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The Post-polyketide Synthase Steps in iso-Migrastatin Biosynthesis Featuring Tailoring Enzymes with Broad Substrate Specificity

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Abstract

The iso-migrastatin (iso-MGS) biosynthetic gene cluster from *Streptomyces platensis* NRRL 18993 consists of 11 genes, featuring an acyltransferase (AT)-less type I polyketide synthase (PKS) and three tailoring enzymes MgsIJK. Systematic inactivation of *mgsIJK* in *S. platensis* enabled us to (i) identify two nascent products (**10** and **13**) of the iso-MGS AT-less type I PKS, establishing an unprecedented novel feature for AT-less type I PKSs, and (ii) account for the formation of all known post-PKS biosynthetic intermediates (**10-17**) generated by the three tailoring enzymes MgsIJK, which possessed significant substrate promiscuities.

Iso-Migrastatin (iso-MGS, **1**) belongs to the glutarimide-containing polyketide family of natural products, and other members of this family include migrastatin (MGS, **2**), dorrigocin A (DGN A, **3**), 13-epi-DGN A (**4**), DGN B (**5**), lactimidomycin (LTM, **6**), cycloheximide (**7**), streptimidone (**8**), and 9-methylstreptimidone (**9**) (Figure 1).¹ While **2** was originally isolated from *Streptomyces* sp. MK929-43F1² and **3** and **5** from *Streptomyces platensis* NRRL 18993,³ re-examination of the *S. platensis* fermentation revealed that this strain also produced **1** and **2**.⁴ We subsequently established that **1** was the only nascent natural product biosynthesized by *S. platensis* and **2-5** resulted from H₂O-mediated, non-enzymatic ring-expansion and ring-opening rearrangements of **1** (Figure 1).⁵

The glutarimide-containing polyketides exhibit a multitude of biological activities.^{1,6} As it was originally discovered, **2** displayed moderate potency in cell migration inhibition assays,² with synthetic mimics of the macrolide moiety displaying significantly improved potency.⁷ We previously generated a focused library of glutarimide-containing polyketides featuring the molecular scaffolds **1-6**, including eight biosynthetic congeners of **1** (**10-17**,

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Supporting Information. A complete description of materials, methods, and detailed experimental procedures, including Tables S1, S2, and Figures S1, S2, and S3. Also included in SI are ¹H and ¹³C NMR data of compounds **11** (Table S3 and Figures S4, S5, S6). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Figure 2) from optimized fermentations of *S. platensis*^{1b} and **6** and three of its biosynthetic congeners from *Streptomyces amphibiosporus* ATCC 53964,^{5b,6a,b} Preliminary screening of this library revealed that 12-membered macrolides, as exemplified by **1** and **6**, were also potent cell migration inhibitors.^{6b} The modes of action that dictate and differentiate cell migration inhibition from cytotoxicity for the glutarimide-containing polyketides remains controversial.^{1a} While the actin-bundling protein fascin has been identified as the target for the cell migration inhibitory activity of **2**,⁸ blocking the translocation step in eukaryotic protein translation initiation has been deduced as the mechanism for the cytotoxicity of **6**.⁹

We have previously cloned and sequenced the *mgs* biosynthetic gene cluster from *S. platensis* NRRL 18993, which consists of 11 genes (*mgsABCDEFGHIJK*) (Figure S1).¹⁰ Inactivation of selected genes in *S. platensis*^{10a} and expression of the *mgs* cluster in heterologous *Streptomyces* hosts^{10b-d} unambiguously established that the 11 genes are necessary and sufficient to encode **1** production. The biosynthetic machinery of **1**, featuring an acyltransferase (AT)-less type I polyketide synthase (PKS), is characterized by several intriguing properties.^{10a,11} On the assumption of **10** as the nascent PKS product, which has been isolated from the wild-type *S. platensis*,^{1b} and the co-linear model for its biosynthesis,¹¹ the iso-MGS AT-less type I PKS minimally lacks a methyltransferase (MT) domain in module-5, a ketoreductase (KR) domain in module-8, and a KR and an enoylreductase (ER) domain in module-10 (Figure 2 and Figure S1).^{10a} Additionally, four tailoring steps, hydroxylation at C-8, O-methylation at HO-C-8, dehydration of the C-17 OH moiety, and enoyl reduction of the C-16/C-17 olefin, are required for converting **10** to **1**; in fact all possible intermediates, *en route* from **10** to **1** have been isolated from wild-type *S. platensis* (Figures 2 and S1).^{1b} However, based on bioinformatics, only three tailoring enzymes were identified within the *mgs* cluster, MgsI (an oxidoreductase), MgsJ (an O-methyltransferase), and MgsK (a P-450 hydroxylase), together accounting for three of the four tailoring steps.^{10a} While it has been proposed that MgsI, MgsK, and MgsJ are responsible for enoyl reduction of the C-16/C-17 olefin, C-8 hydroxylation, and O-methylation of the HO-C-8, respectively, the exact timing for each of the steps is unknown; also unclear is the nature of C-16/C-17 dehydration prior to enoyl reduction of the C-16/C-17 double bond by MgsI (Figures 2 and S1).^{10a}

Here we report systematic inactivation of *mgsIJK* in *S. platensis*, isolation and characterization of the resulting intermediates, with production time courses, from these mutant strains. These studies enabled us to discover both **10** and **13** as the nascent products of the iso-MGS AT-less type I PKS and account for the formation of all post-PKS biosynthetic intermediates known to date (i.e., **10-17**) by the three tailoring enzymes MgsIJK (Figure 2).

First we systematically inactivated *mgsI*, *mgsJ*, and *mgsK* in the *S. platensis* wild-type by replacing them individually or in combinations with an apramycin resistance gene cassette, using the λ -RED-mediated PCR-targeting mutagenesis strategies [Supporting Information (SI)].^{10a,12} The resultant mutant strains were named SB11016 (i.e., $\Delta mgsI$), SB11017 (i.e., $\Delta mgsJ$), SB11018 (i.e., $\Delta mgsK$), SB11019 (i.e., $\Delta mgsIJ$), SB11020 (i.e., $\Delta mgsJK$), and SB11021 (i.e., $\Delta mgsIJK$), whose genotypes were confirmed by Southern analysis (Figure S2).

Next we fermented the *S. platensis* mutant strains, with the wild-type as a control, to investigate the effect of these mutations on **1** biosynthesis. Fermentation of the wild-type and mutant strains, isolation of **1** and intermediates **10-17**, and determination of the metabolite profiles by HPLC analysis followed established procedures (SI).^{1,5,10} Authentic standards of **1** and **10-17** have been isolated from the *S. platensis* wild-type, and their structures were unambiguously established by comprehensive MS and ¹H and ¹³C NMR

analysis, with the exception of **11**, which was produced in trace quantity by the wild-type.^{1b} We re-isolated **11** from SB11019 and unambiguously confirmed its structure by MS and ¹H and ¹³C NMR analysis (Table S1 and Figures S4-S6). Figure 3 summarizes the metabolite profiles of the six mutant strains in comparison with the *S. platensis* wild-type and authentic standards of **1** and **10-17**. Relative to wild-type, all mutants were unable to produce **1**, confirming the indispensability of MgsIJK in **1** biosynthesis.^{10a} Instead, the mutant strains accumulated varying combinations of intermediates **10-17**, the analysis of which enabled us to assign functions to the three tailoring enzymes MgsIJK, and to identify the biosynthesis of two nascent polyketide products by the iso-MGS AT-less type I PKS (Figure 2).

The triple gene mutant strain SB11021 ($\Delta mgsIJK$) accumulated **10** and **13**, with **13** as the dominant metabolite (Figure 3, panel VIII). This finding indicates (i) MgsIJK are responsible for the three tailoring steps in **1** biosynthesis - hydroxylation at C-8, O-methylation at HO-C-8, and enoyl reduction of the C-16/C-17 double bond and (ii) the iso-MGS AT-less type I PKS yields two nascent products, **10** and **13**, and dehydration of C-17 does not require a dedicated tailoring enzyme (Figure 2).^{10a}

The single gene mutant strains SB11016 ($\Delta mgsI$), SB11017 ($\Delta mgsJ$), and SB11018 ($\Delta mgsK$) provided direct evidence supporting their functional assignments.^{10a} Thus, (i) SB11016 accumulated **10**, **11**, **12**, **13**, **14**, and **15**, and all carried either C-17 OH or C-16/C-17 double bond, supporting MgsI as the oxidoreductase responsible for C-16/C-17 enoyl reduction (Figure 3, panel III). (ii) SB11017 accumulated **10**, **11**, **13**, **14**, **16**, and **17**, and all lacked the O-methyl group at HO-C-8, consistent with the assignment of MgsJ as O-methyltransferase responsible for O-methylation of the HO-C-8 (Figure 3, panel IV). (iii) SB11018 accumulated **10**, **13**, and **16**, and all lacked the HO-C-8, confirming MgsK as a P-450 hydroxylase responsible for C-8 hydroxylation (Figure 3, panel V) (Figure 2).

The double gene mutant strains SB11019 ($\Delta mgsIJ$) and SB11020 ($\Delta mgsJK$) further corroborated the findings from the single gene mutants SB11016, SB11017, and SB11018, as well as the three-gene mutant SB11021. Thus, (i) SB11019 accumulated four metabolites, **10**, **11**, **13**, and **14**. Compared to SB11021, which produced **10** and **13** only, the production of **11** and **14**, in addition to **10** and **13**, by SB11019 are consistent with MgsK as a C-8 hydroxylase (Figure 3, panel VI versus panel VIII). (ii) SB11020 accumulated **10**, **13**, and **16**, and this phenotype was identical to that of SB11018 since MgsJ could not function unless C-8 was first hydroxylated by MgsK (Figure 3, panel VII versus V). (iii) Similarly, the double gene mutant $\Delta mgsIK$ was predicted to be functionally equivalent to the triple gene mutant SB11021, and thereby was not pursued. The fact that the six mutant strains and wild-type all accumulated multiple metabolites revealed significant substrate promiscuities of the three tailoring enzymes MgsIJK in **1** biosynthesis (Figure 2).

Finally we carried out extensive time course analyses of the metabolite profiles of the six mutant strains, in comparison with the wild-type, in an attempt to establish the preferred pathway for **1** biosynthesis (Figure S3). We reasoned that intermediates on the preferred pathway should be accumulated as the dominant metabolites in the various mutant strains, but the final profiles may be complicated by substrate promiscuities of the three tailoring enzymes (Figure 2). Thus, **1** biosynthesis is detected on day one, which continued to increase on days two and three, and reached a plateau on day four. While **1** was always the dominant product, significant amount of the various biosynthetic intermediates were noticeable starting from day two, the relative ratios among which and to **1** were nearly constant throughout the time course (Figure S3, panel I). The latter observation is significant, suggesting that (i) **10-12** cannot be dehydrated to **13-15** non-enzymatically and (ii) **10** and **13** are direct products of the iso-MGS AT-less type I PKS, the dehydratase activity of which works only on the ACP-tethered growing polyketide intermediate before it

is cyclized by TE to afford **10** (Figure 2). This was further supported by the time-course of SB12021, in which the ratio of **10** and **13** (~ 5%/95%) was essentially constant throughout the time examined (Figure S3, panel II). Taken together, these findings experimentally establish a novel feature of the iso-MGS AT-less type I PKS. While it is competent in synthesizing **10** as a minor product, the biosynthesis of **13** would require a DH domain in module-4 according to the co-linear model, the missing function of which must have been provided by one of the three DH domains in module-6, -9, or -11, an unprecedented feature for an AT-less type I PKS (Figure 2).¹¹

Examination of the time courses of both the single and double gene mutants also showed near constant ratios among the various metabolites accumulated throughout the time examined. Thus, SB11018 and SB11020, which are functionally equivalent, accumulated **16** as the major product (Figure S3, panels III and IV), while SB11019 (Figure S3, panel V), SB11017 (Figure S3, panel VI), and SB11016 (Figure S3, panel VII) accumulated **14**, **17**, and **15** as the predominant metabolites, respectively. While these findings prevented us from directly establishing the preferred sequence of the three tailoring steps, they support broad substrate promiscuities of the three tailoring enzymes; each enzyme must have been able to catalyze its respective tailoring step efficiently, independent of the functional groups introduced by the other tailoring enzymes (Figure 2).

Lastly, we closely examined the time courses of the SB11017 mutant and the wild-type in an attempt to differentiate the timings of the three tailoring steps. Thus, MgsI and MgsK compete for **13** as a substrate to afford **16** and **14**, respectively, *en route* to **17** in SB11017 (Figure 2). The fact that **14** was observed consistently in higher titer than **16** was therefore indicative of **13** to **14** as the preferred pathway (Figure S3, panel VI). Similarly, MgsI and MgsJ compete for **14** as a substrate to yield **17** and **15**, respectively, *en route* to **1** in the wild-type (Figure 2). The conclusion of **14** to **15**, with MgsI acting last, as the preferred sequence, was consistent with the observation that **15** was produced in higher titer than **17** in the wild-type throughout the time examined (Figure S3, panel I). It should be pointed out that this conclusion is very speculative, and the instability of intermediates **10-17** in aqueous medium⁵ has prevented us from confirming the preferred sequence directly by studying the MgsIJK tailoring enzymes *in vitro*.

In conclusion, systematic inactivation of *mgsIJK* in *S. platensis* has enabled us to discover both **10** and **13** as the nascent products of the iso-MGS AT-less type I PKS, establishing an unprecedented novel feature for AT-less type I PKSs, and to account for the formation of all the post-PKS biosynthetic intermediates known to date by the three tailoring enzymes MgsIJK, which possessed significant substrate promiscuities. Comparative studies of the iso-MGS AT-less type I PKS with the PKSs responsible for biosynthesis of other glutarimide-containing natural products, such as **6-9**, which feature a C-17 OH equivalent group, should now provide an excellent opportunity to probe how AT-less type I PKSs select and control reductive modifications during polyketide chain elongation. The remarkable substrate promiscuities of MgsIJK could be readily exploited for natural product structural diversity via combinatorial biosynthesis strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. a Rajske SR, Shen B. *ChemBioChem*. 2010; 11:1951–1954. [PubMed: 20806307] b Ju J, Lim S-K, Jiang H, Seo J-W, Shen B. *J. Am. Chem. Soc.* 2005; 127:11930–11931. [PubMed: 16117518]
2. a Nakae K, Yoshimoto Y, Sawa T, Homma Y, Hamada M, Takeuchi T, Imoto M. *J. Antibiot.* 2000; 53:1130–1136. [PubMed: 11132958] b Takemoto Y, Nakae K, Kawatani M, Takahashi Y, Naganawa H, Imoto M. *J. Antibiot.* 2001; 54:1104–1107. [PubMed: 11858668] c Nakae K, Yoshimoto Y, Ueda M, Sawa T, Takahashi Y, Naganawa H, Takeuchi T, Imoto M. *J. Antibiot.* 2000; 53:1228–1230. [PubMed: 11132973] d Nakamura H, Takahashi Y, Naganawa H, Nakae K, Imoto M, Shiro M, Matsumura K, Watanabe H, Kitahara T. *J. Antibiot.* 2002; 55:442–444. [PubMed: 12061554]
3. a Karwowski JP, Jackson M, Sunga G, Sheldon P, Poddig JB, Kohl WL, Kadan S. *J. Antibiot.* 1994; 47:862–869. [PubMed: 7928671] b Hochlowski JE, Whittern DN, Hill P, McAlpine JB. *J. Antibiot.* 1994; 47:870–874. [PubMed: 7928672] c Kadam S, McAlpine JB. *J. Antibiot.* 1994; 47:875–880. [PubMed: 7928673]
4. Woo EJ, Starks CM, Carney JR, Arslanian R, Cadapan L, Zavala S, Licari P. *J. Antibiot.* 2002; 55:141–146. [PubMed: 12002995]
5. a Ju J, Lim S-K, Jiang H, Shen B. *J. Am. Chem. Soc.* 2005; 127:1622–1623. [PubMed: 15700980] b Ju J, Lim S-K, Jiang H, Seo J-W, Her Y, Shen B. *Org. Lett.* 2006; 8:5865–5868. [PubMed: 17134292]
6. a Ju J, Rajske SR, Lim S-K, Seo JW, Peters NR, Hoffmann FM, Shen B. *Bioorg. Med. Chem. Lett.* 2008; 18:5951–5954. [PubMed: 18684620] b Ju J, Rajske SR, Lim S-K, Seo J-W, Peters NR, Hoffmann FM, Shen B. *J. Am. Chem. Soc.* 2009; 131:1370–1371. [PubMed: 19132897] c Takemoto Y, Tashiro E, Imoto M. *J. Antibiot.* 2006; 59:435–438. [PubMed: 17025021] d Nakae K, Nishimura Y, Ohba S, Akamatsu Y. *J. Antibiot.* 2006; 59:685–692. [PubMed: 17256466]
7. a Njardarson JT, Gaul C, Shan D, Huang X-Y, Danishefsky SJ. *J. Am. Chem. Soc.* 2004; 126:1038–1040. [PubMed: 14746469] b Gaul C, Njardarson JT, Shan D, Dorn DC, Wu KD, Tong WP, Huang X-Y, Moore MA, Danishefsky SJ. *J. Am. Chem. Soc.* 2004; 126:11326–11337. [PubMed: 15355116] c Shan D, Chen L, Njardarson JT, Gaul C, Ma X, Danishefsky SJ, Huang X-Y. *Proc. Natl. Acad. Sci. USA.* 2005; 102:3772–3776. [PubMed: 15728385] d Metaferia BB, Chen L, Baker HL, Huang X-Y, Bewley CA. *J. Am. Chem. Soc.* 2007; 129:2434–2435. [PubMed: 17295491] e Oskarsson T, Nagorny P, Krauss IJ, Perez L, Mandal M, Yang G, Ouerfelli O, Xiao D, Moore MA, Massagué J, Danishefsky SJ. *J. Am. Chem. Soc.* 2010; 132:3224–3228. [PubMed: 20155906] f Danishefsky SJ. *Nat. Prod. Rep.* 2010; 27:1114–1116. [PubMed: 20383353] g Lecomte N, Njardarson JT, Nagorny P, Yang G, Downey R, Ouerfelli O, Moore MA, Danishefsky SJ. *Proc. Natl. Acad. Sci. USA.* 2011; 108:15074–15078. [PubMed: 21808037]
8. a Chen L, Yang S, Jakoncic J, Zhang JJ, Huang X-Y. *Nature.* 2010; 464:1062–1068. [PubMed: 20393565] b Nagorny P, Krauss I, Njardarson JT, Perez L, Gaul C, Yang G, Ouerfelli O, Danishefsky SJ. *Tetrahedron Lett.* 2010; 51:3873–3875. [PubMed: 20676392]
9. a Schneider-Poetsch T, Ju J, Eyler DE, Dang Y, Bhat S, Merrick WC, Green R, Shen B, Liu JO. *Nat. Chem. Biol.* 2010; 6:209–217. [PubMed: 20118940] b Lee S, Liu B, Lee S, Huang S-X, Shen B, Qian S-B. *Proc. Natl. Acad. Sci. USA.* 2012; 109:14728–14729. E2424–E2432. c Stern-Ginossar N, Weisburd B, Michalski A, Le VTK, Hein MY, Huang S-X, Ma M, Shen B, Qian S-B, Hengel H, Mann M, Ingolia NT, Weissman JS. *Science.* 2012; 338:1088–1093. [PubMed: 23180859] d Kurata S, Shen B, Liu JO, Takeuchi N, Kaji A, Kaji H. *Nucleic Acids Res.* 2013; 41:264–276. [PubMed: 23087377]
10. a Lim S-K, Ju J, Zazopoulos E, Jiang H, Seo J-W, Chen Y, Feng Z, Rajske SR, Farnet CM, Shen B. *J. Biol. Chem.* 2009; 284:29746–29756. [PubMed: 19726666] b Feng Z, Wang L, Rajske SR, Xu Z, Coeffet-LeGal MF, Shen B. *Bioorg. Med. Chem.* 2009; 17:2147–2153. [PubMed: 19010685] c Wu X, Yang D, Zhu X, Feng Z, Lv Z, Zhang Y, Shen B, Xu Z. *Biotechnol. Bioprocess Eng.* 2010; 15:664–669. [PubMed: 21625393] d Yang D, Zhu X, Wu X, Feng Z, Huang L, Shen B, Xu Z. *Appl. Microbiol. Biotechnol.* 2011; 89:1709–1719. [PubMed: 21132287]

11. a Shen B. *Curr. Opin. Chem. Biol.* 2003; 7:285–295. [PubMed: 12714063] b Cheng Y-Q, Coughlin JM, Lim S-K, Shen B. *Methods Enzymol.* 2009; 459:165–186. [PubMed: 19362640] c Piel J. *Nat. Prod. Rep.* 2010; 27:996–1047. [PubMed: 20464003]
12. Gust B, Challis GL, Fowler K, Kieser T, Chater KF. *Proc. Natl. Acad. Sci. USA.* 2003; 100:1541–1546. [PubMed: 12563033]

Figure 1. Structures of selected glutarimide-containing polyketide natural products **1-9** and H_2O -mediated, non-enzymatic ring-expansion and ring-opening rearrangements of **1** to **2-5**.

Figure 2.

Proposed biosynthetic pathway for iso-MGS (**1**) featuring the iso-MGS AT-less type I PKS that lacks a dehydratase (DH) domain in module-4, a MT domain in module-5, a KR domain in module-8, and a KR and an ER domain in module-10 according to the co-linear PKS model for the biosynthesis of the two nascent products, **10** and **13**, and the tailoring enzymes MgsIJK with broad substrate specificity that convert **10** to **12** and **13** to **1**. Biosynthesis of the glutarimide starter unit by the loading module, module-2 and module-3 has been previously proposed.^{10a} Heavy arrows denote the preferred pathway by the tailoring enzymes with thin arrows accounting for metabolites resulted from substrate promiscuity. ACP, acyl carrier protein; AMT, amidotransferase; DH, dehydratase; ER, enoylreductase; KR, ketoreductase; KS, ketosynthase; MT, methyltransferase; TE, thioesterase. [NH₂] depicts the amino donor for the AMT domain, [SAM] denotes S-adenosylmethionine as the methyl donor for the MT domain, the ACP domain with an overlaid X is nonfunctional, the domain with ? denotes an unknown function, green ovals depict AT-docking domains, and the yellow domains highlight the missing domains for the iso-MGS AT-less type I PKS predicted according to the co-linear model for the biosynthesis of **10** and **13**.^{10a,11}

Figure 3. Metabolite profiles of *S. platensis* mutant strains (III-VIII), in comparison with the wild-type (II) and authentic standards **1** and **10-17** (I), upon HPLC analysis. See Figure 2 for structures of **1** and **10-17**, and (•) depicts metabolites unrelated to **1** biosynthesis.