Polyketide Biosynthesis

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A Common Origin for Guanidinobutanoate Starter Units in Antifungal Natural Products**

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The guanidinium group is found in bioactive natural products of both terrestrial and marine origin,[1] and its unique properties in molecular recognition underlie its incorporation into drugs and into molecules designed for supramolecular studies.[2] The natural products include a remarkable subgroup of guanidine-containing macrocyclic polyketides from streptomycete bacteria,[3–7] many of which show potent antifungal activity. In these compounds a 4-guanidinobutanoic acid starter unit is activated and transferred to a modular assembly-line polyketide synthase (PKS) multienzyme for elongation in the standard way.[8] The same starter unit appears also to be involved in the biosynthesis of several giant linear polyene polyketides.[9] We have recently completed sequencing of the genome of the prolific antibiotic-producing strain *Streptomyces violaceusniger* DSM 4137,[4c] which houses no fewer than 15 gene clusters for assembly-line natural product biosynthesis, including several known antifungal compounds.[4c] Intriguingly, two of these clusters (*azl* and *cle*) encode the biosynthetic pathway to different guanidinyl-containing polyketides: the 36-membered macrocyclic polyketides azalomycin F3a (**1a**) and F4a (**1b**) and the giant linear polyenes desulfoclethramycin (**2a**) and clethramycin (**2b**; Figure 1a).

We wished to establish whether both polyketide synthases indeed use 4-guanidinylbutyryl-CoA as starter unit for polyketide chain assembly; and whether this compound originates from arginine, as previously suggested but never proved.[9a,10] We report here that the genome of DSM 4137 contains two copies of genes encoding a three-step precursor pathway leading from L-arginine to 4-guanidinylbutyryl-CoA. These precursor genes are distributed across three different loci, neither the *azl* nor the *cle* cluster housing a complete set (Figure 1b). By heterologous expression of individual genes in *Escherichia coli* and reconstitution of the pathway in vitro, we show that both copies of each of these enzymes are active as indicated in Scheme 1. We have used specific gene knockouts to confirm that the pathway elucidated here is essential for biosynthesis of both metabolites. The *azl* and *cle* acyltransferases, which transfer the aminoacyl group to the respective polyketide synthases for chain initiation, are apparently able to prime either assembly-line. Such crosstalk may have evolved in *S. violaceusniger* as an important feature of its antifungal response.

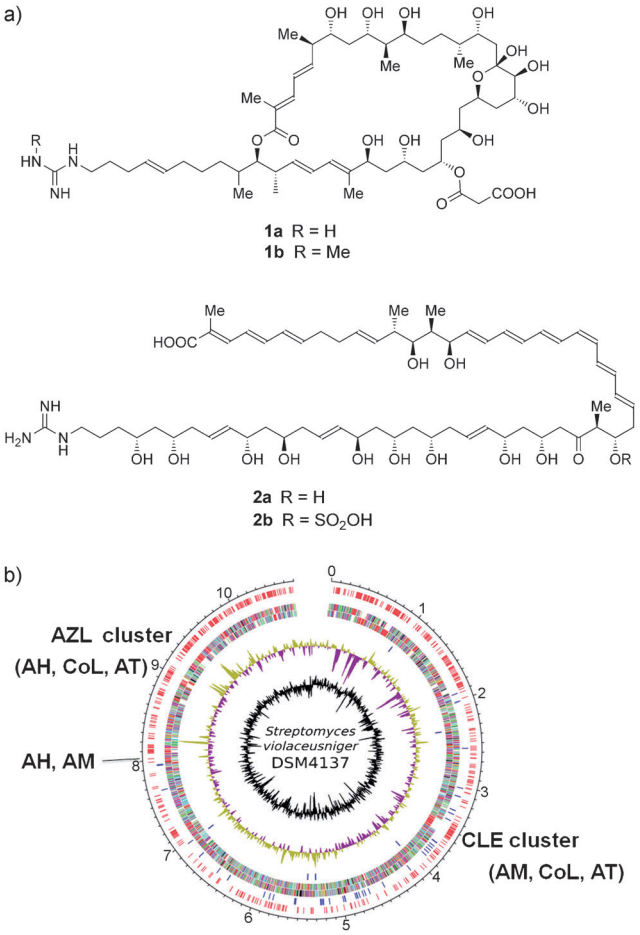

Figure 1. a) The structures of the guanidine-containing antibiotics azalomycin [F3a (**1a**) and F4a (**1b**)] and desulfoclethramycin (**2a**) and clethramycin (**2b**) produced by *Streptomyces violaceusniger* DSM 4137; b) the location of the corresponding *azl* and *cle* gene clusters, and of candidate genes for the synthesis and attachment of their common 4-guanidinobutanoyl starter unit, on the *S. violaceusniger* chromosome. AM, flavin-dependent arginine 2-monooxygenase (decarboxylating); AH, 4-guanidinobutyramide hydrolase; CoL, 4-guanidinobutanoate:CoA ligase; AT, 4-guanidinobutyryl-CoA:ACP acyltransferase.

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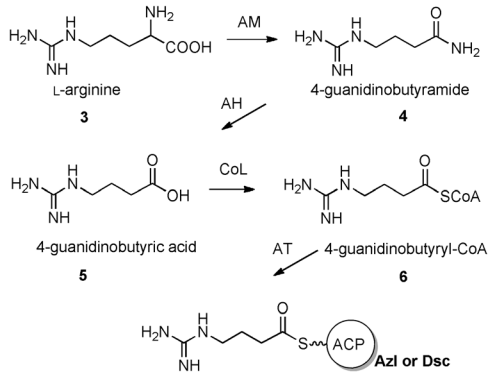

Scheme 1. Proposed pathway in *S. violaceusniger* DSM 4137 for production of 4-guanidinobutyryl-CoA from arginine, and transfer of the guanidinobutanoyl group to the N-terminal acyl carrier protein (ACP) domain of the azalomycin (*azl*) and clethramycin (*cle*) modular polyketide synthases.

The mechanism by which a guanidine-containing starter unit is incorporated into antifungal polyketides has been a matter of conjecture, despite early attempts to incorporate label from plausible precursors.[10a] We considered the mechanistic precedent offered by known examples in which a nonribosomal peptide synthetase (NRPS) module integrated into a hybrid PKS–NRPS multienzyme serves to introduce a specific amino acid unit, either to initiate[11] or to terminate[12] the polyketide chain. However, a better model is provided by recently characterized PKSs[13] in which the N-terminal loading module of the PKS consists of a single ACP, which is aminoacylated by a separate, specific acyltransferase. In particular, the sequencing of two different gene clusters for nitrogen-containing linear polyenes, ECO-02301 from *Streptomyces aizunensis*[10b] and ECO-0501 from *Amycolatopsis orientalis*[14] has revealed a lone ACP domain in the loading module, as well as candidate genes for a starter unit pathway clustered together with the polyketide synthase genes. These genes were predicted to encode an arginine monooxygenase (AM), a 4-guanidinobutanoate:CoA ligase (CoL) and a 4-guanidinobutyryl-CoA:ACP acyltransferase respectively. No 4-guanidinobutyramide hydrolase (AH) candidate was reported from either cluster (Scheme 1).

These putative precursor genes were used to screen the complete genome of *S. violaceusniger* DSM 4137 for homologous sequences (Figure 1 b and the Supporting Information). The *azl* cluster located at 9.2 Mbp on the linear genome houses eight PKS multienzymes (open reading frames (Orfs) STRVN_7492 to STRVN_7499), adjacent to a ligase (CoL; SRTVN_7500) and acyltransferase (AT; STRVN_7501), with a candidate amide hydrolase (AH) nearby (STRVN_7510) but no amino acid monooxygenase. The *cle* cluster at 3.5 Mbp on the genome contains nine PKS multienzymes (Orfs STRVN_2722-2730), clustered near a monooxygenase (STRVN_2699), a ligase (STRVN_2700), and an acyltransferase (STRVN_2704) but no amide hydrolase. The DSM4137 genome also contains, at around 8.0 Mbp, adjacent genes encoding AH (STRVN_6564) and AM (STRVN_6565; Figure 1 b).

We individually expressed in *E. coli* each of AM-2699, AM-6565, AH-6564, AH-7510, CoL-2700, CoL-7500, AT-2704, and AT-7501, as well as the N-terminal ACP domains from the polyene PKS multienzyme CleAI (STRVN_2730) and the macrocyclic polyol PKS multienzyme AzlA1 (STRVN_7492). All were obtained as purified recombinant proteins (Figure S2 in the Supporting Information). For assays of arginine monooxygenase activity, L-arginine (**3**) was incubated with purified AM in phosphate buffer and the reaction was monitored by using HPLC–MS. Arginine monooxygenase, first described in *Streptomyces griseus*,[15] is one of very few flavin-dependent enzymes capable of directly decarboxylating an α-amino acid in the presence of oxygen to yield an amide. With both AM-2699 and AM-6565, rapid conversion to **4** was observed, with no evidence either of further hydrolysis to 4-guanidinobutyric acid (**5**) or of a competing oxidative deamination to form 4-guanidinyl-2-oxo-butanoate (Figure 2 a). In contrast, there was no observable reaction when arginine was replaced by either lysine, glutamine, asparagine, tryptophan, ornithine, or histidine. When fully [^{13}C,^{15}N]-labeled L-arginine was used as substrate, the labeled amide product **4** was obtained in essentially quantitative yield, and its mass was shifted higher by nine mass units, as expected (Figure S3). The structure of labeled product **4** was further confirmed by ^{13}C NMR analysis (Figure S4). The labeled **3** and **4** were then fed to the DSM 4137 strain as potential precursors of the guanidinyl-containing secondary metabolites (Figures S5 and S6). Both precursors were incorporated intact, into both azalomycin and desulfoclethramycin (clethramycin, the final product of the pathway, is a minor coproduct under these conditions). The efficiency of incorporation from **3** and **4** was 10% and 72%, respectively, into azalomycin F3a, and 17.5% and 93%, respectively, into desulfoclethramycin. These results provided the first direct evidence of the importance of the AM-initiated pathway for starter unit biosynthesis in *S. violaceusniger*.

Amide hydrolase activity was also monitored using HPLC–MS, facilitated by the use of labeled **4** as substrate. Purified recombinant AH-6564 and AH-7510 both efficiently catalyzed this reaction (Figure 2 b and Figure S7). Likewise, both of the predicted ligases, CoL-2700 and CoL-7500, efficiently catalyzed the reaction of 4-guanidinobutyric acid (**5**) with ATP and CoASH in the presence of magnesium ions to give the thioester **6** (Figure 2 c and Figure S8). Neither 3-guanidinopropionic acid nor 4-aminobutyric acid were substrates for these ligase enzymes. These results taken together suggest that there is a single intracellular pool of **6** in DSM 4137 providing starter units for both the linear polyene and the macrocyclic polyketide.

We next determined whether the putative *cle* 4-guanidinobutanoyl-CoA:ACP acyltransferase (AT-2704) and its *azl* counterpart (AT-7501) catalyze the reaction predicted for them, and whether they show specificity for their own ACP substrate. Modeling of the AT sequences (Figure S9) showed that both are likely to adopt the fold of well-studied malonyl-CoA:ACP acyltransferases.[16] The recombinant ACPs were converted to the holo form by co-expression with 4′-phosphopantetheinyl transferase, and incubated with 4-guanidinobutyryl-CoA and either of the two ATs. HPLC–MS

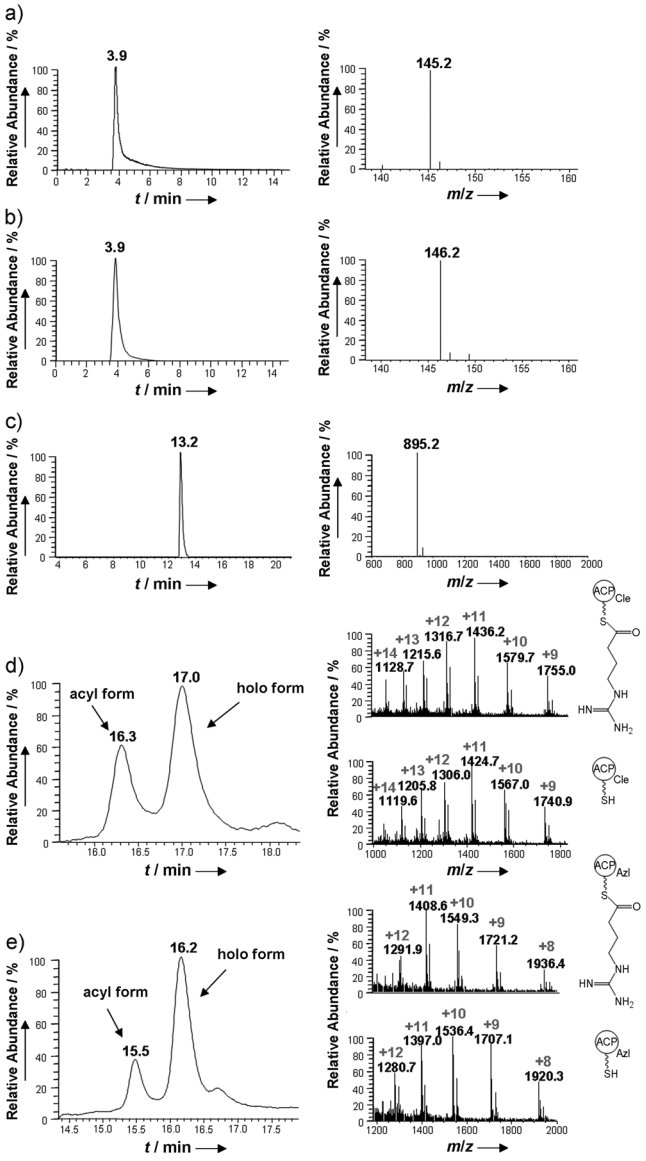

Figure 2. HPLC–MS analysis of reactions catalyzed by the enzymes in the 4-guanidinobutanoate pathway. a) HPLC–MS analysis of 4-guanidinobutyramide (**4**; *m*/*z* 145.2) produced by AM-2699 (AM-6565 behaved identically) using L-arginine as substrate; b) HPLC–MS analysis of 4-guanidinobutyric acid (**5**; *m*/*z* 146.2) produced by AH-6564 (AH-7510 behaved identically) using 4-guanidinobutyramide as substrate; c) LC–MS analysis of 4-guanidinobutyryl-CoA (**6**; *m*/*z* 895.2) produced by CoL-2700 (CoL-7500 behaved identically) using 4-guanidinobutyric acid and free CoA; d) HPLC–MS analysis of guanidinobutyryl-ACP produced by incubation of AT-2704 with 4-guanidinobutyryl-CoA and holo-ACP from the *cle* cluster; e) HPLC–MS analysis of guanidinobutyryl-ACP produced by incubation of AT-2704 with 4-guanidinobutyryl-CoA and holo-ACP from the *azl* cluster.

analysis of the product mixture revealed that under these conditions either AT is able to transfer the guanidinobutanoyl group to the N-terminal ACP domain of both PKSs, thus suggesting crosstalk in the initiation of clethramycin and azalomycin polyketide chain biosynthesis (Figure 2 d,e; and Figures S10, S16, and S19).

From the results of in vitro reconstitution of the pathway from **3** to **6**, it would appear that no single gene for any of the precursor pathway enzymes is essential. To test this, strains were constructed with specific in-frame deletion mutations in each of AM-2699, AH-6564, and AH-7510 (Figure S14) and their ability to produce azalomycin and (desulfo)clethramycin was examined. All three mutant strains produced both types of metabolite at wild-type levels (Table S4). In contrast, a double mutant, deleted in both AH-6564 and AH-7510, did not produce either metabolite (Table S4). The dependence of these distinct biosynthetic pathways on the same unusual starter unit provides a simple way to coordinate the production of these metabolites.

The linear polyene mediomycin A (Figure S11) produced by *Streptomyces mediocidicus* (Figure S12) differs from clethramycin only in that the predicted starter unit is 4-aminobutyrate.[9b] Mediomycin could be derived either by utilization of ornithine instead of arginine as an alternative substrate for an AM-led precursor pathway, or by late-stage unmasking of the amino group, catalyzed by an amidinohydrolase. The gene cluster for the polyene ECO-02301, which also contains a 4-aminobutyryl starter unit, is reported to encode a putative amidinotransferase, which might serve the same function.[14] We have recently sequenced the clethramycin/mediomycin cluster from *S. mediocidicus*, and found that it contains a putative amidinotransferase, as well as a gene, the product of which (AM-medio) has high (82%) sequence identity with AM-2699 (AM-cle) from DSM 4137; and a gene, the product of which (AT-medio) has high (79%) sequence identity with AT-2704 (AT-cle) from DSM 4137. Both these proteins were expressed and purified from *E. coli* (Figure S2c). The recombinant, purified AM-medio in vitro showed the same high specificity for arginine as AM-cle (Figure S13), while recombinant, purified AT-medio showed the same ability to acylate the N-terminal starter ACP domains of both the clethramycin and azalomycin PKSs using 4-guanidinobutyryl-CoA (Figures S17 and S20). These observations provide additional support for a common origin of the 4-guanidinobutanoate starter unit in antifungal polyketides. Further, they raise the intriguing possibility that numerous other metabolites with a starter unit apparently derived from ornithine, such as linearmycin,[17] tetrafibricin,[18] the marginolactones oasomycin and desertomycin,[10a] and monazomycin A,[6] also utilize 4-guanidinobutanoate as a starter unit; and that the primary amino group in these antibiotics is unmasked at a late stage of biosynthesis by the action of a specific amidinotransferase. Clear precedents for use of such a protective group strategy can be found in the biosynthetic pathways to both the aminoglycoside butirosin[19] and the macrolactam vicenistatin.[20]

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