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The Missing C-17 O-Methyltransferase in Geldanamycin Biosynthesis

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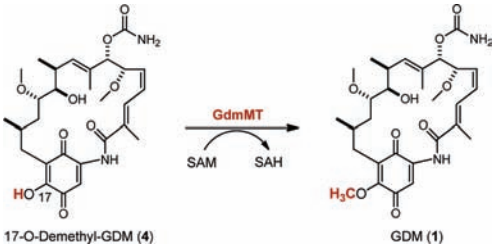
ABSTRACT



The biosynthetic gene clusters for the Hsp90 inhibitor geldanamycin (GDM, 1) have been cloned previously from three different *Streptomyces* strains, but the gene encoding the C-17 O-methyltransferase remains unknown. The cloning and sequencing of a new GDM biosynthetic gene cluster from *Streptomyces autolyticus* CGMCC 0516 was reported, identifying the *gdmMT* gene that encodes the missing C-17 O-methyltransferase for 1 biosynthesis.

Geldanamycin (GDM, **1**, Figure 1C), a member of the ansamycin family of natural products, was first isolated in 1970 from *Streptomyces hygroscopicus* var. *geldanus* var. *nova* in a screen for antiparasitic compounds[1] and is now best known as a potent Hsp90 inhibitor.[2] Mechanistically, **1** competes with ATP for the ATP binding site of Hsp90, inhibiting its ATP-dependent functions.[3] Hsp90 is expressed at significantly higher levels in cancer cells than in normal cells. Consequently, Hsp90 inhibition by **1** leads to rapid degradation of important Hsp90 client proteins resulting in cancer cell-selective cytostatic and cytocidal effects.[2−4] Although **1** itself was not considered a viable clinical candidate due primarily to unacceptable toxicity and poor bioavailability,[5] several analogues of **1** have been developed and are currently in various stages of clinical trials as novel anticancer agents. Such candidates include 17-allylamino-17-demethoxy-geldanamycin (17-AAG or KOS-953), 17-AAG hydroquinone hydrochloride (IPI-504), 17-dimethylaminoethylamino-17-demethoxy-geldanamycin (17-DMAG), and 17-amino-17-demethoxy-geldanamycin (17-AG or IPI-493, **2**).[2,4] Notably, all these analogues are generated by varying the substitution at the C-17 position of **1**.

Aimed at engineering the GDM biosynthetic machinery for improved titers and enhanced structural diversity, the

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(1) (a) DeBoer, C.; Meulman, P. A.; Wnuk, R. J.; Peterson, D. H. *J. Antibiot.* **1970**, *23*, 442. (b) Sasaki, K.; Rinehart, K. L., Jr. *J. Am. Chem. Soc.* **1970**, *92*, 7591.

(2) (a) Buchner, J. *Trends Biochem. Sci.* **1999**, *24*, 136. (b) Uehara, Y. *Curr. Cancer Drug Targets* **2003**, *3*, 325. (c) Porter, J. R.; Ge, J.; Lee, J.; Normant, E.; West, K. *Curr. Top. Med. Chem.* **2009**, *9*, 1386. (d) Fukuyo, Y.; Hunt, C. R.; Horikoshi, N. *Cancer Lett.* **2010**, *290*, 24.

(3) (a) Whitesell, L.; Mimnaugh, E. G.; De Costa, B.; Myers, C. E.; Neckers, L. M. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 8324. (b) Stebbins, C. E.; Russo, A. A.; Schneider, C.; Rosen, N.; Hartl, F. U.; Pavletich, N. P. *Cell* **1997**, *89*, 239.

(4) (a) Mimnaugh, E. G.; Chavany, C.; Neckers, L. *J. Biol. Chem.* **1996**, *271*, 22796. (b) Neckers, L. *Trends Mol. Med.* **2002**, *8*, S55. (c) Isaacs, J. S.; Xu, W.; Neckers, L. *Cancer Cell* **2003**, *3*, 213.

(5) Le Brazidec, J. Y.; Kamal, A.; Busch, D.; Thao, L.; Zhang, L.; Timony, G.; Grecko, R.; Trent, K.; Lough, R.; Salazar, T.; Khan, S.; Burrows, F.; Boehm, M. F. *J. Med. Chem.* **2004**, *47*, 3865.

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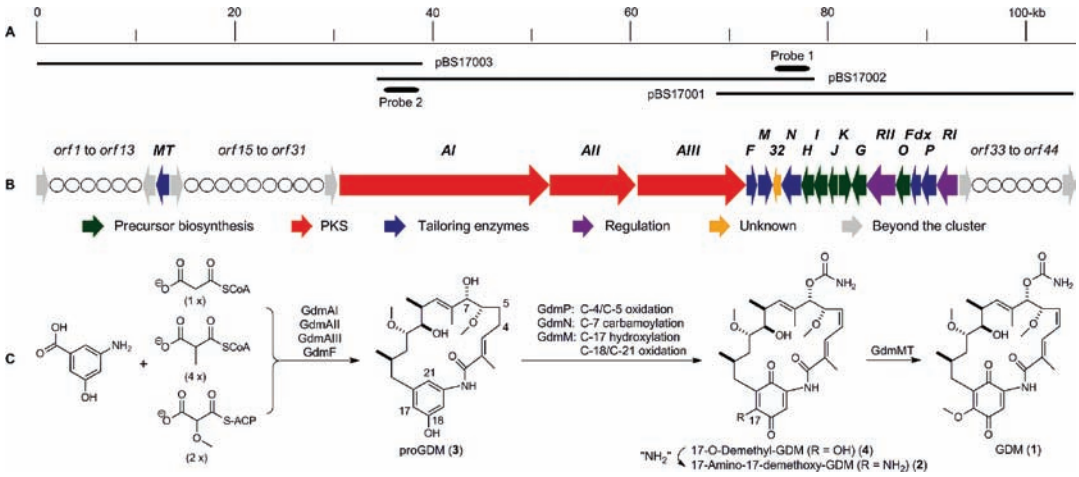

Figure 1. The GDM gene cluster from *S. autolytics* CGMCC 0516 unveiling the *gdmMT* gene that encodes the missing C-17 O-methyltransferase for **1** biosynthesis. (A) The three overlapping cosmids pBS17001, pBS17002, and pBS17003 that cover the sequenced 104.5-kb DNA region. Probes 1 and 2 were used to screen the genomic library to isolate the overlapping cosmids. (B) Genetic organization of the GDM cluster, highlighting the *gdmMT* gene that is 17-kb apart from the upstream boundary of the GDM cluster. Functional assignments of the genes within, as well as the *orfs* beyond, the GDM cluster are summarized in Table S1. (C) Proposed GDM biosynthetic pathway featuring the GdmAI, AII, AIII and F-catalyzed synthesis of the first isolated intermediate **3**, GdmP, N, and M-catalyzed modifications of **3** into 17-O-demethyl-GDM (**4**), and GdmMT-catalyzed C-17 O-methylation of **4** to afford **1**. A trace amount of **2**, which most likely is a shunt metabolite of **4**, was also isolated from the Δ*gdmMT* mutant strain SB17002.

GDM biosynthetic gene cluster has previously been cloned and sequenced from three producers: *Streptomyces hygroscopicus* NRRL 3602,[6] *Streptomyces hygroscopicus* 17997 (incomplete),[7] and *Streptomyces hygroscopicus* JCM4427.[8] The resulting clusters are identical in overall organization, consisting of the same set of genes that encode three type I polyketide synthases (PKSs), four post-PKS tailoring enzymes, six enzymes for precursor biosynthesis, and two regulators (Figure S1 and Table S1). Thus, as depicted in Figure 1C, the polyketide backbone of **1** is assembled from one molecule of 3-amino-5-hydroxylbenzoic acid, one molecule of malonyl CoA, four molecules of methylmalonyl CoA, and two molecules of methoxymalonyl-acyl carrier protein (ACP) by the GdmAI, AII, AIII PKSs. Subsequent macrolactamization of the nascent polyketide intermediate by the GdmF amide synthase affords the first isolated intermediate progeldanamycin (proGDM, **3**). Three dedicated tailoring enzymes have been characterized for the conversion of **3** into **1**: C-4/C-5 desaturation by the GdmP P-450 oxygenase, C-7 carbamoylation by the GdmN carbamoyl transferase, and C-17 hydroxylation and C-18/C-21 oxidation by, minimally, the GdmM flavin-dependent monooxygenase; this leaves C-17 O-methylation as the only step unaccounted for in **1** biosynthesis.[6−8]

We set out to search for the missing, but important, C-17 O-methyltransferase since all clinically important GDM analogues are derived from modifications at the C-17 position of **1**. Here, we report the cloning and sequencing of a new GDM biosynthetic gene cluster from *Streptomyces autolytics* CGMCC 0516. Although the new cluster shares the same overall architecture as the three GDM clusters reported previously, the open reading frames (orfs) flanking the four clusters differ significantly (Figure S1 and Table S1). We discovered the *gdmMT* gene, residing 17-kb apart from the upstream boundary of the newly discovered GDM cluster (Figure 1B), and its function as the dedicated C-17 O-methyltransferase in **1** biosynthesis (Figure 1C) was established by in vivo and in vitro studies.

We first cloned the GDM biosynthetic gene cluster from *S. autolyticus* CGMCC 0516 using PCR-amplified fragments of the *gdmN* (Probe 1) and *gdmA1* (Probe 2) genes as probes and confirmed its involvement in **1** biosynthesis by gene inactivation [Supporting Information (SI)]. We have previously reported *S. autolyticus* CGMCC 0516 as a new **1** producer,[9] a cosmid library of which was constructed and screened by colony hybridization using Probes 1 and 2 (Figure 1A). From ~2000 colonies, nine overlapping cosmids were identified and, together, as exemplified by pBS17001, pBS17002, and pBS17003, they covered a 104.5-kb continuous DNA region, which was subsequently sequenced (Figure 1A). The DNA sequence has

(6) (a) Rascher, A.; Hu, Z.; Viswanathan, N.; Schirmer, A.; Reid, R.; Nierman, W. C.; Lewis, M.; Hutchinson, C. R. *FEMS Microbiol. Lett.* **2003**, *218*, 223. (b) Rascher, A.; Hu, Z.; Buchanan, G. O.; Reid, R.; Hutchinson, C. R. *Appl. Environ. Microbiol.* **2005**, *71*, 4862.

(7) He, W.; Lei, J.; Liu, Y.; Wang, Y. *Arch. Microbiol.* **2008**, *189*, 501.

(8) (a) Hong, Y.-S.; Lee, D.; Kim, W.; Jeong, J.-K.; Kim, C.-G.; Sohng, J. K.; Lee, J.-H.; Paik, S.-G.; Lee, J. J. *J. Am. Chem. Soc.* **2004**, *126*, 11142. (b) Shin, S. J.; Na, Z.; Lee, D.; Kim, W.; Lee, K.; Shen, Y.-M.; Paik, S.-G.; Hong, Y.-S.; Lee, J. J. *J. Microbiol. Biotechnol.* **2008**, *18*, 1101.

(9) Li, M. G.; Wu, S. H.; Zhao, L. X.; Zhang, Q.; Li, W. J.; Cui, X. L.; Xu, L. H.; Wu, D. G.; Jiang, C. L. *Chin. Chem. Lett.* **2001**, *12*, 903.

been deposited into the GenBank database under accession no. HQ840740. The overall GC content of the sequenced region is 72.8%. Among the 60 *orfs* identified, 18 were annotated as *gdm* genes (Figure 1B), the proposed functions of which are summarized in Table S1. The new cluster consists of the same set of genes and shares the same overall architecture as the three GDM clusters reported previously[6−8] (Figure S1). Comparisons between the PKSs, the tailoring enzymes, and the regulators among the four clusters showed high sequence identities ranging from 84% to 100% (Table S1). To unambiguously confirm that the cloned gene cluster encodes **1** biosynthesis, we inactivated the *gdmAI* gene in *S. autolyticus* CGMCC 0516 using the PCR-targeting and λ-RED-mediated mutagenesis method[10] (SI). The resultant Δ*gdmAI* mutant strain was named SB17001, and its genotype was confirmed by Southern analysis (Figure S2). When cultured under the standard conditions for **1** biosynthesis using the wild-type strain as a control, SB17001 failed to produce **1** (Figure 2, panels I−VI), as would be expected for the indispensable role that GdmAI plays in **1** biosynthesis (Figure 1C).

We next located the *gdmMT* gene residing 17-kb apart from the upstream boundary of the GDM cluster and confirmed its function as the missing C-17 O-methyltransferase in **1** biosynthesis in *S. autolyticus* CGMCC 0516 by gene inactivation and complementation experiments. Although the genes within the GDM clusters are highly conserved among the four clusters, *orfs* flanking the clusters differ significantly[6−8] (Figure S1). This observation serves as another illustration for how conserved secondary metabolite biosynthetic clusters are inserted into varying loci of different hosts as the result of horizontal gene cluster transfer. Remarkably, close examination of the 31 *orfs* upstream of the GDM cluster from *S. autolyticus* CGMCC 0516 unveiled *gdmMT*, 17-kb apart from the upstream boundary of the GDM cluster, whose deduced gene product showed significant sequence homology to known O-methyltransferases involved in secondary metabolite biosynthesis, such as ChlB5 (41% identity and 59% similarity) for chlorothricin biosynthesis in *Streptomyces antibioticus* DSM 40725[11] and MmcR (26% identity and 40% similarity) for mitomycin biosynthesis in *Streptomyces lavendulae* NRRL 2564.[12] GdmMT contains the three conserved motifs characteristic for SAM-dependent methytransferase[13] as well as the DXDXD motif for divalent cation binding.[14]

To directly probe the role of *gdmMT* in **1** biosynthesis, we first inactivated the *gdmMT* gene in *S. autolyticus* CGMCC 0516 using the PCR-targeting and λ-RED-mediated mutagenesis method[10] to afford the Δ*gdmMT* mutant strain SB17002 (SI), whose genotype was

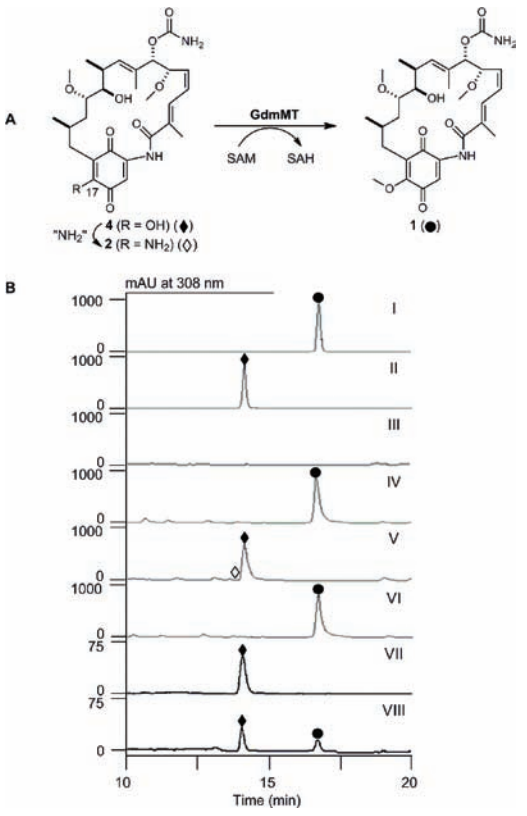

Figure 2. In vivo and in vitro confirmation of GdmMT catalyzed C-17 O-methylation of **4** to afford **1** in *S. autolysis* CGMCC 0516. Metabolite profile analysis by HPLC: I, **1** standard (●); II, **4** standard (◆); III, SB17001 (i.e., Δ*gdmAI*); IV, wild-type; V, SB17002 (i.e., Δ*gdmMT*); VI, SB17003 (i.e., SB17002/pBS17008); VII, complete assay of **4** with boiled GdmMT and SAM; and VIII, complete assay of **4** with GdmMT and SAM. A trace amount of **2** (◇) isolated from the Δ*gdmMT* mutant strain SB17002 as a shunt metabolite.

confirmed by Southern analysis (Figure S3). When cultured under the standard conditions for **1** biosynthesis using the wild-type strain as a control, SB17002 failed to produce **1** but instead accumulated one new metabolite (Figure 2, panel V), which was subsequently identified as 17-O-demethyl-geldanamycin (**4**) (Figure 1C) by mass and ^{1}H and ^{13}C NMR spectroscopic analyses (SI and Figures S4 and S5). While the wild-type strain produced 178 ± 12 mg L^{-1} of **1**, the Δ*gdmMT* mutant strain SB17002 produced 154 ± 37 mg L^{-1} of **4**. These results provided the first experimental evidence that *gdmMT* encodes the missing C-17 O-methyltransferase responsible for C-17 O-methylation as the last step in **1** biosynthesis (Figure 1C). Intriguingly, a trace amount of **2**, less than 1/250 to that of **4** based on HPLC analysis, was also detected from the fermentation culture of SB17002 (Figure 2, panel V), which was subsequently isolated and its structure confirmed by mass and ^{1}H and ^{13}C NMR analyses (SI and Figures S6 and S7). To our knowledge, this is the first time **2** has been isolated as a natural product

(10) Gust, B.; Challis, G. L.; Fowler, K.; Kieser, T.; Chater, K. F. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 1541.
(11) Jia, X.-Y.; Tian, Z.-H.; Shao, L.; Qu, X.-D.; Zhao, Q.-F.; Tang, J.; Tang, G.-L.; Liu, W. *Chem. Biol.* **2006**, *13*, 575.
(12) Gruschow, S.; Chang, L.-C.; Mao, Y.; Sherman, D. H. *J. Am. Chem. Soc.* **2007**, *129*, 6470.
(13) Ingrosso, D.; Fowler, A. V.; Bleibaum, J.; Clarke, S. *J. Biol. Chem.* **1989**, *264*, 20131.
(14) Bist, P.; Rao, D. N. *J. Biol. Chem.* **2003**, *278*, 41837.

from **1** producers or their variants. We propose that **2** is a shunt metabolite of **4**, most likely resulting from adventitious activities during SB17002 fermentation (Figure 1C).

We next carried out complementation experiments to eliminate the possibility of polar effects due to *gdmMT* inactivation in SB17002. Plasmid pBS17008, containing the intact *gdmMT* gene whose expression was under the control of the strong, constitutive *ErmE** promoter,[15] was introduced into SB17002 to afford the recombinant strain SB17003 (SI). Upon fermentation under the standard conditions with the wild-type strain as a control, **1** production was essentially restored in SB17003 (138 ± 12 mg L^{-1}) (Figure 2, panel VI) consistent with the intermediacy of **4** in **1** biosynthesis (Figure 1C).

Finally, we overproduced and purified GdmMT to homogeneity and demonstrated its ability to catalyze C-17 O-methylation of **4** to render **1** in vitro. GdmMT was overproduced in *E. coli* BL21 as an N-His_6-tagged fusion protein and purified to homogeneity by affinity chromatography (SI). The purified GdmMT migrated as a single band on 12% SDS-PAGE with an apparent molecular mass of ~26 kDa, consistent with the predicted size of 26.2 kDa (Figure S8). To determine the conditions for optimal activity, the pH dependence of GdmMT was first investigated with **4** and SAM as substrates in 50 mM potassium phosphate (pH 5.5−8.0) or 50 mM bis-tris-HCl (pH 8.0−9.5) buffer (SI). GdmMT displayed optimal activity at pH 7.0 (Figure S9). The effect of divalent metal ions on GdmMT activity was next examined (SI). The presence of EDTA or Cu^{2+} completely abolished methyltransferase activity; inclusion of Ca^{2+}, Mn^{2+}, and Zn^{2+} in the reactions resulted in a slightly lower activity, whereas addition of Mg^{2+} afforded a slight increase in enzyme activity (Figure S10). Similar patterns of metal ion-dependent enzyme activity have been reported for other SAM-dependent methyltransferases involved in microbial secondary metabolite biosynthesis.[12,16] Thus final assays were carried out in 50 mM potassium phosphate buffer, pH 7.0, in the presence of 5 mM $MgCl_2$, with **4** and SAM as substrates (SI).

GdmMT was found to be very unstable under all conditions examined, including those containing up to 20% glycerol in storage solution. The enzyme completely loses activity within a few hours at either −20 or 4 °C. Although the intrinsic instability prevented meaningful kinetic analyses of the enzymatic reaction, GdmMT catalyzed C-17 O-methylation of **4** was readily demonstrated in vitro using freshly prepared enzyme. Thus, as depicted in Figure 2, GdmMT catalyzed time-dependent formation of **1** from **4** and SAM (Figure 2, panel VIII), with no formation of **2** in controls containing either SAM alone or with both **4** and SAM in the presence of heat-denatured GdmMT (Figure 2, panel VII).

In conclusion, the current study completes what we know of the GDM biosynthetic machinery by finally identifying the *gdmMT* gene, residing 17-kb apart from the rest of the GDM cluster. The gene encodes the missing C-17 O-methyltransferase, which was unambiguously demonstrated to catalyze C-17 O-methylation of **4** as the last step in **1** biosynthesis[6−8] (Figure 1C). The 17-kb DNA separating *gdmMT* from the rest of the GDM cluster may have resulted from gene insertion or chromosomal rearrangement. An extra 15-kb and 20-kb DNA, upstream of the GDM cluster from *S. hygroscopicus* JCM4427[8] and *S. hygroscopicus* NRRL 3602,[6] respectively, were also sequenced, but no *gdmMT* was discovered. The missing *gdmMT* gene may reside in completely different chromosomal locations in these other producers. The observation that genes encoding secondary metabolite production are often clustered in one region of the bacterial chromosome has greatly facilitated their cloning, sequencing, and characterization to date, but exceptions to this rule are becoming increasingly evident. GDM now joins the growing list of microbial natural products that includes ansamitocin,[17] clavam,[18] moenomycin A,[19] and meilingmycin,[20] whose biosynthetic genes are scattered on the bacterial chromosome, thus posing significant challenges to complete cluster characterization efforts.

All analogues of **1** currently in clinical trials as novel anticancer drugs are generated by installation of various modifications at the C-17 position.[2] The Δ*gdmMT* mutant strain SB17002 engineered in this study provides a novel means of producing **4**, which may constitute an alternative substrate for preparation of C-17 modified GDM analogues. The production of **2**, albeit in low titers, by SB17002 is also noteworthy. It has been shown recently that **2** is the major active metabolite of 17-AAG, is more potent than 17-AAG, and has a longer biological half-life in vivo.[2] Thus, as **2** continues to show promise in clinical development, it is tempting to speculate that further optimization of SB17002 may afford a fermentation approach for **2** production.

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Supporting Information Available. Experimental procedures, Table S1, and Figures S1−S10. This material is available free of charge via the Internet at http://pubs.acs.org.

(15) Kieser, T.; Bibb, M. J.; Buttner, M. J.; Chater, K. F.; Hopwood, D. A. *Practical Streptomyces Genetics*; The John Innes Foundation: Norwich, United Kingdom, 2000.

(16) (a) Xu, J.; Mahmud, T.; Floss, H. G. *Arch. Biochem. Biophys.* **2003**, *411*, 277. (b) Luo, Y.; Lin, S.; Zhang, J.; Cooke, H. A.; Bruner, S. D.; Shen, B. *J. Biol. Chem.* **2008**, *283*, 14694.

(17) Yu, T. W.; Bai, L.; Clade, D.; Hoffmann, D.; Toelzer, S.; Trinh, K. Q.; Xu, J.; Moss, S. J.; Leistner, E.; Floss, H. G. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 7968.

(18) Tahlan, K.; Park, H. U.; Jensen, S. E. *Can. J. Microbiol.* **2004**, *50*, 803.

(19) Ostash, B.; Saghatelian, A.; Walker, S. *Chem. Biol.* **2007**, *14*, 257.

(20) He, Y.; Sun, Y.; Liu, T.; Zhou, X.; Bai, L.; Deng, Z. *Appl. Environ. Microbiol.* **2010**, *76*, 3283.