the growing chain covalently attached to the enzyme as a thioester as well as the exact protein sequence. Although understanding of the biosynthesis of polyketides is advancing rapidly, knowledge of the detailed programming by iterative PKSs is still limited.[8,9] A key requirement for understanding the mechanisms of PKS enzymes is the determination of the structures of intermediates that remain enzyme-bound during numerous successive steps of elongation and modification. Chemical termination at intermediate steps in conjunction with mass spectrometry (MS) has been effective for some modular bacterial PKS systems.[10−12] In fungal systems, direct FT-ICR-MS has been used to examine potential intermediates loaded on the PKS.[13,14] However, MS has limitations for elucidation of the stereochemistry of intermediates and analysis of possible isomers. An alternative approach pioneered by Cane, Hutchinson, and co-workers to probe the mechanism of modular bacterial PKS systems relies on the use of *N*-acetylcysteamine (SNAC) thioesters of partially assembled precursors.[15−17] Using PKS domain inactivation followed by addition of putative intermediate SNAC thioesters also allows bacterial polyketides to be produced in cell-free systems.[18,19] However, it is challenging to apply this advanced precursor feeding approach to iterative PKS systems because domain inactivation abolishes the production of polyketides. In addition, this approach often fails with whole or disrupted fungal cells because of catabolism of the precursors, except in some isolated cases.[20−22] Therefore, the development of new methods to probe fungal PKS function using partially assembled intermediates could aid the elucidation of these complex machineries.

Hypothemycin biosynthesis has been studied in considerable detail.[23−25] Two iterative PKS proteins, Hpm8 and Hpm3, build the polyketide backbone of hypothemycin. Hpm8, a highly reducing PKS (HRPKS), first assembles a reduced hexaketide intermediate. With the assistance of a starter unit acyl-carrier protein transacylase (SAT) domain, this newly formed hexaketide is transferred to Hpm3, a nonreducing PKS (NRPKS), where it is further extended to a nonaketide, which then undergoes regioselective cyclization and macrolactonization to afford (6′*S*,10′*S*)-7′,8′-dehydrozearalenol (DHZ).[24] Subsequent post-PKS modifications of DHZ by other enzymes afford hypothemycin. Although the general functions of the two PKSs have been assigned, it remains unresolved how Hpm8 controls the tailoring of the intermediates en route to its hexaketide product using its reductive domains (KR, DH, and ER) in a permutative fashion. To probe the programing rules of HRPKSs, confirmation of the structures of enzyme-bound intermediates and the way they interact with HRPKSs would be desirable. Here we report efforts to confirm such intermediate structures by in vitro incorporation of partly assembled precursors into Hpm8.

On the basis of the accepted mechanism for polyketide biosynthesis,[1] we propose that there are 14 acyl carrier protein (ACP)-tethered intermediates en route to the hexaketide that is eventually transferred to Hpm3 [Figure 1; also see the Supporting Information (SI)].[24] Among these 14 compounds, four are classified as "ready" precursors because they have the correct functionality to proceed to the next round of chain elongation by Claisen condensation. The other 10 putative

Received: January 7, 2013
Published: January 28, 2013

 © 2013 American Chemical Society dx.doi.org/10.1021/ja4001823 | *J. Am. Chem. Soc.* 2013, 135, 1735−1738

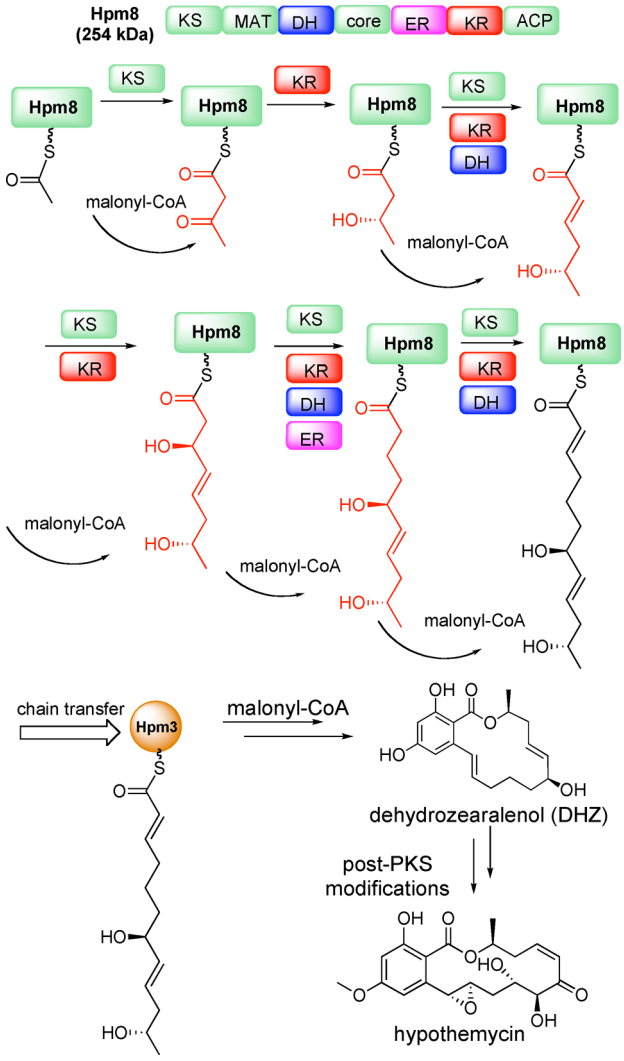


Figure 1. Biosynthesis of hypothemycin. Hpm8 assembles a hexaketide intermediate, which is then transferred to Hpm3. Hpm3 extends the hexaketide intermediate to DHZ. Intermediates shown in red were synthesized as ^{13}C-labeled SNAC thioesters.

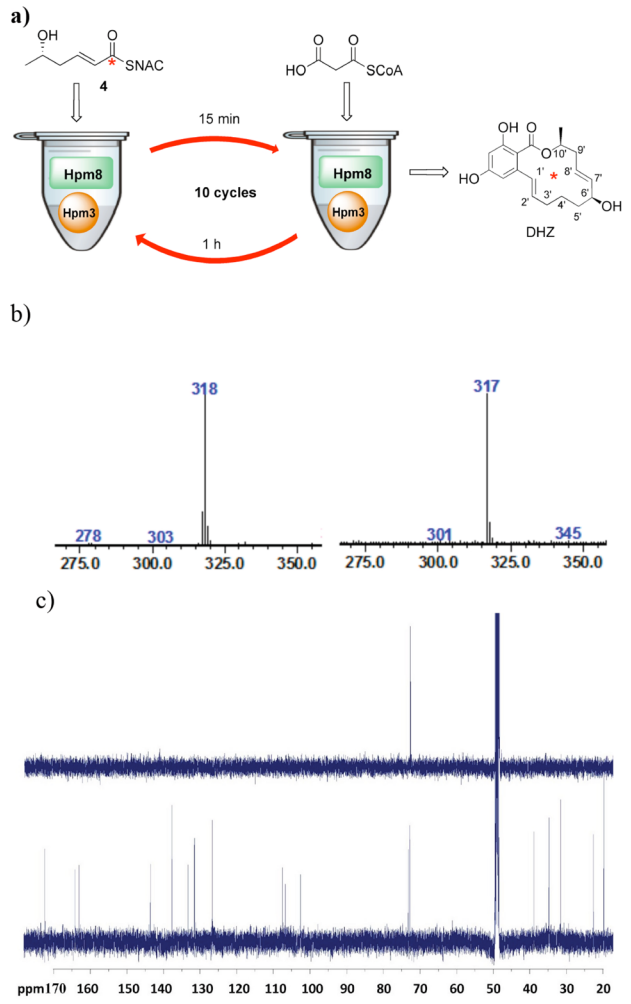


Figure 2. (a) Incorporation of triketide **4** into DHZ. (b) Mass spectra of (left) DHZ incorporating **4** and (right) the unlabeled DHZ standard. (c) ^{13}C NMR spectra of (top) DHZ incorporating **4** and (bottom) the unlabeled DHZ standard.

intermediates are "unready" precursors because they must first be modified by KR, DH, or ER domains prior to chain extension.

Our idea was to synthesize of all of the "ready" precursors (**2**, **4**, **6**, and **8**) and several "unready" precursors (**1**, **3**, **5**, and **7**) as ^{13}C-labeled SNAC thioesters (see Table 1) and determine whether these compounds could be linked to Hpm8 to give corresponding enzyme-bound intermediates that could be elaborated to the correct hexaketide and ultimately to ^{13}C-labeled DHZ. We selected the "unready" precursors **1**, **3**, **5** and **7** because they cover the scope of possible oxidation states at the β-position of the intermediates. The stereochemistry of the β-hydroxyl of "unready" precursor **3** is based on the stereoselectivity of the KR domain of Hpm8.[25]

The first substrate tested was "ready" triketide **4**, which was labeled with ^{13}C at the carbonyl that is proposed to become C6′ in DHZ (Figure 2a; for syntheses, see the SI). To avoid a large background of unlabeled DHZ that could be formed by initial self-loading of unlabeled malonyl-CoA,[26] we designed a "preloading" assay consisting of (1) incubation of Hpm8 (10 μM) with a large excess of labeled substrate (e.g., **4**) (0.2 mM) for 15 min (preload); (2) addition of 10 μM Hpm3, 4 mM NADPH, 0.4 mM malonyl-CoA, incubated for 1 h; and (3) subsequent addition of 0.4 mM malonyl-CoA at 1 h intervals (nine times) and addition of 0.2 mM labeled substrate every 2 h (three times). Analysis of the reaction mixture by LC−MS after incorporation of **4** generated a compound identical to DHZ in terms of LC retention time and UV absorbance. This compound showed a major peak in the mass spectrum at m/z 318 ($[M - H]^-$) (Figure 2b), indicating that it consists primarily of a singly ^{13}C-labeled DHZ. The incorporation ratio was calculated to be 78% on the basis of this mass spectrum.[27]

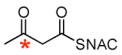
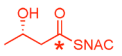
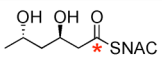
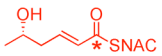
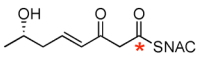
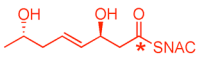
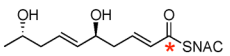
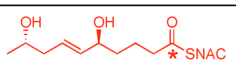
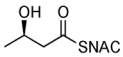
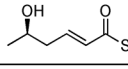
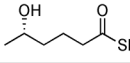
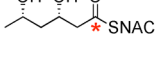
To provide further confirmation of the location of the ^{13}C enrichment, we isolated ~20 μg of ^{13}C-labeled DHZ (based on UV absorbance). The ^{13}C NMR spectrum (Figure 2c) showed only a single resonance at 72.8 ppm, corresponding to the C6′ position of DHZ. This demonstrates that C6′ of DHZ is ^{13}C-enriched, consistent with the specific incorporation of the triketide portion of **4** and its subsequent conversion into the final product. These data illustrate that the ketosynthase (KS) domain of Hpm8 can be primed by trans-thioesterification with the "ready" precursor **4**, which can then be elongated to the hexaketide moiety that is subsequently transformed by the NRPKS Hpm3 into DHZ. This represents the first example of

dx.doi.org/10.1021/ja4001823 | J. Am. Chem. Soc. 2013, 135, 1735−1738

the incorporation of a labeled advanced intermediate by a purified iterative HRPKS.

We next examined the incorporation of the putative intermediates shown in Table 1: diketides **1** and **2**, triketide **3**, tetraketides **5** and **6**, and pentaketides **7** and **8**. The "ready" precursors **2**, **6**, and **8** were incorporated efficiently, with ^{13}C enrichments of 37, 27, and 39%, respectively, based on mass spectra (Table 1). The relative amounts of the resorcylic acid lactone (RAL) produced, compared with that obtained with no precursor addition, ranged from 1.0 to 2.6 based on UV absorbance. Interestingly, the more advanced the precursor, the more DHZ was produced, possibly because advanced precursors require fewer additional reactions to reach the final product. Alternatively, the higher yields may also be due to higher rates of priming (trans-thioesterification) of the more advanced precursors onto the cysteine thiol of the KS domain.

Table 1. Incorporation of Partially Assembled Precursor Analogues

	Intermediate Analogue[a,b]	^{13}C Enrichment	Relative RAL yield[c]
0	none	none observed	1.0
1		41%	0.4
2		37%	1.0
3		15%	0.6
4		78%	1.0
5		19%	0.4
6		27%	2.0
7		8%	0.9
8		39%	2.6
	Unnatural analogue[d]	Production[e]	Relative RAL yield[c]
9		24%	1.3
10		25%	1.5
11		30%	2.0
12		0%	0.4

[a] * indicates the location of the ^{13}C label. [b]Structures of "ready" precursors are shown in red. [c]The relative resorcylic acid lactone (RAL) yields include DHZ and, for unnatural precursor analogues, the amount of the corresponding DHZ analogues. [d]Compounds **9**–**12** have incorrect stereochemistry or functionality for conversion to DHZ and (except for **12**) were therefore transformed to the corresponding DHZ analogues. [e]Production is the amount of DHZ analogue formed, expressed as a percentage of total RAL production.

In contrast, with exception of diketide **1**, which may also mimic malonyl-CoA, conversions of the "unready" precursors **1**, **3**, **5**, and **7** gave relatively lower ratios of incorporation into DHZ (41, 15, 19, and 8%, respectively) and poorer relative RAL production (0.4, 0.6, 0.4, and 0.9, respectively). For example, the lack of α,β-dehydration in **3** compared with **4** and the unrealized enoyl reduction of **7** compared with **8** both resulted in a >5-fold decrease in the incorporation of the labeled precursor. Therefore, the Hpm8 machinery clearly prefers precursor analogues that are structurally comparable to the correctly tailored intermediates ready for the next round of chain extension. The differences between the incorporation efficiencies obtained in the "ready" and "unready" precursor feeding experiments provide insights into the mechanisms of the Hpm8 multidomain enzyme. For the "ready" precursors, the subsequent enzymatic steps can be initiated immediately after priming (trans-thioesterification) of the KS domain.[28] The carbon chains of "ready" precursors are correctly functionalized, so they can easily be primed on KS domains and serve as the starting material for next round of chain elongation. For incorporation of "unready" precursors, several alternative mechanisms can be considered. One possibility is that the "unready" precursors load directly onto the ACP by trans-esterification and are then further converted into the "ready" forms in the normal fashion. However, as free ACPs are normally loaded with malonyl-CoA by the MAT domain, competition for the vacant ACP thiol is likely to be very inefficient. An alternative path would involve loading on the active-site thiol of the KS domain with subsequent trans-esterification to the ACP domain followed by modification into the "ready" precursors. This "KS domain to ACP domain" transfer of incompletely tailored fragments does not occur naturally, so this process may be very slow or impossible. The most likely pathway may be direct action of reducing/tailoring domains on the "unready" SNAC esters to transform them into "ready" SNAC derivatives. In our previous studies,[25] we found that "unready" SNAC esters such as **1** and **5** can be reduced by Hpm8 to give the "ready" precursors **2** and **6**, respectively. The KR domain can recognize the SNAC moiety, and this recognition can correctly direct the acyl chain to the active site for reduction. The analogous concept may apply for the direct transformation of **7** to **8** by the ER domain or of **3** to **4** by the DH domain. The DH domain has been shown to be inefficient at utilizing SNAC precursors,[29] which may account for the significantly lower incorporation of **3**.

We also observed that the "unready" β-keto analogues **1** and **5** produced much less DHZ, perhaps because they inhibit the natural chain extension. With the unlabeled DHZ production inhibited, the incorporation ratio (^{13}C enrichment) of **1** and **5** would also be relatively high, as observed.

To probe further the substrate tolerance of the KS domain of Hpm8, the unnatural precursors **9**–**12** were chemically synthesized for use in the assay to see whether they would produce DHZ analogues. Compounds **9** and **10** have an epimeric distal alcohol, whereas **11** lacks the conjugated double bond. As the functions of the tailoring domains of PKS

dx.doi.org/10.1021/ja4001823 | J. Am. Chem. Soc. 2013, 135, 1735–1738

enzymes are processive and limited to the two carbons adjacent to the thioester carbonyl,[1−7] such "incorrect" functionality cannot be modified to give the natural DHZ product. Hpm8 is able to accept **9** or **10** to form 10′-*epi*-DHZ, but with much lower efficiency (the relative ratio of *epi*-DHZ to DHZ formed directly from malonate was 25:75; Table 1). This is consistent with our in vivo observation that Hpm8 showed discrimination toward the stereochemistry of that hydroxyl group.[25] Compound **11**, which represents an over-reduced precursor, was similarly incorporated by this system to generate *β*-zearalenol (the relative ratio of *β*-zearalenol to DHZ was 3:7). Compound **12** is a diastereomer of the "unready" precursor **3**. Our previous work[25] showed that the KR domain of Hpm8 displays strict stereospecificity toward *β*-keto intermediates that is dependent on the chain length. We selected **12**, which is not an expected intermediate, to test whether the DH domain is able to eliminate a triketide alcohol with unnatural stereochemistry. The results showed that very little if any **12** was incorporated into DHZ. This demonstrates that the (*S*)-hydroxyl group is not a good substrate in the DH active site, which is consistent with results for other PKS and FAS systems.[30] The overall decrease in RAL yield suggests that the system is inhibited by this unnatural precursor analogue.

In summary, a series of ^{13}C-labeled intermediate SNAC thioesters were chemically synthesized and incorporated into DHZ using purified Hpm8 and Hpm3. Our results show an interesting pattern of the incorporation of partially assembled precursor analogues at the HRPKS stage: (1) "ready" precursors are easily recognized and taken up by Hpm8; (2) "unready" precursors are incorporated less effectively by Hpm8, but some incorporation is still observed, albeit with lower yields of DHZ; (3) unnatural precursor analogues can be incorporated, but the efficiency is dependent on the nature of the structural changes. Our findings not only further support the processive nature of polyketide biosynthesis but also provide guidelines for precursor-directed biosynthesis to generate novel polyketides with improved biological profiles.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthetic procedures for labeled precursors and experimental details for incorporations by Hpm8 and Hpm3. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

yitang@ucla.edu; john.vederas@ualberta.ca

Author Contributions

‖Z.G. and J.W. contributed equally.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Mark Miskolzie for assistance with NMR experiments and the Natural Sciences and Engineering Research Council of Canada, the Canada Research Chair in Bioorganic and Medicinal Chemistry, and the U.S. National Institutes of Health (1R01GM085128 and 1DP1GM106413) for financial support. Dedicated to Prof. Christoph Tamm (Univ. Basel) on the occasion of his 90th birthday.

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