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A Long-Range Acting Dehydratase Domain as the Missing Link for C17-Dehydration in iso-Migrastatin Biosynthesis

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Abstract

The dehydratase domains (DHs) of the iso-migrastatin (iso-MGS) polyketide synthase (PKS) were investigated by systematic inactivation of the DHs in module-6, -9, -10 of MgsF (i.e., DH6, DH9, DH10) and module-11 of MgsG (i.e., DH11) in vivo, followed by structural characterization of the metabolites accumulated by the mutants, and biochemical characterization of DH10 in vitro, using polyketide substrate mimics with varying chain lengths. These studies allowed us to assign the functions for all four DHs, identifying DH10 as the dedicated dehydratase that catalyzes the dehydration of the C17 hydroxy group during iso-MGS biosynthesis. In contrast to canonical DHs that catalyze dehydration of the β -hydroxy groups of the nascent polyketide intermediates, DH10 acts in a long-range manner that is unprecedented for type I PKSs, a novel dehydration mechanism that could be exploited for polyketide structural diversity by combinatorial biosynthesis and synthetic biology.

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Long-rang acting domain: inactivation of dehydratase domains of iso-migrastatin polyketide synthase, followed by in vitro characterization of DH10 toward substrate mimics with varying chain lengths, allowed us to identify DH10 as the dedicated dehydratase for elimination of a remote hydroxy group at C17 position during the biosynthesis of isomigrastatin, the function of which was “missing” in module-4 according to the collinear model.

Keywords

biosynthesis; dehydratase; iso-migrastatin; natural products; polyketides

iso-Migrastatin (iso-MGS, **1**), first isolated from *Streptomyces platensis* NRRL 18993,^[1] is a 12-membered macrolide belonging to the glutarimide-containing polyketide family of natural products. Members of this family feature varying oxidation states such as lactimidomycin (LTM, **2**),^[2] different polyketide chain lengths as exemplified by streptimidone (**3**),^[3] 9-methylstreptimidone (**4**),^[4] and cycloheximide (**5**),^[5] an enlarged 14-membered macrolide like migrastatin (MGS, **6**),^[6] and acyclic dorrigocin-type polyketides (e.g. dorrigocin A, **7**)^[7] (Figure 1). While **1**, **6**, and **7** were originally isolated from the same *S. platensis* NRRL 18993 strain independently,^[1,6,7] it was subsequently established that **6** and **7** resulted from **1**, via a H₂O-mediated ring expansion and ring-opening rearrangement, respectively, revealing a common biosynthetic origin for iso-MGS, MGS, and the dorrigocins.^[8]

The glutarimide-containing polyketide family of natural products exhibits multifaceted biological activities, among which the cell migration inhibitory activity originally discovered from **6** has attracted broad interest.^[6,9] Albeit moderate for the natural product, cell migration inhibition potency has been significantly improved by preparing analogs mimicking the 14-membered macrolide moiety of **6**.^[10] Members of the 12-membered macrolides, such as **1** and **2**, exhibited potent cytotoxicity, although their inhibitory activities toward tumor cell migration remained controversial.^[11] Although the detailed modes of action that dictate and differentiate cell-migrating inhibition from cytotoxicity remain elusive for this family of natural products,^[9] the discoveries of actin-bundling protein fascin as the target for cell migration inhibitory activity of **6**^[12] and of blocking the translocation step in initiation of eukaryotic protein translation as the mechanism for cytotoxicity of **1**^[13]

are exciting, serving as great inspiration for the development of this family of natural products as small molecule probes^[13] or promising anticancer drug leads.^[9–12]

We have previously cloned and sequenced the *mgs* biosynthetic gene cluster from *S. platensis* NRRL 18993 and confirmed that the 11-gene cluster, consisting of *mgsABCDEFGHIJK*, is necessary and sufficient for production of **1**.^[14] We subsequently discovered that the iso-MGS acyltransferase (AT)-less type I polyketide synthase (PKS) produced two nascent polyketide intermediates, **8** (>95%) and **11** (<5%), which were further converted by three tailoring enzymes, MgsIJK, to **1** (from **8**) as the major product, together with **12** and **13** (from **11**) as minor products (Figures 2 and S1).^[15] Characterization of the *ltm* biosynthetic gene cluster from *Streptomyces amphibiosporus* ATCC 53964 revealed a similar LTM AT-less type I PKS, with high sequence similarity to that of iso-MGS, producing **9** as the nascent product.^[16] Only one tailoring enzyme, LtmK, was found for LTM biosynthesis, which catalyzed the conversion of **9** to **2** (Figures 2 and S2).^[16]

Comparative analysis of the iso-MGS and LTM AT-less type I PKSs revealed several common features involving “missing” domains in corresponding modules (Figures 2 and S1–S3), which deviate from the canonical collinear model of type I PKS.^[17] Only two mechanisms, known as module skipping and module stuttering,^[18] have been proposed to account for the loss of type I PKS collinearity at the modular level, while deviations from the collinear model at the domain level appear to be quite common, in particular for AT-less type I PKSs,^[19a,b] the molecular mechanisms of which however remain poorly understood.^[19] Thus, recognizing the fact that the varying nascent polyketide scaffolds result from iso-MGS and LTM PKSs with similar modular and domain organizations, we reason that **1** and **2** biosynthesis provides an outstanding opportunity to discover new molecular logic for type I PKS catalysis. In particular, module-4 of both iso-MGS and LTM PKSs consists of the same ketosynthase (KS)-ketoreductase (KR)-acyl carrier protein (ACP) domains (Figure 2), and a survey of the AT-less PKSs responsible for the biosynthesis of **1**, **2**, **4**, and **5** in fact revealed an absolutely conserved domain organization for the first six modules (Figure S3).^[14,16,20] Considering that the presence of the hydroxy group at C17 for **2**, or its equivalents C9 for **4** and C7 for **5**, correlates well with the domain organization of module-4 according to the collinear model (Figures 1 and 2), the “missing” dehydratase activity, responsible for C16–C17 double bond formation in **8**, en route to the eventual methylene group at C17 of **1**,^[15] therefore must have been provided by one of the four dehydratase domains (DHs) in PKS module-6, -9, -10 of MgsF (i.e., DH6, DH9, DH10) or PKS module-11 of MgsG (i.e., DH11) during **1** biosynthesis (Figures 2 and S1).

Here we report systematic inactivation of DH6, DH9, DH10, and DH11 of iso-MGS PKS in vivo, followed by isolation and structural characterization of the metabolites accumulated by the mutants, and biochemical characterization of MgsF-DH10 in vitro, using N-acetylcysteamine (SNAC) thioesters with varying polyketide chain lengths as substrate mimics of the nascent ACP-tethered polyketide intermediates. Findings from these studies allowed us to make functional assignments for the four DHs, revealing DH10 to provide the “missing” dehydratase activity by catalyzing dehydration of the C17 hydroxy group to form the C16–C17 double bond in biosynthesis of **8** in a long-range acting manner that acts on **21** as the preferred substrate.

We first systematically inactivated DH6, DH9, and DH10 of MgsF and DH11 of MgsG by mutating their active sites^[21] Asp and/or His to Ala on pBS11001 (SI), a BAC vector carrying the complete *mgs* gene cluster that have been constructed for **1** production in several heterologous *Streptomyces* species.^[14b] The resultant variants (pBS11030–pBS11037) with the desired mutations (Figure S4) were introduced into *Streptomyces albus* J1074, yielding mutant strains SB11027 [DH6*, MgsF (D2038A)], SB11028 [DH9*, MgsF (H6136A)], SB11029 [DH10*, MgsF (H7705A)], SB11030 [DH10*, MgsF (D7895A)], SB11031 [DH11*, MgsG (H806A)], SB11032 [DH11*, MgsG (D975A)], SB11033 [DH11*, MgsG (H806A and D975A)], and SB11034 [DH10*DH11*, MgsF (D7895A) and MgsG (H806A)] (SI).

We next fermented each of the mutant strains, with SB11001 [i.e., *S. albus* J1074 (pBS11001)] as a control, under the previously established conditions^[14b] to investigate the effects of DH mutations on biosynthesis of **1** (SI). Figure 3 summarizes representative metabolite profiles of the DH mutants, in comparison with the control, and in total, six new metabolites (**10**, **14–18**), as well as three known iso-MGS analogs (**11–13**), were isolated from the DH mutant strains (SI), the structures of which were established by HR-ESI-MS, 1D and 2D NMR analysis (Tables S3–S7 and Figures S5, S10–17).

We subsequently made functional assignments to the four DHs based on the phenotypes of their corresponding mutants. Thus, a functional DH6 was crucial to the biosynthetic machinery of **1** since the corresponding mutant SB11027 completely abolished the production of **1**, without accumulating any detectable biosynthetic intermediates (Figure 3B, panels I vs II). DH9 catalyzed the canonical β -dehydration of the C7 hydroxy group of the ACP-tethered growing polyketide intermediate, supported by the accumulation of **10** as the predominant product by the corresponding mutant SB11028 (Figure 3B, panels I vs III). The fact that **10** was isolated as a full length and cyclized congener of **8** also revealed that the downstream PKS module-10 and -11 had broad substrate promiscuity, capable of accepting and processing biosynthetic intermediates with a C7 hydroxy group to completion. The DH10 mutants SB11029 and SB11030 completely abolished production of **1** but accumulated **11**, **12**, and **13**, with the latter as the major product, all of which featured a C17 hydroxy group (Figure 3B, panels I vs IV and see Figure S6 for SB11030). This was unexpected since **11–13** had previously been found from the wild-type only as the minor products.^[15] The exclusive accumulation of **11–13** in SB11029 and SB11030 therefore, for the first time, suggested DH10 to provide the “missing” dehydratase activity in module-4 for the formation of C16–C17 double bond in **8**, acting in a long-range manner that is unprecedented for type I PKS (Figure 2). DH11 catalyzed the canonical β -dehydration of the C3 hydroxy group of the ACP-tethered full length polyketide intermediate, as evidenced by the isolation of **14** as the major metabolite, featuring a C3 hydroxy group, together with **1** and **15**, from the corresponding mutants SB11031, SB11032, and SB11033 (Figure 3B, panels I vs V and Figure S6 for SB11032 and SB11033). The fact that DH11 mutants still produced **1**, albeit at a reduced titer, indicated that the function of DH11 could be partially complemented by a yet to be identified dehydratase activity. The accumulation of **15** and **14** revealed a subtle reliance of PKS module-11 on the fate of the C3 hydroxy group of the ACP-tethered growing polyketide intermediate for the last cycle of chain elongation and TE-

catalyzed cyclization of the resultant full polyketide intermediate, respectively (Figures 2 and S1).

Functional assignments for DH10 and DH11 were further supported by the double-DH mutant SB11034, which accumulated **11–13**, all with a C17 hydroxy group, **16** and **18**, with both C17 and C3 hydroxy groups, and **17**, a prematurely derailed polyketide intermediate with a C17 hydroxy group (Figure 3B, panels I vs VI). The accumulation of **11–13**, **16**, and **18** provided further evidence supporting DH10 and DH11 to catalyze regio-specific dehydration of the C17 and C3 hydroxy groups, respectively. Co-isolation of **11–13** was consistent with the findings that the function of DH11 could be partially complemented, while that of **17** agreed with the characterization of **15** from SB11031, SB11032, and SB11033, both of which most likely resulted from the reduced efficiency of PKS module-11 to catalyze the last cycle of chain elongation and subsequent TE-catalyzed cyclization of the ACP-tethered full length polyketide intermediate with a C3 hydroxy group (Figures 2 and S1).

Finally, we biochemically characterized DH10 in vitro, using SNAC thioesters with varying polyketide chain lengths as substrate mimics of the nascent ACP-tethered growing polyketide intermediates, to confirm its activity and determine the timing of the dehydration step of the C17 hydroxy group in biosynthesis of **8** (Figure 4). Thus, MgsF-DH10 was first overproduced in *E. coli* BL21 as an N-His₆-tagged fusion protein and purified (SI and Figure S7). Three SNAC thioesters **19**, **21**, and **23**, featuring varying chain lengths, were synthesized (SI and Figures S18–26), which mimicked the growing polyketide intermediates attached to the ACPs in PKS module-4, -10 and -11, respectively (Figure S1). MgsF-DH10 catalyzed efficient dehydration of the C17 hydroxy group in **21** to afford **22** (Figure 4B), exhibiting an optimal activity at pH 7.8 in 100 mM sodium phosphate (Figure S7), while no activity for dehydration of the β -hydroxy group in **19** was detected (Figure 4A). When **23** was subjected to the same MgsF-DH10-catalyzed reaction conditions, dehydration of both the C17 and C3 hydroxy groups were observed to afford **24**, **25**, and **26**, preferring the C17 hydroxy group to yield **24** as the predominant product (Figure 4C). These findings indicated that DH10 catalyzed the dehydration on the substrate that was tethered to ACP in PKS module-10 rather than that in PKS module-4.

To shed additional insights into the catalytic mechanism, we carried out steady-state kinetic studies of MgsF-DH10, comparing the kinetic parameters for dehydration of the C17 hydroxy groups between **21** and **23** and between the C17 and C3 hydroxy groups in **23** (Figure S7). MgsF-DH10 exhibited similar catalytic efficiencies in catalyzing dehydration of the C17 hydroxy group between **21** (affording **22** with $k_{\text{cat}} = 10.35 \pm 0.47 \text{ min}^{-1}$ and $K_{\text{m}} = 7.90 \pm 0.75 \text{ mM}$) and **23** (affording **24** with $k_{\text{cat}} = 9.30 \pm 0.51 \text{ min}^{-1}$ and $K_{\text{m}} = 6.97 \pm 0.82 \text{ mM}$). These findings suggested that MgsF-DH10 has relatively broad tolerance in the distance for the hydroxy group that will be acted on, as exemplified by the C17 hydroxy groups in **21** and **23**, which were 15- and 17-carbons away, respectively, from the ACP domain they were tethered (Figures 2 and S1). In contrast, MgsF-DH10 apparently preferred the long-range dehydration of the C17 hydroxy group (**23** to **24**) to the canonical β -dehydration of the C3 hydroxy group (**23** to **25** with $k_{\text{cat}} = 5.82 \pm 0.43 \text{ min}^{-1}$ and $K_{\text{m}} = 9.83 \pm 1.41 \text{ mM}$). The preference of the long-distant dehydration activity was further supported

by comparing MgsF-DH10-catalyzed formation of **26** from **24** (dehydrating the C3-hydroxy group) or **25** (dehydrating the C17-hydroxy group), and under identical assay conditions, significantly higher conversion to **26** was observed from **25** than **24** (Figure S7).

To further probe the molecular mechanism of MgsF-DH10 preferring long-range dehydration to the canonical β -dehydration, sequence comparison between MgsF-DH10 and other canonic DHs was carried out, revealing intriguing differences. While all DHs contained the conserved His-Asp catalytic dyad, an extra sequence consisting of 23 amino acids was found within the HxxxGxxxxP motif in MgsF-DH10 (Figure S8).^[19c,21] Mutating one of the active site residue Asp to Ala, as exemplified by MgsF-DH10* (D7895A), or deleting the extra 23 amino acids, as exemplified by MgsF-DH10* (del23aa), afforded soluble MgsF-DH10 mutant proteins (SI and Figure S7), both of which completely lost their activity when assayed with **21** or **23** as a substrate (Figure S7). While the former would support a conserved His-Asp catalytic dyad for all DHs, the latter would suggest that the extra 23 amino acid segment may play a crucial role in the long-range acting dehydratase activity, a finding that will surely inspire future effort to investigate the molecular mechanism and exploit the regio-selectivity of DH domains for engineering polyketide natural product structural diversity.

Based on these new findings, we now propose that during the biosynthesis of **1**, no dehydration occurs on iso-MGS PKS module-4 with the resultant polyketide intermediate continued to be elongated until it reaches iso-MGS PKS module-10, where DH10 catalyzes the long-range dehydration of the C17 hydroxy group. The DH10-catalyzed long-range dehydration may not be complete. The growing polyketide intermediates, with or without the C17 hydroxy group, could both undergo the final round of elongation and cyclization, catalyzed by iso-MGS PKS module-11, to afford the nascent polyketide intermediates **8** and **11**, as has been observed previously during the fermentation of SB11021.^[15] Modification of **8** and **11** by the tailoring enzymes MgsIJK finally complete the biosynthesis of **1** and **13** (Figures 2 and S1). It should be noted that while identifying DH10 as a long-range acting dehydratase provided the missing link for C17-dehydration, it also raised a new question about the cognate dehydratase in the iso-MGS PKS module-10, which would be needed to account for the formation of the methylene group at C5 of the nascent polyketide intermediates **8** and **11** (Figures 2 and S1). Together with the other missing domains predicted according to the collinear model of type I PKS (Figures S1–S3), comparative analysis of iso-MGS PKS and LTM PKS promises to uncover new chemistry and enzymology for type I PKSs.

In conclusion, systematic inactivation of DH6, DH9, DH10, and DH11 of iso-MGS PKS in vivo and biochemical characterization of the wild-type and mutant MgsF-DH10 enzymes in vitro has enabled us to identify DH10 as the dedicated DH to provide the “missing” dehydratase activity in the iso-MGS PKS module-4. MgsF-DH10 catalyzes the dehydration of the C17 hydroxy group to afford the C16-C17 double bond in biosynthesis of **8** in a long range acting manner that is unprecedented for type I PKSs. Together with the DHs that catalyze the canonical β -dehydration^[17] and the recently reported vinylogous δ -dehydration^[19e] (Figure S9), MgsF-DH10 further enriches PKS chemistry and enzymology

and expands the PKS engineering and synthetic biology toolbox for polyketide natural product structural diversity.

Supplementary Material

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Figure 1. Selected glutarimide-containing polyketide family of natural products with or without a hydroxy group at the C17-positions (**1**, **2**, **6**, **7**) or equivalents (**3**, **4**, **5**).

Figure 2.

Comparative analysis of the iso-MGS and LTM PKSs identifying the “missing” dehydratase activity for **1** biosynthesis. The iso-MGS and LTM PKSs produce **8** and **9** as the nascent polyketide intermediates that are transformed to **1** and **2** by MgsIJK and LtmK, respectively, and four DHs are predicted according to type I PKS collinear model for **8** and **9** biosynthesis. The “missing” dehydratase activity for C16–C17 double bond formation in **8** is shown in this study to be provided by DH10 in a long-range acting manner. TE, thioesterase.

Figure 3. Systematic inactivation of DH6, DH9, DH10, and DH11 of iso-MGS PKS allowed functional assignment of their roles in biosynthesis of **1**. (A) Structures of the metabolites (using same numbering as **1** with the varying groups) characterized from the DH mutants. (B) Metabolite profiles of selected DH mutants (II–VI) in comparison with the control (I)

Figure 4.

In vitro assays demonstrating MgsF-DH10 as a dehydratase acting in a long-range acting manner. HPLC profiles of MgsF-DH10 catalyzed (ii) dehydration in comparison with (i) the negative control using SNAC thioesters (A) **19**, (B) **21**, and (C) **23** mimicking the growing polyketide intermediates tethered to the ACPs in PKS module-4, -10, and -11, respectively.