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Nonlinear Biosynthetic Assembly of Alpiniamide by a Hybrid *cis/trans*-AT PKS-NRPS

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Abstract

Alpiniamide A is a linear polyketide produced by *Streptomyces* endophytic bacteria. Despite its relatively simple chemical structure suggestive of a linear assembly line biosynthetic construction involving a hybrid polyketide synthase-nonribosomal peptide synthetase enzymatic protein machine, we report an unexpected nonlinear synthesis of this bacterial natural product. Using a combination of genomics, heterologous expression, mutagenesis, isotope-labeling, and chain terminator experiments, we propose that alpiniamide A is assembled in two halves and then ligated into the mature molecule. We show that each polyketide half is constructed using orthogonal biosynthetic strategies, employing either *cis*- or *trans*-acyl transferase mechanisms, thus prompting an alternative proposal for the operation of this PKS-NRPS.

Graphical Abstract

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Supporting Information

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Information regarding the experimental methods as well as supplemental figures, NMR, and LC-MS data (PDF)

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Streptomyces and allied actinobacterial genera are responsible for the production of the majority of known bacteria bioactive natural products.^{1,2} In particular, their large genomes encode diverse biosynthetic gene clusters housing polyketide synthases (PKS) and nonribosomal peptide synthetases (NRPS), chain-building megasynthases that use a modular paradigm for the construction of some of the most potent and structurally diverse chemicals in microbiology.

A large number of PKS and NRPS multienzymes exhibit a colinear arrangement between gene sequence and the encoded modules and domains, allowing relatively confident prediction of gene structure from the chemical nature of the product, and *vice versa*. In a canonical modular PKS, such as the PKSs for biosynthesis of erythromycin,³ nystatin,⁴ and candicidin,⁵ each extension module contains core ketosynthase (KS), acyl carrier protein (ACP), and acyltransferase (AT; *cis*-AT) domains, which select the extender unit and promote a specific cycle of polyketide chain elongation. The optional presence of amending ketoreductase (KR), dehydratase (DH), and enoylreductase (ER) domains accounts for the vast number of structurally complex and diverse metabolites accessible by this common pathway.^{6,7}

Typical modular NRPSs^{8–10} follow a similar processive and flexible assembly logic to produce nonribosomal peptides, using core condensation (C), adenylation (A), and thiolation (peptidyl carrier protein) (T) domains, together with optional modifying domains including methyltransferase (MT), epimerase (E), and cyclization (Cy) domains which act on the chain-extension intermediates.⁷ A number of colinear PKS–NRPS modular systems have also been uncovered in which ketide and amino acids are incorporated in a single hybrid assembly line.^{11–14}

The assembly line paradigm also embraces so-called *trans*-AT (or AT-less) PKSs,¹⁵ whose hallmark is the absence of AT domains integrated into each extension module. In these systems, the AT activity is supplied by a *trans*-acting AT enzyme encoded within the gene cluster, which docks at a specific site (ATd) in each extension module.^{16–18} The free-standing AT shows high specificity for malonyl-CoA extender units.^{15,19} *Trans*-AT PKS (and PKS–NRPS) multienzymes typically feature unusual domain order, modules shared

between adjacent proteins, and enzymatic activities acting iteratively across module boundaries.^{15,19–23} Nevertheless, detailed phylogenetic analysis of KS domains has permitted increasingly accurate assignment of function to domains and modules.²⁴ Deciphering these and other notable departures from colinearity^{25–27} provides valuable insights into the evolution and scope of assembly line enzymology.

Whole genome sequencing (WGS) has become an essential tool to identify and decipher biosynthetic assembly lines,^{28,29} as fragmented draft sequences have increasingly been supplanted by near-finished sequence data.³⁰ We have previously identified 35 putative biosynthetic gene clusters in the genome of *Streptomyces* sp. CBMAI 2042, an endophytic bacteria from *Citrus sinensis*.³¹ Within this extensive biosynthetic potential, we were particularly intrigued by an apparently novel hybrid PKS–NRPS assembly line with both *cis*- and *trans*-AT domains, an organization for which there are few precedents.¹⁵

During our study, a nearly identical cluster was reported from *Streptomyces* sp. IB2014/011-12 isolated from Lake Baikal sediments³² and shown to govern the biosynthesis of alpiniamide A (**1**, Figure 1) and several congeners. This (or a closely related compound) was previously isolated from the endophytic *Streptomyces* sp. YIM66017 in *Alpinia oxyphylla*.³³

These authors have proposed³² that **1** is assembled by a mechanism in which a *cis*-AT domain recruits a propionate starter unit, and a *trans*-AT domain dictates the incorporation of four malonyl-CoA extender units flanking an NRPS-specified glycyl unit. These malonate units are methylated by integrated methyltransferase domains to provide the additional three-carbon “propionate” units of alpiniamide. However, their data left open the possibility of an alternative origin of these units. In the present work, we describe the production of alpiniamide A by the endophytic strain *Streptomyces* sp. CBMAI 2042. Our analysis of this system establishes a different biochemistry for alpiniamide A biosynthesis that provides a rare example of mixed origins of propionate units to provide the methyl-branched polyketide scaffold.

RESULTS AND DISCUSSION

Genome Mining.

We previously reported that the genome-sequenced endophytic strain *Streptomyces* sp. CBMAI 2042³¹ produces cyclodepsipeptide derivatives, valinomycin³⁴ and its analogues,^{35,36} and the peptide blue pigment indigoidine.³¹ Of the eight PKS gene clusters identified by antiSMASH analysis,³¹ a DNA region located on contig1 (GenBank: [RCOL000000000](#), from 122974 to 152587 bp) was assigned with an unusual *cis/trans*-AT PKS–NRPS cluster arrangement (Figure S1a). Bioinformatic analysis indicated three biosynthetic genes encoding, respectively, a type I PKS, housing a single *cis*-AT module and a *trans*-AT module (ctg1_117 – *alpA1*); a *trans*-AT-like pentadomain protein (ctg1_118 – *alpA2*); and a hybrid *trans*-AT PKS–NRPS (ctg1_119 – *alpA3*). Gene ctg1_115 (*alpT*) encodes a malonyl-CoA-specific bidomain protein (AT-ACP), suggesting a free-standing AT function as required in *trans*-AT type systems^{15,37} (Figures 2a and b, Table S1).

PKS multienzymes containing both *cis*- and *trans*-AT are rare,¹⁵ and at the time of our initial investigation of the *alp* locus in CBMAI 2042, it was an orphan gene cluster. When the *alp* gene cluster from *Streptomyces* sp. IB2014/011-12 (GenBank: [QEIK00000000.1](#)) was published and connected to alpiniamides,³² it was evident that it shared 90% sequence similarity with the gene cluster in *Streptomyces* sp. CBMAI 2042 and had an identical organization of modules and domains (Figures 2c and S1b). However, our independent bioinformatic analysis suggested the possibility of a different assembly logic for the ketide units in alpiniamide than earlier reported by Luzhetskyy and co-workers.³² The crude extract of *Streptomyces* sp. CBMAI 2042 was very complex, and our attempts to connect the gene cluster to a chemical entity in wild type (WT) cultures during the initial studies were unsuccessful. In order to test our hypothesis, we captured the CBMAI 2042 *alp* gene cluster for heterologous expression in an optimized host.

Whole Genome Cluster Reconstitution and Heterologous Expression of Alpiniamide A.

Initial bioinformatic analysis identified a region comprising 29.8 kb from *alpF* to *alpQ* (Figure 2b, Table S1) as likely to be involved in assembly of alpiniamide. PCR-based transformation-associated recombination (TAR) cloning^{38–42} was used to capture the gene cluster into the pCAP03_p21 vector,^{42,43} with insertion of the strong constitutive p21 promoter⁴⁴ upstream of the cloning site. The 29.8 kb *alp* BGC was divided into seven DNA fragments ranging from 3.5 kb to 5 kb containing ~150 bp homology overlaps between the fragments and 40 bp arms homologous to the linearized vector. The amplified PCR products were confirmed by restriction analysis (Figures S2 and S3) and transformed with the linear vector in *Saccharomyces cerevisiae* VL6–48N³⁸ for recombination. After restriction analysis (Figure S4) to confirm the correct construct of assembled plasmids, pRSalp_6B (~40.3 kb) was selected for conjugation with *Streptomyces coelicolor* M1146⁴⁵ and heterologous expression. The recombinant strain *S. coelicolor* M1146_RSalp was confirmed by PCR for chromosomal integration of the cluster (Figure S5).

S. coelicolor M1146_RSalp, *Streptomyces* sp. CBMAI 2042 (WT), and *S. coelicolor* M1146 (empty strain, control) were each cultivated in triplicate in an MP medium for 7 days. As an additional control, in-frame deletion^{46,47} of the adenylation (A) domain from the NRPS gene *alpA3* resulted in the mutant RSalp A_{domain}, expected to be specifically disrupted in alpiniamide production (Figure S6). From the comparative analysis using a C₁₈ reversed phase HPLC-MS, we observed a distinct metabolite in extracts of both the native and heterologous M1146_RSalp strains evidencing that the *cis/trans*-AT product was produced by the wild type strain. As expected, extracts of the RSalp A_{domain} showed no production of alpiniamide (Figures 2d and S7).

HPLC isolation provided approximately 3 mg of the major expressed product from a 500 mL culture extract of *S. coelicolor* M1146_RSalp. Based on (+)-HR-ESI-MS analysis (Figure S8), we identified a molecular formula of C₁₇H₂₉NNaO₆ (four degrees of unsaturation) corresponding to a sodium adduct ion at *m/z* 366.1886 [M + Na]⁺ and the molecular formula C₁₇H₂₇NO₅ (five degrees of unsaturation) corresponding to the dehydrated molecule at *m/z* 326.1960 [M-H₂O+H]⁺ (Table S2). ¹H NMR spectroscopic data (Table S3 and Figure S9) and additional COSY, HSQC, and HMBC (Figures S10, S11, and

S12) cross-peak analysis, together with SciFinder and Streptome DB⁴⁸ searches, allowed elucidation of the structure of alpiniamide A (Figure S13) as the main product, with analytical and spectroscopic data consistent with those reported for **1** isolated from *Streptomyces* sp. IB2014/011-12³² and *Streptomyces* sp. YIM66017.³³ Luzhetskyy and colleagues were able in their work to establish the structures of additional alpiniamides B–E, coproduced as minor components together with **1**,³² affording valuable evidence of plasticity of the alpiniamide assembly line. HPLC-MS and NMR analysis did detect trace levels of such species in extracts of *S. coelicolor* M1146_RSalp, but our analysis presented below relates specifically to the mechanism of production of **1**.

***cis/trans*-AT Assembly Line in Alpiniamide A Biosynthesis.**

Previous studies have shown that *trans*-AT domains are highly specific for malonyl-CoA as substrate^{15,16,37,50} and that the introduced two-carbon unit may subsequently undergo α -methylation by *S*-adenosylmethionine (SAM)-dependent integrated MT domains to yield a methyl-branched extension unit. In contrast AT domains of *cis*-ATs are highly specific for recruiting either malonyl- or (2*S*)-methylmalonyl-CoA or (more rarely) other alkylmalonyl-CoA esters.

The results of heterologous cluster expression, as well as previous deletion analysis of the *alpD* and *alpR* genes in IB2014/011-12³² showing them to be dispensable, confirmed that the region from *alpF* to *alpA3* is likely to be essential for alpiniamide A biosynthesis. AlpA1 encodes a multidomain type I PKS with predicted domain order KS, AT, ACP, KS, DH, KR, and ACP. It comprises a candidate starter module (KS^s, *cis*-AT, ACP) predicted to recruit a propionate unit, followed by a single extension module lacking an AT domain and also lacking a docking domain for a discrete *trans*-AT enzyme (ATd). Multienzyme AlpA2 encodes a *trans*-AT-like protein with domains cMT, ACP, KS, ATd, ACP, and a thioesterase (TE) domain. AlpA3 is a multidomain hybrid *trans*-AT PKS-NRPS comprising domains C, A (glycine-specific), PCP, KS, ATd, cMT, ACP, KS, DH, and KR. AlpT provides a candidate external malonyl-CoA-specific *trans*-AT activity.

To identify the respective roles of the annotated *trans*- and *cis*-AT enzymes in alpiniamide A biosynthesis, feeding experiments were performed with labeled precursors, L-methionine-(methyl-¹³C) and sodium propionate-1-¹³C, followed by comprehensive MS/MS analyses (Figure 3). By performing these experiments in the heterologous strain *S. coelicolor* M1146_RSalp, the undesired competition for these biosynthetic precursors was reduced.

LC-MS analysis revealed a reproducible pattern of incorporation: when L-methionine-(methyl-¹³C) was added to M1146_RSalp cultures, strong M+1 and M+2 isotopic peaks were observed, indicating that two methyl groups of alpiniamide were derived from SAM, rather than from propionate, the direct precursor of methylmalonyl-CoA (Figure 3a). This is convincing evidence that conventional *trans*-AT enzymology incorporates only two of the five ketide units in alpiniamide, not four as in the previously advanced mechanistic proposal.³²

Crucially, when *S. coelicolor* M1146_RSalp was grown in the presence of sodium propionate-1-¹³C, the results suggested the incorporation of three three-carbon methyl-

branched building blocks from methylmalonyl-CoA, as shown by strong M+1, M+2, and M+3 isotopic peaks (Figure 3b). Labeled propionate was not fed in the previous work,³² and so this feature of alpinamide biosynthesis would have been overlooked.

To confirm the location of the introduced ¹³C labels, tandem mass (MS/MS) spectra were obtained from labeled and unlabeled samples of alpinamide A. Consistently altered isotopic patterns were seen in MS/MS when compared to unlabeled alpinamide A (Figure S14). From L-methionine-(methyl-¹³C) feeding experiments (Figure 3b), MS/MS spectra showed the presence of fragment ions (m/z 137.0594 (M+2) and m/z 154.0852 (M+2)) corresponding to the presence of two methyl-¹³CH₃ in accordance with L-methionine-(methyl-¹³C) incorporation promoted by the (SAM)-dependent integrated MT domains only in the “post-NRPS subunit” (red portion, Figure 3a). The ion fragments from the “pre-NRPS subunit” (m/z 109.1002, m/z 137.0955, and m/z 154.1213; blue portion, Figure 3a) evidence no incorporation of a labeled methyl group. The reciprocal labeling pattern was observed after sodium propionate-1-¹³C feeding (fragment ions m/z 109.1015 (M+2), m/z 137.0953 (M+2), and m/z 154.1233 (M+3); Figure 3c) demonstrating the presence of either two or three ¹³C-labels in the “pre-NRPS subunit” (blue portion, Figure 3a). Here, the ion fragments from the “post-NRPS subunit” (m/z 137.0600 and m/z 154.0860; red portion, Figure 3a) display no presence of a label.

Taken together, these results are consistent with the discrete AT AlpT providing extender units *in trans* to each of two post-NRPS extension modules (one in AlpA2 and one in AlpA3). Both these multienzymes have an ATd domain, proposed to accommodate the docking of a *trans*-AT enzyme (Figure S1), while AlpA1 has none. Furthermore, both extension module 3 in AlpA3 and extension module 4 (split between AlpA3 and AlpA2) have an integral methyltransferase (MT) domain, which would methylate the growing polyketide chain at these positions. In contrast, the three propionate units in the pre-NRPS part of the assembly line are likely to be introduced by incorporation of methylmalonyl-CoA. The N-terminal loading module of AlpA1 contains a methylmalonyl-CoA-specific AT domain, an ACP, and a so-called KS^s domain, in which the active site cysteine (Cys) residue of a ketosynthase is replaced by serine (Ser; Figure S15). As in several polyene biosynthetic pathways,^{51–53} KS-Ser is proposed to act as a decarboxylase converting methylmalonyl-ACP into propionyl-ACP to provide the starter unit for polyketide chain synthesis.

Conceivably, the loading module AT could also service the adjacent extension module of AlpA1. In the assembly line biosynthesis of enacyloxin⁵⁴ in *Burkholderia ambifaria*, two *cis*-AT domains located in extension modules 1 and 6 appear to act *in trans* to incorporate all malonyl extender units into the polyketide chain.⁴² An alternative possibility is suggested by the presence in the *alp* cluster of the essential gene *alpE*,³² which encodes a 3-oxoacyl-ACP synthase III (KASIII) enzyme. The sequence of AlpE most closely resembles that of specialized KS domains that transfer alkylmalonate units onto PKS ACPs, such as DpsC in daunorubicin biosynthesis⁵⁵ and OzmC in oxazolomycin biosynthesis.⁵⁵ AlpE may therefore act as a methylmalonyl-CoA:ACP acyltransferase to provide propionate extension units for AlpA1.

In this revised proposal for the alpiniamide assembly line, the multienzymes are used in the order AlpA1-AlpA3-AlpA2 (Figure 4). The single extension module of AlpA1 is used iteratively to create a branched triketide acyl chain on the “pre-NRPS” left-hand side of 1. Such iterative behavior in a modular PKS is uncommon but has been invoked in the biosynthesis of the fatty acid synthase inhibitor thiolactomycin⁵⁶ and in the pathway to lankacidin antibiotics.⁵⁷

Detailed bioinformatic analysis of the domains of the Alp PKS-NRPS was undertaken to assess how closely their predicted functions match the requirements of the revised scheme in Figure 4. Key active site sequence motifs were initially detected using the automated genome annotation pipeline antiSMASH^{58,59} and then curated manually. The Alp PKS-NRPS was also analyzed by the *trans*-AT PKS polyketide structure predictor TransATor (<https://transator.ethz.ch>).²⁴ This software exploits the previous finding that in *trans*-AT PKSs KS phylogeny reports directly on the chemical nature of the growing chain substrate.⁶⁰ The results showed that, in accordance with the scheme of Figure 4, the *N*-terminal KS of AlpA1 is predicted as a starter KS, and the second KS of AlpA1 is predicted to act on either β -hydroxy- α -methylated chains or α,β -unsaturated, α -methyl chains. Similarly, the KS of module 3 in AlpA3 is predicted to prefer an aminoacyl substrate and the KS of module 4 (shared between AlpA3 and AlpA2) to prefer β -d-hydroxy- α -L-methylated chains. All the KSs in the Alp assembly line contain the Cys-His-His triad of active site residues essential for condensing enzyme activity. However, TransATor²⁴ analysis revealed that the KS of AlpA2 is found in a clade of nonelongating KS (KS^o – module 5) domains, in good accordance with the scheme in Figure 4.

The active site sequences of KR domains in modular PKSs provide information about the configuration of the β -hydroxacyl thioester product.⁶¹ antiSMASH analysis showed in accordance with the proposed scheme that the KR of AlpA1 is predicted to be active (B1 type). The KR of AlpA3 should from the scheme of Figure 4 be active but is predicted to be inactive (C2 type) because an essential active site asparagine residue is substituted by cysteine. The reason for this discrepancy is unclear.

All the essential active site sequence motifs are present in the two annotated Alp PKS dehydratase (DH) domains. The scheme illustrated in Figure 4 requires that the DH domain of AlpA1 (module 1) is active in the first extension cycle but inactive in the second. A precedent for such conditional use of a domain is found for example in the 2-fold use of the first extension module of the azalomycin PKS⁶² where an enoylreductase (ER) domain is active only in the second extension cycle. Similarly, the DH of module 4 in AlpA3 is predicted to be active but is apparently skipped in order to produce 1.

A more radical feature of the proposed scheme in Figure 4 is that it envisages that the two halves of the alpiniamide molecule are separately assembled and the two chains are then joined in amide linkage. In the assembly of all previously characterized hybrid PKS–NRPS products in which a glycine unit is flanked by polyketide units, such as oxazolomycin,⁶³ jamaicamide,⁶⁴ and pederin/onnamide,^{65,66} a single chain-building process has been invoked. This conventional strategy in particular prevents the production of short enzyme-bound thioester intermediates having an exposed *N*-amino group, which might abort chain

synthesis through intramolecular nucleophilic attack on the thioester. Such considerations have been used to rationalize the use of natural protective group strategies in the biosynthesis of butirosin,⁶⁷ macrolactams,⁶⁸ and marginolactones.⁶⁹ On the other hand, there are now well-documented modular PKS and PKS/NRPS systems in which separately assembled chains are then connected. For example, malleilactone/burkholderic acid^{70,71} arises from distinct polyketide chains further linked through an ester bond catalyzed by a condensation (C) domain. In other systems, a KASIII-type ketosynthase enzyme catalyzes head to head Claisen-like condensation of two chains,⁷² for example, in myxopyronin⁷³ and coallopyronin⁷⁴ biosynthesis.

In an effort to confirm the proposed noncolinear arrangement of modules in alpiniamide biosynthesis, and to investigate whether the alpiniamide backbone might be synthesized as two separate chains, we tested malonyl- and methylmalonyl-mimic probes (chain terminators) in feeding experiments^{75–77} to offload and detect enzyme-bound intermediates from the assembly line(s) *in vivo*.

Evidence that Alpiniamide A Biosynthesis Involves Postassembly Connection of Two Separate Chains.

Streptomyces M1146_RSalp was grown in the presence of either of the chemical probes 2 and 3 (Figure 5), mimics, respectively, of the extender units malonyl- and methylmalonyl-CoA.^{75–78} The method relies upon successful competition by the mimic for condensation with the growing chain, and offloading of shorter chains is generally found to be more effective. A summary of the intermediates intercepted *in vivo* and detected by LC-HRMS analyses is given in Figure 5. Using the malonyl-mimic probe 2, several off-loaded intermediates were observed from the *trans*-AT (post-NRPS) multienzymes, as judged by the detection of ions with *m/z* 201.1228 (Figure S16) corresponding to the capture of glycine; *m/z* 243.1339 (Figure S17) corresponding to the malonyl-mimic probe 2 plus an acetate unit condensed to glycine; and the ion with *m/z* 257.1500 (Figure S18), corresponding to an intermediate in which the acetate unit is methylated, evidence of the action of the integral *C*-methyltransferase in AlpA3. These data provide the first evidence for chain extension on AlpA3 initiated by a free glycine unit, a key feature of the revised scheme (Figure 4). From feeding experiments using the methylmalonyl-mimic 3, we observed the ion *m/z* 214.1440 (Figure S19) corresponding to offloading of a propionyl unit from the start of the *cis*-PKS chain in AlpA1.

However, we cannot yet exclude the possibility that the detected species arise from a low-level aberrant functioning of a single assembly line, the normal intermediates from which were not captured.

The nature of the enzyme that would link two separate chains to form alpiniamides is unclear. The KASIII-like protein AlpE has already been referred to as a candidate AT supplying extender propionate units to AlpA1. This enzyme was previously proposed³² to furnish the propionate starter unit for AlpA1. Interestingly, in the benzoxazole antibiotic A33853⁷⁹ formed from two moieties of 3-hydroxyanthranilic acid (3-HAA) and one 3-hydroxypicolinic acid (3-HPA), a KASIII-like protein (BomK) has been shown to catalyze amide bond formation between 3-HPA and the 3-HAA dimer as its thioester. Against this, as

previously noted,³² phylogenetic analysis of AlpE places it in a distinct clade of KASIII enzymes acting as specific short-chain acyltransferases and condensing enzymes (Figure S20). Alternatively, the *N*-terminal C domain of AlpA3 may catalyze amide linkage between the amino-diketide released from AlpA3/AlpA2 and the AlpA1-tethered triketide.

CONCLUSIONS

This work presents a new proposal for alpiniamide A biosynthesis based on the surprising finding that the five propionate units of this natural product arise by two entirely different mechanisms. The type I modular PKS encoded by *alpA1* is proposed to incorporate a propionate starter unit using an integrated *cis*-AT domain, as well as incorporating propionate extender units catalyzed by a discrete KASIII enzyme acting as a dedicated methylmalonyl-CoA:ACP acyltransferase. In contrast, the *trans*-AT PKS-NRPS multienzyme AlpA2–AlpA3 recruits extension units from malonyl-CoA that are then methylated by integrated S-adenosylmethionine (SAM)-dependent methyltransferase domains. Such an arrangement is highly unusual but not unprecedented: the cyanobacterial natural product nosperin⁸⁰ has a pre-NRPS portion predicted to incorporate acetate, and methyl branches arise from SAM-dependent methylation, while the post-NRPS final extension module recruits malonyl-CoA by a classical *cis*-AT route. We have also obtained initial evidence that alpiniamide assembly may take place on two parallel assembly lines, the products of which are then linked together, an unusual arrangement which has interesting evolutionary implications. These insights, together with the independent findings of Luzhetskyy and colleagues,³² provide a valuable foundation for future biochemical exploration of this intriguing biosynthetic pathway.

METHODS

DNA Fragments.

Genomic DNA (gDNA) extracted from *Streptomyces* sp. CBMAI 2042 was used as a template for all PCR reactions to obtain seven PCR fragments covering the *alp* gene cluster. Primer pairs were designed using Geneious v. 10.2.3 (Table S4). Fragments were concentrated with a DNA Clean & Concentrator kit (Zymo Research), purified from agarose gel with QIAquick Gel Extraction Kit (Qiagen). Vector pCAP03_p21 was linearized with restriction enzymes (NdeI and EcoRI) and recovered from a 0.5% agarose gel with QIAquick Gel Extraction Kit (Qiagen).

TAR Cloning.

A single colony of *S. cerevisiae* VL6–48N strain was inoculated in 10 mL of YPD liquid medium (glucose 2%, peptone 1%, yeast extract 0.5%) supplemented with adenine hemisulfate salt (100 mg/L) at 30 °C and 220 rpm overnight, and a previously optimized protocol was followed.^{38,43,81} A total of 20 μ L of transforming DNA mix (120 ng linearized vector and ~100 ng of each PCR amplified fragments) was prepared for yeast cell transformation according to Table S5. Isolated colonies were selected and transferred to a fresh YPD agar plate containing 5-fluorootic acid (5-FOA).⁸² After 48 h, six random selected yeast colonies were selected and grown on YPD liquid medium for 24 h at 30 °C

and 200 rpm for DNA extraction with Zymoprep Yeast Plasmid Miniprep II (ZYMO RESEARCH) and screened by PCR (Table S4). Positive plasmids (Figure S4) were transferred into electrocompetent *E. coli* DH10B cells. Plasmids were then purified from antibiotic resistant *E. coli* clones with a Qiagen Plasmid extraction kit, and resulting constructions were confirmed by restriction analysis (BamHI; EcoRI+EcoRV; KpnI +HindIII; BamHI+HindIII).

Intergeneric Triparental Conjugation.

pRSalp_6B plasmid (~41 kb) was transferred to chemically competent *E. coli* ET12567. For conjugation, ET/pRSalp_6B and ET1257/pUB307 (helper strain) colonies were inoculated into 10 mL of 2TY medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl) with appropriated antibiotics at 37 °C and 250 rpm overnight. A total of 100 μ L of inoculum was transferred to 10 mL of fresh 2TY medium containing antibiotics, incubated at 37 °C until A_{600} was 0.4–0.6. The cells were spun down and washed twice with 2TY medium and resuspended in 0.4 mL of 2TY. *S. coelicolor* M1146 mycelia from a 36 h culture in TSBY liquid medium (3% tryptic soy broth, 10.3% sucrose, 0.5% yeast extract) were washed twice with 2TY and resuspended in 1 mL. For conjugation, 0.2 mL of *E. coli* cells and 0.5 mL of *S. coelicolor* M1146 were mixed, and 150 μ L of mixture was plated on SFM agar (2% D-mannitol, 2% soya flour, 2% agar, 10 mM MgCl₂). Plates were incubated for 16 h at 30 °C and followed by overlay with 1 mL of antibiotic solution (final concentration 40 μ g mL⁻¹ of kanamycin and 20 μ g mL⁻¹ nalidixic acid). Incubation at 30 °C for 5–7 days resulted in exconjugants, which were streaked on an SFM agar plate containing nalidixic acid (25 μ g mL⁻¹) and kanamycin (50 μ g mL⁻¹).

Heterologous Expression.

M1146_RSalp was routinely precultivated in TSBY liquid medium containing a half concentration of kanamycin and nalidixic acid at 30 °C for 48 h. A total of 400 μ L of the preculture was inoculated into 40 mL of MP medium (1% glucose, 1% soluble starch, 0.5% NaCl, 0.3% yeast extract, 0.3% soytone, 0.2% peptone, 0.2% malt extract, 25 mM TES pH 7.2) and incubated for 7 days at 30 °C (250 mL flasks) and 250 rpm.

Alpiniamide A Isolation and Purification.

Extraction of cultures was performed by adding 20 g L⁻¹ of Amberlite XAD7HP resin (SIGMA) to the cultures followed by rotary shaking (250 rpm) for 30–60 min. The resin and cell pellet were recovered from the fermentation broth by centrifugation at 8000 rpm, for 20 min. The supernatant was removed, and 15 mL of methanol was added and stirred for 1–2 h to elute adsorbed metabolites from the resin. Solvent was evaporated under a vacuum followed by lyophilization. The extract was dissolved in MeOH and separated on a HyperSep C18 SPE Cartridge (Thermo Fisher Scientific), which was washed with increasing MeOH concentrations. Fractions containing alpiniamide A were combined, and organic solvent was evaporated under a vacuum followed by lyophilization. Further purification was achieved by preparative high-performance liquid chromatography (HPLC) using a Phenomenex Luna C18 column (5 mm, 100 mm $\times$ 2 mm) coupled to an Agilent Technologies system composed of a PrepStar pump, a ProStar 410 autosampler, and a ProStar UV. The solvent systems used was as follows: deionized water (A) and acetonitrile

(B) buffered with 0.1% trifluoroacetic acid (TFA; v/v). The HPLC separation profile consisted of 5 min of isocratic development at 5% B, 20 min of linear gradient from 5% to 70% B, 5 min of linear gradient from 70% to 100% B, 2 min of isocratic development at 100% B, and 3 min of linear gradient from 100% to 5% B, 15 mL/min flow rate and monitored UV at 200–600 nm. Fractions of interest were combined; solvent was evaporated to completion *in vacuo* and lyophilized, yielding 3 mg of brown oil, for NMR analysis.

In Frame Gene Deletion.

For A domain knockout, a deletion plasmid was obtained by cloning ~2 kb regions flanking the targeted gene into the suicide plasmid pYH7.^{46,47} The two fragments and linearized pYH7 (NdeI restriction enzyme) were isothermally assembled according to the Gibson Assembly protocol.⁸³ Final construction was confirmed by restriction analyses with NdeI endonuclease and Sanger sequencing. pRSalp A_{domain} plasmid was transformed into *E. coli* ET12567/pUZ8002 and then was transferred into the *Streptomyces* sp. CBMAI 2042 chromosome by conjugation. Exconjugants obtained on an SFM plate were streaked on fresh agar containing apramycin (Apr) and nalidixic acid (Nal) for selection of the first crossover event. Next, apramycin-resistant colonies were restreaked on antibiotic-free SFM agar plates for a second crossover. Single colonies from nonselective plates were replica-plated on Apr-free and Apr-containing plates. Six clones that were sensitive to Apr (Apr^S) were screened by PCR resulting in two double-crossover mutants containing the desired gene deletion (Figure S6B).

Feeding Experiments with Labeled Precursors.

An isolated colony of M1146_RSalp was precultivated in TSBY containing a half concentration of kanamycin and nalidixic acid at 30 °C for 48 h. A total of 1% of the preculture was inoculated into different flasks containing 30 mL of MP medium and supplemented with labeled precursor every 10–12 h (four time points) up to a final concentration of 1 mM L-methionine-(methyl-¹³C) (Cambridge Isotope Laboratories) and 12.9 mM sodium propionate-1-¹³C (Sigma-Aldrich). Duplicates were prepared for each labeled feeding experiment. After 7 days of incubation, the cultures were extracted as previously described. The extracts were evaluated by LC-MS.

Feeding Experiments with Chain Termination Synthetic Probes.

An isolated colony of M1146_RSalp was precultivated in TSBY containing a half concentration of kanamycin and nalidixic acid at 30 °C for 48 h. A total of 1% of the preculture was inoculated into different flasks containing 25 mL of MP liquid medium. After 24 h of incubation, daily additions of chain termination synthetic probes (dissolved in methanol, up to 50 μ L) were carried out over the next 4 days to reach a 5 mM final concentration as previously described.⁷⁷ Control experiments were set up with no probes. Feeding experiments were set up in duplicate. After 7 days of incubation, the cultures were extracted as previously described. The extracts were evaluated by LC-MS.

LC-MS Analysis of Alpiniamide A.

Methanol extracts were filtered with 0.2 mm PTFE filters for LC-MS analysis. A 2 μ L aliquot was injected onto a Phenomenex Luna C18 reversed-phase HPLC column (5 μ m, 150 $\times$ 4.6 mm) and analyzed with an Agilent Technologies 1200 Series system coupled to an Agilent Technologies 6530 Accurate-Mass Q-TOF mass spectrometer in positive ionization mode. Data were analyzed with Agilent MassHunter software B.05.01. A solvent system of water (A) and acetonitrile (B) buffered with 0.1% formic acid (v/v) and the following analytical method were used: 0.75 mL min⁻¹ flow rate, 0–2 min 95% A/5% B; 2–27 min, 95% A/5% B to 0% A/100% B; 27–32 min, 0% A/100% B; 32–32.5 min, 0% A/100% B to 95% A/5% B; 32.5–35 min, 95% A/5% B. Data acquisition for feeding experiments is shown in the Supporting Information.

NMR Analysis.

¹H NMR spectra and 2D correlation maps (COSY, HSQC and HMBC) were recorded on a JEOL ECA 500 spectrometer (500.16 MHz) using deuterated methanol (CD₃OD) as a solvent. ¹³C chemical shifts were inferred from 2D correlation maps HMBC/HSQC. Chemical shifts were recorded using an internal deuterium lock for ¹³C and residual ¹H in CD₃OD (δ H 3.31, δ C 49.0) and are given in parts per million on a scale relative to δ TMS = 0. NMR data were analyzed with MestReNova V.12.0 software (Table S3).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Alpiniamide A (1)

Figure 1.

Structure of alpiniamide A, the product of the *alp cis/trans*-AT PKS-NRPS biosynthetic gene cluster from *Streptomyces* spp.

Figure 2.

(a) Organization of the alpiniamide (*alp*) biosynthetic gene cluster (BGC) in *Streptomyces* sp. CBMAI 2042. The designation *alp* is used here following the previous report,³² but attention is needed to avoid confusion with the well-studied kinamycin (*alp*) cluster of *Streptomyces ambofaciens*.⁴⁹ (b) Candidate genes for alpiniamide A biosynthesis selected for TAR cloning, comprising the *cis/trans*-AT PKS-NRPS system. % identity: CBMAI 2042 genes aligned with IB2014/011-12 (Geneious v. 10.2.3 software; Biomatters Ltd.). (c) Organization of the *alp* gene cluster from *Streptomyces* sp. IB2014/011-12.³² The dotted line below represents the deduced boundaries of the *alp* gene cluster (*alpF* to *alpA3*). (d) Comparative production of alpiniamide by *alp* BGC. Base peak chromatogram (BPC, *m/z* 200–1800) corresponding to methanol extracts of (i) *Streptomyces coelicolor* M1146, (ii) M1146_RSalp, (iii) RSalp A_{domain}, and (iv) *Streptomyces* sp. CBMAI 2042 cultures. A common peak (rt 13.4 min) corresponding to alpiniamide A (1) is highlighted in the dashed rectangle.

Figure 3.

LC-HR-MS/MS analysis of extracts from cultures of M1146_RSalp strain, supplemented with stable isotope-labeled precursors. (a) Proposed fragmentation scheme for alpiniamide m/z 326.19 $[M-H_2O+H]^+$. Isotopic distribution and MS/MS spectrum after feeding with (b) *S*-[methyl- ^{13}C]methionine and MS/MS fragmentation evidencing ion fragments m/z 154, 155, and 156 and m/z 137, 138, and 139 in the post-NRPS assembly. The ion fragment m/z 109 is derived from the pre-NRPS subunit showing no labeled methyl group at this portion; (c) $[1-^{13}C]$ sodium propionate and MS/MS fragmentation into ion fragments m/z 154, 155, 156, and 157; m/z 137, 138, and 139; and m/z 109, 110, and 111 in the pre-NRPS assembly. Blue: pre-NRPS assembly. Red: post-NRPS assembly.

Proposed assembly line pathway for biosynthesis of alpiniamide A. Domains highlighted in blue correspond to pre-NRPS triketide unit assembly from *cis*-AT PKS AlpA1. The single extension module of AlpA1 is used iteratively to generate the AlpA1-linked triketide intermediate. Domains highlighted in red are involved in the post-NRPS ketide assembly, first by *trans*-AT PKS AlpA3 and then release of the aminoketide by AlpA2. Domains in green belong to the NRPS module. C-methyltransferases catalyzing methyl incorporation into acetate units are highlighted in orange. Alpiniamide A (1) then arises from the condensation of the two chains catalyzed either by the C domain at the N-terminus of AlpA3 or possibly by AlpE. Domains: decarboxylase KS-Ser (KS^S); ketosynthase (KS); acyltransferase (AT); acyl carrier protein (ACP); dehydratase (DH); ketoreductase (KR); C-methyltransferase (MT); *trans*-AT docking domain (ATd); condensation (C); adenylation (A); peptidyl carrier protein (PCP); thioesterase (TE). DH* denotes an inactive domain.

Figure 5. Overview of intermediates capture via chemical probes (malonyl- (2) and methylmalonyl-mimic (3)) during alpiniamide A assembly in *Streptomyces* M1146_RSalp.