Biosynthesis

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Divergent Mechanistic Routes for the Formation of *gem*-Dimethyl Groups in the Biosynthesis of Complex Polyketides**

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Abstract: *The gem-dimethyl groups in polyketide-derived natural products add steric bulk and, accordingly, lend increased stability to medicinal compounds, however, our ability to rationally incorporate this functional group in modified natural products is limited. In order to characterize the mechanism of gem-dimethyl group formation, with a goal toward engineering of novel compounds containing this moiety, the gem-dimethyl group producing polyketide synthase (PKS) modules of yersiniabactin and epothilone were characterized using mass spectrometry. The work demonstrated, contrary to the canonical understanding of reaction order in PKSs, that methylation can precede condensation in gem-dimethyl group producing PKS modules. Experiments showed that both PKSs are able to use dimethylmalonyl acyl carrier protein (ACP) as an extender unit. Interestingly, for epothilone module 8, use of dimethylmalonyl-ACP appeared to be the sole route to form a gem-dimethylated product, while the yersiniabactin PKS could methylate before or after ketosynthase condensation.*

Approximately 10% of all approved drugs contain a geminal dimethyl group. The introduction of this group into compounds can decrease their rates of chemical and metabolic degradation, thus improving the efficacy of drugs.[1] Consequently, there is great potential utility for the regiospecific incorporation of *gem*-dimethyl groups in engineered polyketide products. Regiospecific methylation of polyketide products using organic semisynthesis is challenging. To enable biobased approaches to form new *gem*-dimethyl-containing pharmaceutical agents, and to clarify discrepancies with the current mechanistic understanding, we sought to determine the reaction mechanism of PKS-based *C*-methylation.[2]

Type I polyketide synthases catalyze Claisen condensations and tailoring reactions in an assembly line fashion to elaborate a remarkably diverse collection of secondary metabolites, many of which have medicinal and industrial applications. Polyketide chains are extended by sequential homologation reactions between ketosynthase (KS)-bound thioesters and α-carboxy building blocks (traditionally malonyl- or methylmalonyl-acyl carrier protein (ACP)) to form β-keto-polyketides. Tailoring reactions, such as the reduction of the β-keto group on the growing polyketide chain, and the subsequent sulfonation or *O*-methylation of the corresponding β-hydroxy group, customarily follow condensation. Incorporation of methyl or *gem*-dimethyl groups by methyltransferase (MT) containing PKS modules is thought to follow this biosynthetic paradigm.[2,3]

The currently accepted view of PKS-based reactions is that KS-mediated condensation precedes all subsequent events that take place within the module, including mono- or dimethylation of the β-ketoacyl-ACP when an MT domain is present (Scheme 1B, Route 1).[2] However, another route is possible in which methylation precedes the condensation (Scheme 1B, Route 2). A pK_a-based argument supports Route 1, as the pK_a of a β-ketoacyl-ACP intermediate of Route 1 is about two units lower than the analogous malonyl-ACP of Route 2 and therefore more easily deprotonated.[4] In further support of the proposed Route 1, attack of the enolate on the upstream acyl-KS should occur more readily with the less sterically hindered acetyl enolate, than the more sterically hindered isobutyryl enolate of Route 2. On the other hand, if methylation precedes condensation as in Route 2, the observed production of isobutyryl-ACP in the yersiniabactin PKS (see below) is rationalized by a similar argument that malonyl-ACP is significantly easier to deprotonate than acetyl-ACP (approximate ten unit difference in pK_a) and decarboxylation of dimethylmalonyl-CoA would be facilitated by the presence of electron-donating methyl groups.

There is precedent for the involvement of dimethylmalonyl moieties in biochemical reactions. When provided in vitro to carboxymethylproline synthase (CarB), a member of the crotonase superfamily, a dimethylmalonyl moiety has been shown to be a source of a nucleophilic enolate for C–C bond-forming reactions, despite its steric bulk.[5] Dimethyl-

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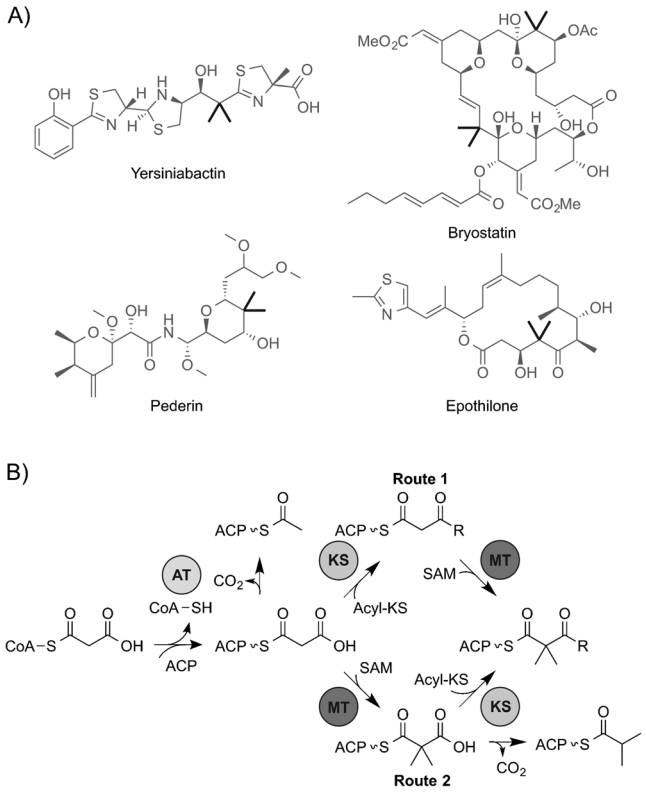

Scheme 1. A) Representative *gem*-dimethyl group containing compounds. B) Potential mechanistic routes for the methylation and condensation in *gem*-dimethyl group producing polyketide synthase domains. Route 1: KS-catalyzed condensation precedes methylation. Route 2: Methylation precedes KS condensation. Acetyl-ACP and isobutyryl-ACP represent potential side products of unproductive decarboxylation (shown in gray). AT = acyltransferase; KS = ketosynthase; MT = methyltransferase. R represents the acyl chain transferred from the previous module.

malonyl groups are also naturally added to sugar residues in the biosynthesis of cervimycin,[6] and have been proposed as a source of isobutyryl groups in polyketide synthesis, but the latter has not been conclusively demonstrated.[7]

Examples of polyketides containing *gem*-dimethyl groups include the potential anticancer agents epothilone, pederin, and bryostatin, and the siderophore yersiniabactin (Scheme 1 A).[8] Currently, the biochemical characterization of *gem*-dimethyl group producing PKSs is limited to the yersiniabactin PKS. Using an in vitro system, Miller and coworkers[9] showed that the yersiniabactin PKS used malonyl-CoA and two *S*-adenosyl methionine (SAM) molecules to produce its *gem*-dimethyl group containing extended product, 3-(hydroxyphenylthiazolinylthiazolinyl) [HPTT]-β-keto-2,2-dimethyl-ACP. They also established that the yersiniabactin AT domain has a 500-fold kinetic preference for malonyl-CoA over methylmalonyl-CoA. Additionally, Mazur and coworkers[10] observed that the condensation reaction was dependent on SAM and that isobutyryl-ACP was a major side product of the reaction; approximately 75 % of the ACP domains of the PKS were occupied by an isobutyryl moiety following the reaction. The observed dependence of the condensation reaction on SAM[10] could be explained by the MT domain generating dimethylmalonyl-ACP, which participates in the decarboxylative homologation reaction. Epothilone PKS module 8 (EpoM8) forms a similar *gem*-dimethyl group based on the final structure of epothilone, but this module has not been previously studied biochemically. The AT domain of EpoM8 is bioinformatically predicted to prefer methylmalonyl-CoA over malonyl-CoA.[11] This prediction was verified biochemically in a competition assay, though a limited amount of malonyl-ACP was formed when EpoM8 was incubated with malonyl-CoA in the absence of methylmalonyl-CoA (Figure SI-2.1 in the Supporting Information). Thus, the MT domain in the module is predicted to naturally carry out only a single C methylation. Despite these initial studies, the exact mechanism and biosynthetic timing that PKSs employ to produce *gem*-dimethyl groups remains unclear.

In order to test if Route 2 is used, we performed in vitro chain elongation reactions with dimethylmalonyl-ACP, generated through directed acylation of the apo-ACP domain using two *gem*-dimethyl group producing PKS modules: yersiniabactin and EpoM8. In addition, we sought to understand if the KS domains of these PKSs demonstrated a preference for the methylation state of the ACP-bound substrate, as reported by Mazur and co-workers.[10] Determination of these parameters would provide a clearer picture of which path(s) are operative in the production of this unusual group. Accordingly, we characterized these *gem*-dimethyl group producing PKS clusters using a well-established tandem mass spectrometry method[12] that can detect intermediates covalently attached to ACP domains in a single-turnover in vitro assay.

The yersiniabactin and EpoM8 PKSs were expressed in *Escherichia coli* strains BLR and BAP1,[13] respectively. These proteins were purified using Ni-NTA (nickel nitrilotriacetate) chromatography. The purified yersiniabactin PKS was approximately 70 % holo form, while the EpoM8 was only the holo form. As both malonyl-CoA and SAM are present in the expression strains, we analyzed the acylation state of each PKS immediately after purification to determine if acylation or methylation occurred during expression. Examination of the freshly purified yersiniabactin PKS and EpoM8 ACPs showed isobutyryl-ACP (a decarboxylation product of dimethylmalonyl-ACP) on EpoM8 (Figure 1 A) and both dimethylmalonyl-ACP and isobutyryl-ACP appended to the yersiniabactin PKS (Figure 1 B). This observation shows that at least the first half of Route 2 is viable for the yersiniabactin PKS. Interestingly, these results suggest that EpoM8 MT is able to methylate malonyl-ACP loaded during in vivo expression in *E. coli*, even though the preferred substrate for the EpoM8 AT is methylmalonyl-ACP (which is not natively present in *E. coli*). Furthermore, in vitro studies of the yersiniabactin PKS showed that acetyl-ACP was not methylated in the presence of SAM (Figure SI-2.2), contrary to previous proposals, which suggested the methylation of acetyl-ACP accounted for the production of isobutyryl-ACP.[10] These facts strongly suggest that isobutyryl-ACP is derived from dimethylmalonyl-ACP and implies that Route 2 contributes in the overall formation of a *gem*-dimethyl group.

To unambiguously determine if the dimethylmalonyl moiety loaded on the yersiniabactin and EpoM8 PKSs can

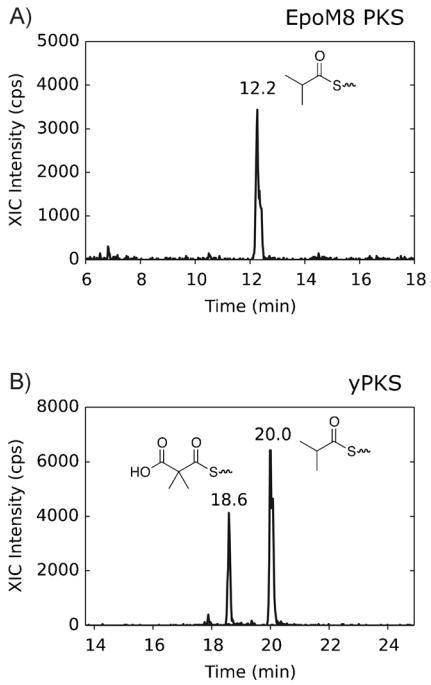

Figure 1. Analysis of the acylation state of epothilone module 8 PKS (EpoM8 PKS) and yersiniabactin PKS after Ni-NTA purification. Retention times are shown on top of peaks. A) EpoM8 isobutyryl-ACP chromatogram. B) Yersiniabactin PKS dimethylmalonyl-ACP chromatogram and isobutyryl-ACP chromatogram. All XIC *m/z* transitions monitored in this study are summarized in the Supporting Information. XIC = extracted ion chromatogram; cps = counts per second.

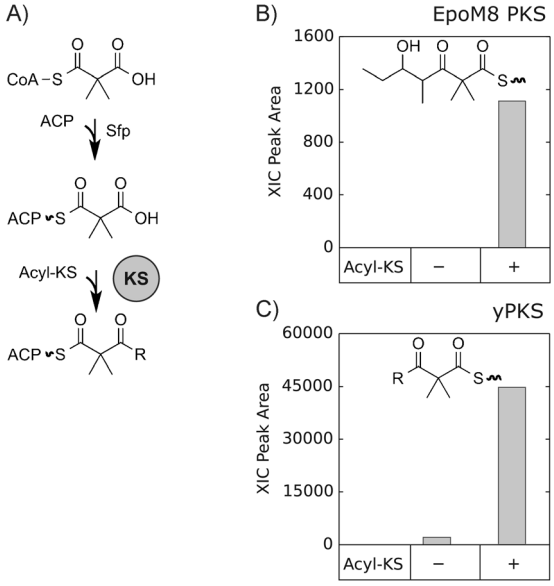

Figure 2. Dimethylmalonyl-ACP is extended by the EpoM8 and yersiniabactin PKS. A) Experiment overview: dimethylmalonyl-ACP was formed using Sfp, dimethylmalonyl-CoA, and apo-PKS. Then, substrates to form acyl-KS were added to generate extended products (experimental details in the Supporting Information). Extracted ion chromatogram (XIC) peak areas for dimethylmalonyl-ACP extension experiments for B) EpoM8 and C) yersiniabactin PKS. For the yersiniabactin case, R represents the acyl chain transferred from the previous NRPS module: a HPTT moiety. All XIC *m/z* transitions monitored in this study are summarized in the Supporting Information.

be utilized by the KS domain in an in vitro extension reaction, we expressed apo-PKS (expressed in *E. coli* strain BLR, this resulted in approximately 100% apo form of the EpoM8). We then synthesized authentic dimethylmalonyl-CoA[5,6] and loaded the ACP domains with the dimethylmalonyl moiety using the phosphopantetheinyl transferase Sfp.[14] Loading of dimethylmalonyl-ACP was verified by LC-MS/MS (Figure SI-2.3). The acyl group to be extended was provided to the appropriate KS domain by either a diketide-*S*-*N*-acetylcysteamine substrate,[15] the acyl group of which mimics the structure of the native intermediate in the case of EpoM8, or by reconstitution of the upstream nonribosomal peptide synthetase (NRPS) enzymes in the case of the yersiniabactin PKS. As shown in Figure 2, both EpoM8 and yersiniabactin extended dimethylmalonyl-ACP in the absence of SAM to produce the correct fully extended and methylated product with retention times matching those observed during fully reconstituted in vitro reactions (Figure SI-2.4). Additionally, the side product isobutyryl-ACP was produced from dimethylmalonyl-ACP by both enzymes (Figure SI-2.5). A large amount of isobutyryl-ACP was produced by both enzymes relative to the extended methylated product, suggesting that Sfp loading experiments may not fully capture the precisely orchestrated catalytic cycle of these enzymes. The formation of acyl-KS also seems to have activated the KS for decarboxylation of dimethylmalonyl-ACP to isobutyryl-ACP (Figure SI-2.5), potentially indicating a conformational shift in the enzyme upon KS acylation.

To determine if Route 1 is also operative for each PKS, we attempted to form the β-keto unmethylated (yersiniabactin PKS) or monomethylated (EpoM8 PKS) products by incubating malonyl-CoA with acyl-KS, while omitting SAM and subsequently adding SAM (Figure 3A). We then examined products before and after SAM addition using LC-MS/MS. Contrary to the results of Mazur et al.[10] the yersiniabactin KS domain was found to use malonyl-ACP as a substrate for condensation in the absence of SAM, and the MT domain methylated the resulting β-keto acyl-ACP moiety after SAM was added (Figure 3B). Small amounts of the unmethylated and singly methylated yersiniabactin-derived PKS product were also observed during reactions with the native malonyl-CoA substrate in the presence of SAM (Figure SI-2.6). EpoM8 was unable to extend methylmalonyl-ACP or malonyl-ACP in the absence of SAM, but it was able to form the dimethyated final product for both extender units in the presence of SAM (Figure 4 and SI-2.7). This suggests that EpoM8 methylates methylmalonyl-ACP and then uses dimethylmalonyl-ACP as the substrate in the condensation reaction. Additionally, EpoM8 did not form the singly methylated intermediate (i.e. an intermediate resulting solely from the extension of methylmalonyl-ACP with no action by the MT) when full reconstitution reactions were performed in the presence of methylmalonyl-CoA and SAM. Together, these findings demonstrate that in vitro, the yersiniabactin PKS is able to employ both pathways in Scheme 1, while EpoM8 is restricted to using solely Route 2.

We have clearly demonstrated that the yersiniabactin and epothilone PKSs are capable of forming dimethylmalonyl-

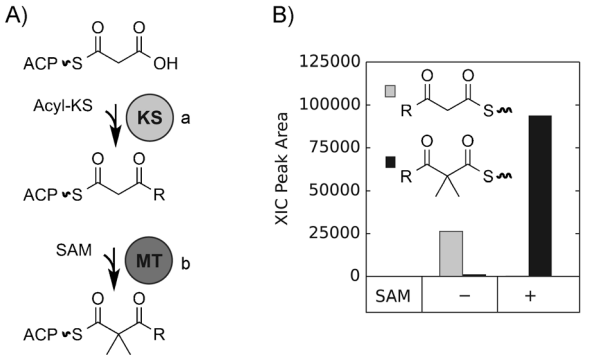

Figure 3. Characterization of Route 1 (KS-catalyzed condensation precedes methylation) for the yersiniabactin PKS. A) Experiment overview: in part a, SAM was omitted, while malonyl-CoA and acyl-KS were included to allow the formationof unmethylated extended-ACP. In part b, SAM was added (experimental details in the Supporting Information). B) Unmethylated extended-ACP extracted ion chromatogram (XIC) peak areas (gray) and methylated extended-ACP peak areas (black). R is a HPTT moiety. All XIC *m/z* values monitored in this study are summarized in the Supporting Information.

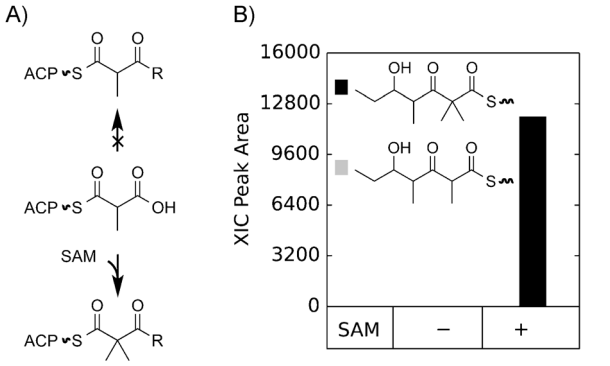

Figure 4. Characterization of Route 1 (KS catalyzed condensation precedes methylation) for the epothilone PKS. A) Experiment overview: using methylmalonyl-CoA as an extender unit, SAM was omitted from the overall reaction (no product formed), or SAM was included, allowing the complete reaction to take place. B) Unmethylated extended-ACP extracted ion chromatogram (XIC) peak areas (gray) and methylated extended-ACP peak areas (black). All XIC *m/z* values monitored in this study are summarized in the Supporting Information.

ACP and isobutyryl-ACP, its decarboxylated derivative, and, contrary to the traditional understanding of methylation in type I PKSs, that both enzymes can use dimethylmalonyl-ACP as an extender unit in acyl chain elongation. For EpoM8, formation of dimethylmalonyl-ACP through Route 2 appeared to be the only means to form a *gem*-dimethyl group (at least in vitro), while the yersiniabactin PKS could methylate before or after condensation. The increased decarboxylation of dimethylmalonyl-ACP we observed upon acylation of the KS suggests that conformational changes in the enzyme are important during the catalytic cycle of these *gem*-dimethyl group producing PKSs, as recently demonstrated for pikromycin module 5 using cryo electron microscopy.[16] Future studies will employ cryo electron microscopy to examine the spatial relationship between MT with the ACP during the catalytic cycle to determine the partition between Routes 1 and 2 for the yersiniabactin PKS. Based on our results, we would suggest that PKS engineering strategies to incorporate this group using combinatorial biosynthesis should swap whole modules, instead of swapping individual MT domains into modules unaccustomed to a particular order of reactions. Ultimately, an improved understanding of this remarkable reaction will allow easier integration of this monomer unit in novel, useful PKS-based compounds.

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