Polyketide Biosynthesis

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Insights into the Pamamycin Biosynthesis

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Abstract: *Pamamycins are macrodiolides of polyketide origin with antibacterial activities. Their biosynthesis has been proposed to utilize succinate as a building block. However, the mechanism of succinate incorporation into a polyketide was unclear. Here, we report identification of a pamamycin biosynthesis gene cluster by aligning genomes of two pamamycin-producing strains. This unique cluster contains polyketide synthase (PKS) genes encoding seven discrete ketosynthase (KS) enzymes and one acyl-carrier protein (ACP)-encoding gene. A cosmid containing the entire set of genes required for pamamycin biosynthesis was successfully expressed in a heterologous host. Genetic and biochemical studies allowed complete delineation of pamamycin biosynthesis. The pathway proceeds through 3-oxoadipyl-CoA, a key intermediate in the primary metabolism of the degradation of aromatic compounds. 3-Oxoadipyl-CoA could be used as an extender unit in polyketide assembly to facilitate the incorporation of succinate.*

Pamamycins (**1**) are a group of macrodiolide antibiotics produced by several *Streptomyces* species (Figure 1, S1). They were identified due to their ability to stimulate the formation of aerial mycelia in *Streptomyces alboniger* DSMZ40043 and were demonstrated to inhibit the growth of Gram-positive bacteria and fungi.[1] Initially, the compound with the structure designated as pamamycin-607 (R^1–R^4 = Me, R^5 = H) was isolated from *S. alboniger*;[1b] however, a re-examination of extracts revealed a broader chemical diversity of **1** differing in terms of the side-chain substituents (Figure 1, S1).[2] Compounds **1** are composed of two asymmetrical parts named hydroxy acids small (**3**) and hydroxy acids large (**4**) (Scheme 1). Due to the pronounced bioactivities and challenging molecular structure, these natural products have stimulated intense synthetic efforts,[3] which have already culminated in several total syntheses of pamamycin-607[4] and some homologues.[5] Feeding experiments clearly demonstrated the polyketide origin of **1** by the incorporation of ^{13}C-labeled acetate, propionate, and succinate.[6] This fact is especially interesting since succinate cannot directly participate in a Claisen condensation. The corresponding biochemical reaction as well as the enzyme in charge remained mysterious till now.

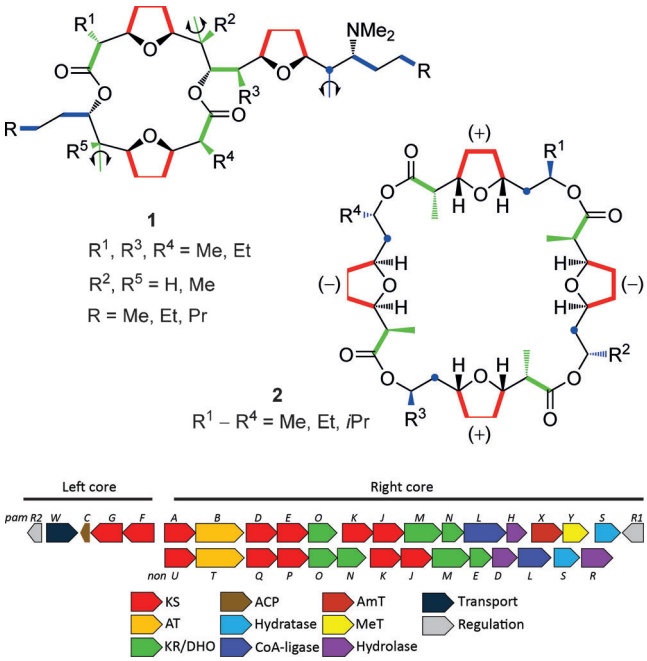

Figure 1. Structures of **1** and **2** and the genetic organization of the respective biosynthesis gene clusters. The origin of carbon atoms is color coded: blue–acetate, green–propionate, red–succinate.[6,7] Arrows indicate decarboxylation. The deduced function of genes is based on studes of **2** biosynthesis and sequence homology. KS: ketosynthase, AT: acyltransferase, KR/DHO: ketoreductase/dehydrogenase, ACP: acyl-carrier protein, AmT: aminotransferase, MT: methyltransferase.

The only other natural products known to utilize succinate as a building block are the macrotetrolide antibiotic nonactin (R^1–R^4 = Me) (**2**) produced by *S. griseus* and *S. fulvissimus* (Figure 1).[7,8] Compounds **2** consist of two enantiomers of hydroxy acids with tetrahydrofuran rings, which are stereospecifically assembled into the final molecule. The gene cluster for the biosynthesis of **2** (*non*) contains five ketosynthase KS genes.[9]

These unusual KSs were classified as noniterative type II PKS based on sequence similarity despite the lack of ACP proven by the heterologous expression and deletion experiments.[9a,b] Extensive studies using ^{13}C-labeled precursors showed utilization of acetate and succinate as building blocks

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for assembly of **2**[7] as well as direct incorporation of 3-oxoadipate as a precursor of **2**,[10] leading to idea that the first committed step in the biosynthesis is condensation of succinate and malonate. However, the labeling pattern of 3-oxoadipate used in this study did not explain how this intermediate is made by the **2** biosynthesis machinery. Lastly, the acetate incorporated in positions of **1** and **2** that correspond to the 3-oxoadipate location proceed through the double decarboxylation contributing only one carbon atom to the structure of final molecules. These unusual features distinguish the biosynthesis of **1** and **2** from that of other polyketides. Despite the excellent insights into the origins of the precursors of **1** and **2**, feeding experiments are not sufficient to establish their biosynthetic route. Moreover, the *non* genes' inactivation experiments further complicated the hypothesized biosynthesis of macrotetrolides.[9a,11] The aim of this project was to elucidate the biosynthetic pathway leading to production of **1** with a focus on the enzyme(s) responsible for the incorporation of succinate. We endeavor 1) to identify the enzymes capable of utilizing succinate as an intact four-carbon building block in the biosynthesis of **1** and **2**; 2) to explain the incorporation of only one carbon atom from acetate into polyketide backbone of **1** and **2**; 3) to identify enzyme(s) responsible for elongation of **3** to **4** by addition of the second succinate unit; 4) to establish the entire biosynthetic route leading to the assembly of **1**.

To identify the biosynthetic genes for **1**, the genomes of two producing strains, *S. alboniger* DSMZ40043 and *S.* sp. HKI 118, were sequenced, searched for secondary-metabolism gene clusters with AntiSMASH,[12] and aligned to identify clusters present in both strains. Only two regions coding for PKS and terpenoid biosynthesis were identical in *S. alboniger* and *S.* sp. HKI118. The PKS gene cluster from *S. alboniger* contains 20 genes with counterparts in *S.* sp HKI118 and share a high degree of homology with the *non* clusters of *S. fulvissimus*[8] and *S. griseus* subsp. *griseus*[9] (Figure 1, Table S3). This cluster (*pam*-cluster) consists of two "cores" of KS genes. The "right core" highly resembles the *non* gene clusters and includes five KS (*pamA*, *D*, *E*, *K*, *J*), three KR/DHO (*pamO*, *M*, *N*), AT (*pamB*), *nonS*-like enoyl-CoA hydratase (*pamS*, putatively involved in tetrahydrofuran ring closure[13]), acyl-CoA ligase (*pamL*), and putative resistance (*pamH*) genes (Figure 1). In addition, genes for an aminotransferase (AmT) and a methyltransferase (MT) (*pamX* and *Y*) are located in this part of the cluster. The "left core" includes two KS (*pamF* and *G*) and ACP (*pamC*) genes. Genes encoding a putative transporter protein and two transcriptional regulators of the TetR and LuxR families were also found flanking the biosynthetic genes. All KS in the cluster, except for PamA, possess the characteristic CHN active site triad with N predicted as the acyl-CoA binding site (Table S4). This architecture of catalytic residues is more typical for type III PKS enzymes than for type I where the CHH motif is conserved.[14]

The *pamD* gene, encoding a KS from the "right core" of the cluster (Figure 1), was deleted from the chromosome of *S. alboniger*. This mutant failed to produce **1**, proving that the identified region is indeed responsible for its biosynthesis (Figure S2). An *S. alboniger* cosmid library was created and screened for clones containing the entire set of *pam* genes using two probes flanking the cluster. Expression of one of the positive clones, termed R2, in *S. albus* J1074 resulted in the production of **1** (Figure S3).

To determine the sequential biosynthetic steps resulting in the production of **1**, a set of mutant cosmids with deletions of individual *pam* genes was created. Metabolites produced by recombinant *S. albus* strains harboring these cosmids were analyzed by LC-MS (Table 1). In contrast to the deletions of *pamB*, *pamD*, and *pamO*, which led to the complete cessation of biosynthesis, *S. albus* containing cosmids with mutations in *pamK*, *pamJ*, and *pamL* retained the ability to produce **3** and **4** (Table 1, Figure S4). This indicates that PamB, D, and O are responsible for the initial steps of **1** biosynthesis, which are shared for both **3** and **4**, while PamK, PamJ, and PamL are involved in the final steps of **1** formation. Deletion of the aminotransferase and methyltransferase genes *pamX* and *pamY* resulted in accumulation of **3** and hydroxy acid K (**5**) (Table 1, Figure S5),[15] the non-aminated precursor of **4**. This proves that amination and methylation occur before the closure of the macrodiolide ring as proposed previously.[15] In contrast, deletion of the KS genes *pamF* and *pamG* from the "left core" of the cluster caused accumulation of **3**, whereas neither **1** nor **4** were found, indicating that these enzymes are necessary for the extension of **3** to give **4**. Deletion of *pamC*, which encodes ACP, perturbed the structural range of **1**, with a shift toward the accumulation of lower-molecular-weight compounds (Table 1, Figure S6). This result indicates that PamC participates in the delivery of starter units as described in several cases of bacterial type III PKS.[16]

Deletion of *pamA* caused a significant decrease in **1** production as well as changes in the structural range of the accumulated compounds (Table 1; Figures S7 and S8). Because PamA was proposed to participate in the first condensation step of the pathway, any perturbation of its function should lead to the complete cessation of **1** production. PamA-catalyzed condensation of succinate and malonate should result in the production of 3-oxoadipyl-CoA, which is one of the key intermediates in the degradation of

Table 1: Accumulation of **1** and their precursors produced by *S. albus* strains harboring cosmids with various *pam* gene deletions.

Cosmid\Compound	**1**	**3**	**4**	**5**
R2	+	+	+	−
pamC[a]	+	+	+	−
pamG	−	+	−	−
pamF	−	+	−	−
pamA[a]	+	+	+	−
pamB	−	−	−	−
pamD	−	−	−	−
pamO	−	−	−	−
pamK	−	+	+	−
pamJ	−	+	+	−
pamL	−	+	+	−
pamX	−	+	−	+
pamY	−	+	−	+

[a] The t_R value is different from that of **1**, **3**, and **4** from *S. albus* R2.

aromatic compounds in bacteria.[17] A KEGG COMPOUND search[18] of the *S. albus* genome revealed genes that could cause accumulation of this metabolite, including the gene for 3-oxoadipate:succinyl-CoA transferase. Thus, in the absence of PamA, the biosynthesis machinery for **1** draws 3-oxoadipyl-CoA from the primary metabolism resulting in the residual accumulation of **1**. *S. albus* lacking the 3-oxoadipate:succinyl-CoA transferase gene was generated. This mutant remained able to produce **1** when the *pamA*-deficient cosmid was introduced (Figure S8). However, the level of production was severely reduced. Thus, 3-oxoadipyl-CoA is an intersection point for primary metabolism and the **1** biosynthesis pathway. The fact that the mutant strain was still able to produce **1** indicates the existence of an alternative mechanism of 3-oxoadipate production via a process independent of 3-oxoadipate:succinyl-CoA transferase, most likely through the phenylacetic acid degradation pathway.[17]

The function of PamA in pamamycin biosynthesis was further demonstrated by in vitro reconstitution. PamA was overexpressed in *E. coli* and purified (Figure S9). The enzyme's activity was tested in an assay containing malonyl-CoA and succinyl-CoA. The reaction was monitored by HPLC (Figure 2). Fractions corresponding to the detected peaks were collected and subjected to LC-MS analysis using a HILIC approach.[19] Formation of a new compound with an HPLC retention time (t_R) of 16.2 min and an absorption spectrum typical of CoA-esters was observed in the reaction mixture after 30 min of incubation (Figure 2E), and the amount of this compound was further increased by increasing the reaction time. HILIC-LC-MS analysis of this product

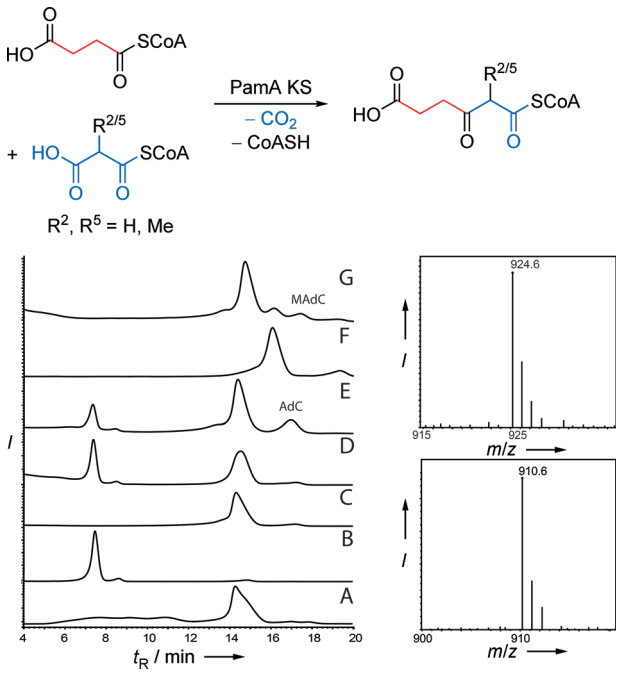
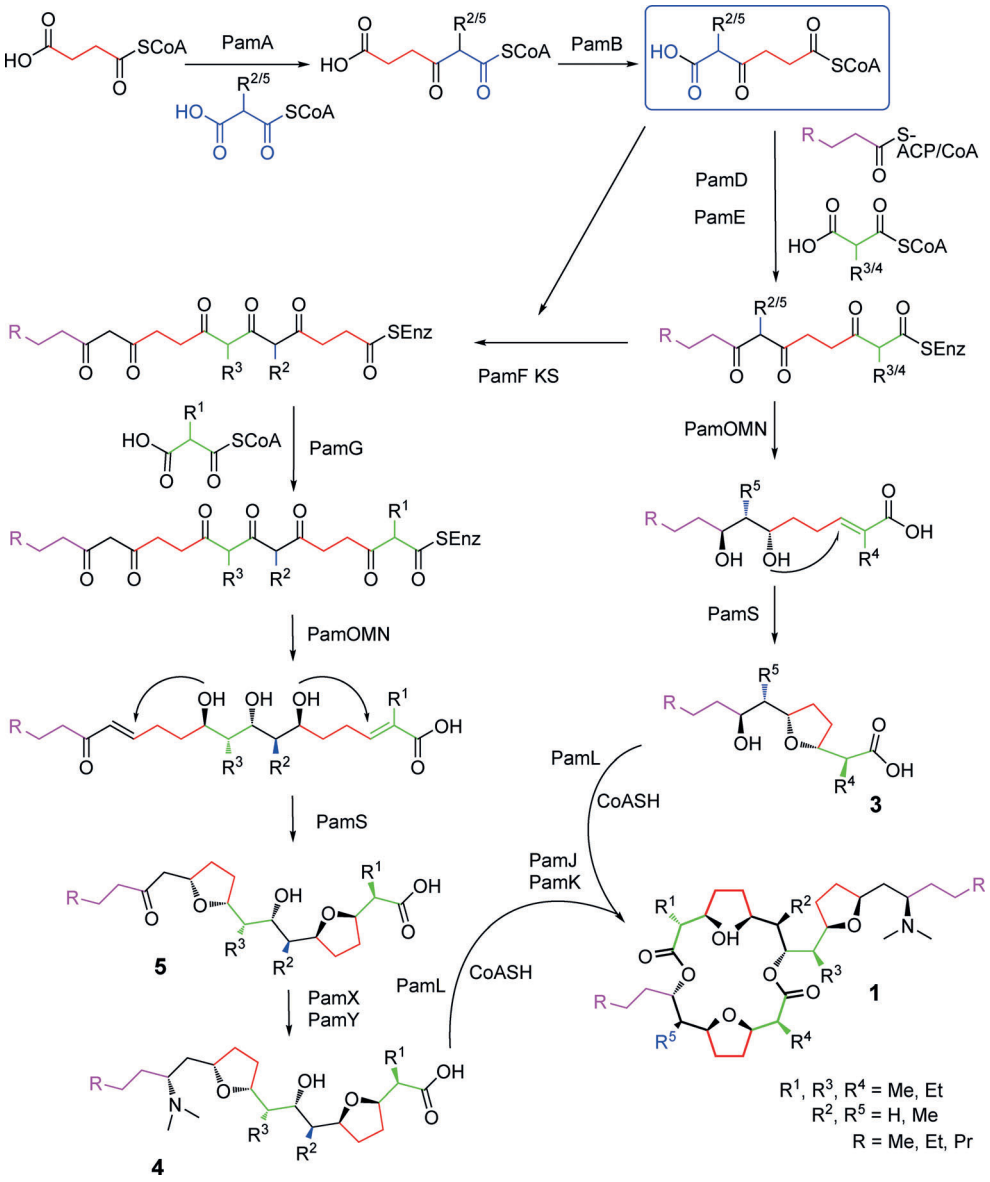


Figure 2. HPLC profiles of CoA-ester standards and reaction mixtures containing PamA protein. A) CoA-SH, B) malonyl-CoA, C) succinyl-CoA, D) reaction mixture with heat-inactivated PamA, E) reaction mixture with PamA, F) methylmalonyl-CoA, G) reaction mixture with PamA and malonyl-CoA substituted with methylmalonyl-CoA. AdC: 3-oxoadipyl-CoA, MAdC: 2-methyl-3-oxoadipyl-CoA.

yielded a signal at *m/z* 909.6, which corresponds to the mass of 3-oxoadipyl-CoA (calculated: *m/z* 909.61) (Figure 2 and Figure S10). The side chains R^2 and R^5 in several **1** contain methyl groups (Figure 1) that are predicted to be incorporated by the PamA reaction, suggesting enzyme–substrate promiscuity. Accordingly, PamA incubated with succinyl-CoA and methylmalonyl-CoA instead of malonyl-CoA produced a new compound with $t_R = 17.1$ min and *m/z* 924. (Figure 2, Figure S11). This *m/z* value corresponds to the predicted mass of 2-methyl-3-oxoadipyl-CoA (calculated: *m/z* 924.64). When the reaction was performed in the presence of both malonyl- and methylmalonyl-CoA substrates, the ratio of the products was found to depend on the initial ratio of the substrates (Figure S12). Thus, unlike NonU, which appears to be specific to malonate because no version of **2** with side-chain substituents is known, PamA is more flexible in its choice of substrates. Furthermore, introduction of *nonU* into the *pamA* mutant strain did not restore the native range of **1** produced, due to the inability of NonU to produce 2-methyl-3-oxoadipate (Table S5). The ability of NonU to synthesize 3-oxoadipyl-CoA is masked by its supply from the primary metabolism.

Using the obtained genetic and biochemical data, we propose the entire pathway leading to the formation of **1** (Scheme 1). The first reaction in the pathway is the PamA-catalyzed condensation of succinyl-CoA with malonyl- or methylmalonyl-CoA. The resulting compounds are then rotated by PamB acyltransferase as proposed by Rong and co-workers.[20] The resulting 4-oxoadipyl-CoA and 5-methyl-4-oxoadipyl-CoA are key intermediates in the biosynthetic pathway for **1** and are used as extenders for a Claisen condensation facilitating the incorporation of succinate. PamD catalyzes the first extension of a short-chain acyl starter unit with one of these compounds followed by the PamE-catalyzed addition of malonyl-CoA. The starter units are most likely supplied as ACP-esters.[16] After this step, the biosynthetic pathway divides into two branches. In one, the activity of the KRs PamO, M, and N coupled with the closure of the tetrahydrofuran ring by PamS, results in the formation of **3**. In the other branch, PamF adds the second molecule of adipate followed by the final extension with malonate catalyzed by PamG. Ketoreduction and closure of the tetrahydrofuran rings by PamO, M, N, and S result in the formation of **5**, which is further reductively aminated and methylated by PamX and PamY, respectively, to produce **4**. Both **3** and **4** are re-activated by PamL, an acyl-CoA ligase. The feeding of a mixture of free acids to the *S. albus* culture expressing the *pamL*, *pamJ,* and *K* genes produced **1** (Figure S13). The closure of the macrodiolide ring of **1** is performed by PamJ and PamK KSs, which catalyze an unusual C–O condensation reaction.[11b]

In summary, the biosynthetic gene cluster for the unusual polyketide secondary metabolite **1** was cloned, and the steps leading to its production were elucidated. In particular, the mechanism of succinate incorporation into the polyketide backbone was demonstrated to occur through 3-oxoadipyl-CoA, which represents a new node intermediate between the secondary and primary metabolism. To the best of our knowledge, PamA is the first studied enzyme responsible



Scheme 1. Proposed pathway for the biosynthesis of **1**.

for the incorporation of succinate into a polyketide molecule. The same condensation reaction occurs in the biosynthesis of **2** and the macrodiolide compound that consists of two molecules of homononactic acid.[21] The biosynthetic genes responsible for the production of the latter compound may be early predecessors of the *pam* and *non* gene clusters. The biosynthesis of these compounds is an interesting example of the utilization of unusual substrates to increase the structural diversity of polyketide natural products. In addition, the characterized *pam* genes will greatly expand the toolbox for the combinatorial biosynthesis of new polyketides.

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