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Engineering Fungal Nonribosomal Peptide Synthetase-like Enzymes by Heterologous Expression and Domain Swapping

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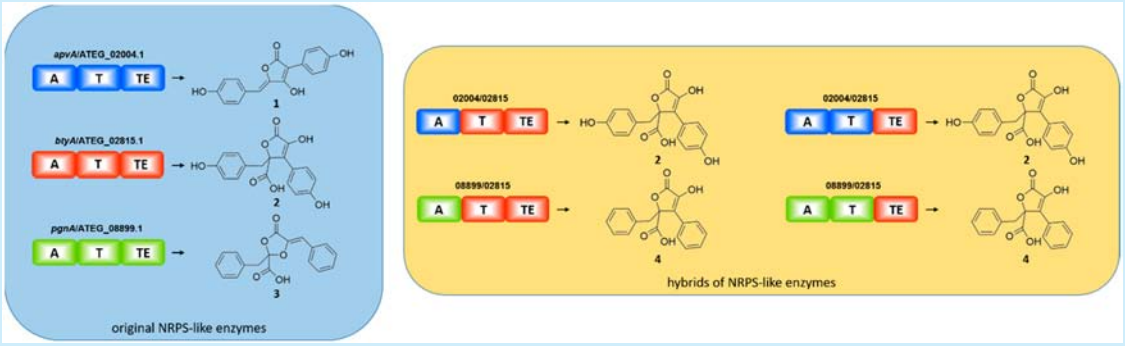
Supporting Information



ABSTRACT: A facile genetic methodology in the filamentous fungus *Aspergillus nidulans* allowed the exchange of various domains in nonribosomal peptide synthase (NRPS)-like enzymes from *Aspergillus terreus*. The newly generated engineered enzymes are capable of producing compounds with different chemical structures than its parent enzyme *in vivo*. This work provides insight in the programing of nonribosomal peptide biosynthesis in filamentous fungi.

Genome sequencing projects have demonstrated that filamentous fungi contain far more secondary metabolite gene clusters than compounds ever identified from these organisms. *Aspergillus terreus*, the industrial source of the cholesterol-lowering drug lovastatin, contains 28 polyketide synthase (PKS) genes, 22 nonribosomal synthetase (NRPS) genes, one hybrid PKS/NRPS gene, two PKS-like genes, and 15 NRPS-like genes. NRPS-like enzymes contain an adenylation domain (A), a thiolation domain (T), and a thioesterase (TE) or reductase domain (R), but do not contain the peptide bond forming condensation domain (C) found in canonical NRPS enzymes.[1−5] In *A. terreus* five single-module NRPS-like enzymes with the A−T−TE domain structure have been identified: AtqA, ApvA, BtyA, AtmelA, and PgnA. In NRPSs, the function of the A domain is to recognize and activate specific amino acid residues. In these NRPS-like enzymes, instead of an amino acid, an α-keto acid is recognized and activated. It is then transferred to the 4′-phospho-pantethine arm on the T domain. From there it is transferred to the TE domain. When a second residue is activated and transferred to the now vacated T domain, the two monomers dimerize to form a cyclic core and the resulting molecule is released from the enzyme.[1] As part of our larger effort to characterize fungal secondary metabolite genes in *A. terreus*, our group previously examined the five NRPS-like genes that contain the A−T−TE domain structure using knockout or overexpression *A. terreus* strains or heterologous expression *A. nidulans* strains.[6,7]

The biosynthesis of didemethylasterriquinone D by AtqA has been studied in detail through its homologue TdiA in *A. nidulans* in 2007.[1] In 2015, the spatial regulation of *apvA* and *atmelA* was elucidated, and it was shown that each gene can be heterologously expressed in *A. nidulans* to produce aspulvinone E (**1**) (Figure 1). More recently, the product of PgnA was determined by Tet-on overexpression. It was shown that phenguignardic acid (**3**) could also be produced via heterologous expression of *pgnA* in *A. nidulans*. Though BtyA was confirmed to play a role in butyrolactone biosynthesis

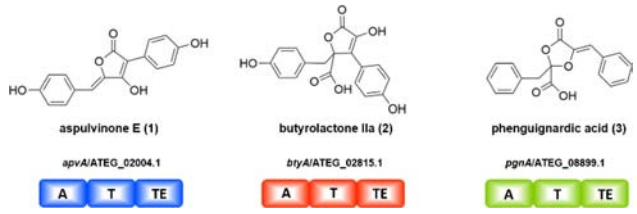


Figure 1. Three NRPS-like enzymes from *Aspergillus terreus* and their products.

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through knockout studies, the product of BtyA was not confirmed through heterologous expression. Therefore, we first developed a heterologous expression strain of *btyA* in the *yA* locus of *A. nidulans*, the same locus as *pgnA* was expressed in. *ApvA* was previously expressed in the *wA* locus, so to generate a comparable set, *apvA* was also expressed in the *yA* locus.[11] For this study we used a heterologous expression approach using the well-characterized and heavily engineered LO4389 strain of *A. nidulans* as a host system.[8] This strain has the 25 genes involved in production of the major secondary metabolite sterigmatocystin eliminated. One of the advantages of using this *A. nidulans* strain is that we can easily identify and isolate the products of the heterologously expressed genes from *A. terreus* strain NIH2624. Another advantage is the strength of the *alcA* promoter which should allow secondary metabolite detection even when engineered enzymes display lowered activity. The α-keto acid forms of phenylalanine and tyrosine are part of the shikimate pathway that is highly conserved in fungi, plants, and bacteria.[9] Therefore, it is assumed that they are readily available as starting material for the enzymes. The *A. nidulans* NRPS-like enzyme, MicA, is thought to use phenylpyruvic acid as well.[5] To create both constructs, the genes were amplified from *Aspergillus terreus* genomic DNA. Genes were fused via fusion PCR with an *alcA* promoter on the 5′ end, an *AfpyrG* marker on the 3′ end, and flanking fragments for heterologous recombination in the yellow locus (*yA*) of *Aspergillus nidulans* (Figure S1A). As a control we fused only the *AfpyrG* marker to the flanking fragments. Thus, the control strain can be cultured in the same media as the other mutant strains. The purified fusion PCR products were used in a previously described transformation protocol,[10] and the generation of gene construct insertions was confirmed by diagnostic PCR (Figure S2). Transformed strains were cultured in Glucose Minimal Media for 42 h and induced with 2-butanone for an additional 3 days, followed by ethyl acetate extraction. The extract of the *apvA* in *yA* strain was analyzed by Liquid Chromatography−Mass Spectrometry (LC-MS), and the data were identical to those reported for *apvA* in *wA*.[11] For confirmation of the product of the *btyA* in *yA* heterologous expression strain, we scaled up to a 1 L culture. The main product was purified using column chromatography followed by preparative High Performance Liquid Chromatography (HPLC), and its identity was confirmed by Nuclear Magnetic Resonance (NMR) spectroscopy to be butyrolactone IIa (**2**) (Figure S6). In Figure 2, HPLC traces of extracts of the two new strains (*apvA*, panel A and *btyA*, panel B) and the previously generated strain (*pgnA*, panel C) show three clearly different products from enzymes with a similar domain structure. It was previously proposed that ApvA and BtyA both utilize two 4-hydroxyphenylpyruvic acid (HPPA) building blocks to produce **1** and **2**, respectively.[6] Along the same lines, the biosynthesis of **3** was proposed to be through dimerization of two phenylpyruvic acid (PPA) building blocks instead of HPPA.[7] In Figure 1 it can be seen that, despite the similarity in building blocks used and in the domain structure of the enzymes, the products have different cyclization patterns. In this study we were interested in identifying which of the three domains in NRPS-like enzymes is responsible for the different cyclization. Second, we were interested in testing whether we could rationally swap domains in NRPS-like enzymes to change the structure of the final products as has been done before with a nonreducing polyketide synthetase.[12] The modular architecture of the NRPS-like enzymes should in theory allow for the recombination of modules to generate

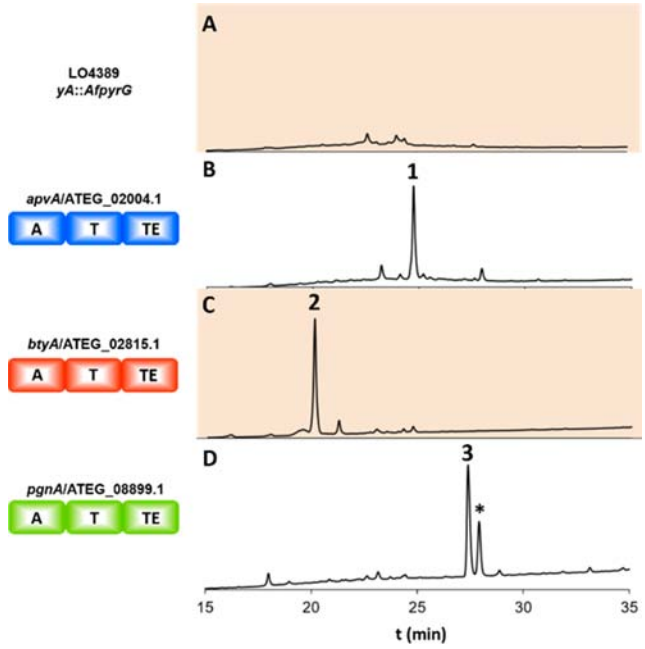

Figure 2. HPLC traces of ethyl acetate extracts of the strains with different heterologous constructs expressed at total spectrum scan by DAD detector. (A) Control strain with the *AfpyrG* marker shows only minor products, most likely siderophores produced by *A. nidulans*. (B) Strain with *apvA* under the *alcA* promoter shows aspulvinone E (**1**) production. (C) Strain with *btyA* under the *alcA* promoter shows butyrolactone IIa (**2**) production. (D) Strain with *pgnA* under the *alcA* promoter shows phenguignardic acid (**3**) production. * denotes a side product that was previously undetected. The production of * varies per culture iteration and condition, and it has no identifiable *m*/*z* peaks in either positive or negative mode in the range 100−1500. UV absorption of * can be found in Figure S5.

novel, artificial nanomachines that produce complex molecules. First, we asked whether protein domains of these three enzymes could be swapped while retaining activity. Since it is known that the function of the A domain in fungal NRPS and NRPS-like enzymes is to activate specific amino acids and α-keto acids, we started the engineering effort by substituting the A domain. T domains have been exchanged before to generate functional mutants in the single-module NRPS IndC, but without changing the product that was formed.[13]

We created two different hybrid constructs with the A domains replaced. In one construct we replaced the A domain of BtyA with the A domain of ApvA since both enzymes utilize the same HPPA building blocks and differ only in their cyclization pattern. We expected that the novel hybrid enzyme with the A domain from ApvA and the T and TE domains from BtyA would still produce butyrolactone IIa (**2**). We expected the second hybrid enzyme, with the A domain from PgnA and the T and TE domains from BtyA, to produce a novel compound: butyrolactone IIa constructed with PPA instead of HPPA, phenylbutyrolactone IIa (**4**). To create the hybrid enzymes, we first analyzed the NRPS-like genes and determined the domain borders using the Conserved Domain Database (or Pfam). Exact positions of the substitutions can be found in the alignment data (Figure S3). Hybrids were generated using genomic DNA of the heterologous expression strains. The A domains were amplified together with the *alcA* promoter and the 5′ *yA* flanking region. The T and TE domains were amplified together with the *AfpyrG* and the 3′ *yA* flanking

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region (Figure S1B). The two fragments were fused to generate hybrid NRPS-like genes, and the fusion constructs were used for transformation. Diagnostic PCR confirmed insertion of the fragment at the correct locus, and sequencing confirmed the correct substitution location in the hybrid (Figures S2, S4). The first hybrid enzyme ApvA with the BtyA T and TE produced butyrolactone IIa, as verified by LC/MS. This showed that our hypothesis was indeed correct that A domains can be substituted without completely losing activity (Figure 3A).

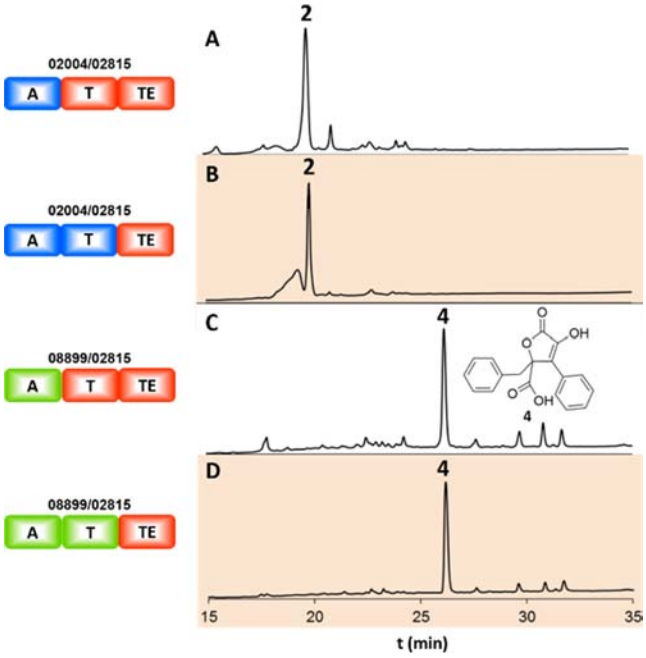

Figure 3. HPLC traces of ethyl acetate extracts of the strains with different heterologous constructs expressed at total spectrum scan by DAD detector. (A, B) Both ApvA/BtyA hybrids produce butyrolactone IIa (**2**). (C, D) Both PgnA/BtyA hybrids produce phenylbutyrolactone IIa (**4**).

The second hybrid, PgnA with the BtyA T and TE, produced a compound that was neither phenguignardic acid nor butyrolactone IIa, based on LC/MS analysis (Figures 3C, S5). To determine the chemical structure, we scaled up the culture of the mutant strain with the hybrid gene to 1 L. However, the compound was not sufficiently stable during the purification process, with significant degradation even in neutral buffers, despite being a single large peak in the LC/MS analysis. The product seemed to degrade into at least four different compounds, so a specific mechanism for that process is not straightforward. Possibly keto−enol tautomerism in the five-membered ring plays a role in the instability of the molecule. We therefore obtained High Resolution MS and NMR data from the crude extract (~90 mg) of the 1 L culture and showed that the hybrid enzyme produced the expected compound with phenyl side chains and a heterocyclic core like butyrolactones (**4**) (Table S4, Figures S7, S8). This result showed that newly engineered combinations of enzymatic domains from NRPS-like enzymes can produce novel secondary metabolites in sufficient quantities for characterization.

Having established the exchangeability of A domains in NRPS-like enzymes, we next wanted to extend the swapping strategy to replace thioesterase (TE) domains in NRPS-like enzymes to elucidate their functions. The TE domain in NRPS-like enzymes is thought to play a role in the cyclization process, but whether or not it can perform its function as independently as the A domain was not clear. To answer this question, we engineered two different hybrid enzymes. In one construct we combined the A and T domains of ApvA with the TE domain of BtyA. In the second construct we combined the A and T domains of PngA with the TE domain of BtyA (Figure 3B and D). Domain borders were again determined using the Conserved Domain Database (or Pfam), and the substitution position was arbitrarily chosen within the domain linkers (Figure S3). Hybrids were generated in the same method as described above for the A domain substitutions. Both hybrid strains were cultured, and the extracts were analyzed using LC/

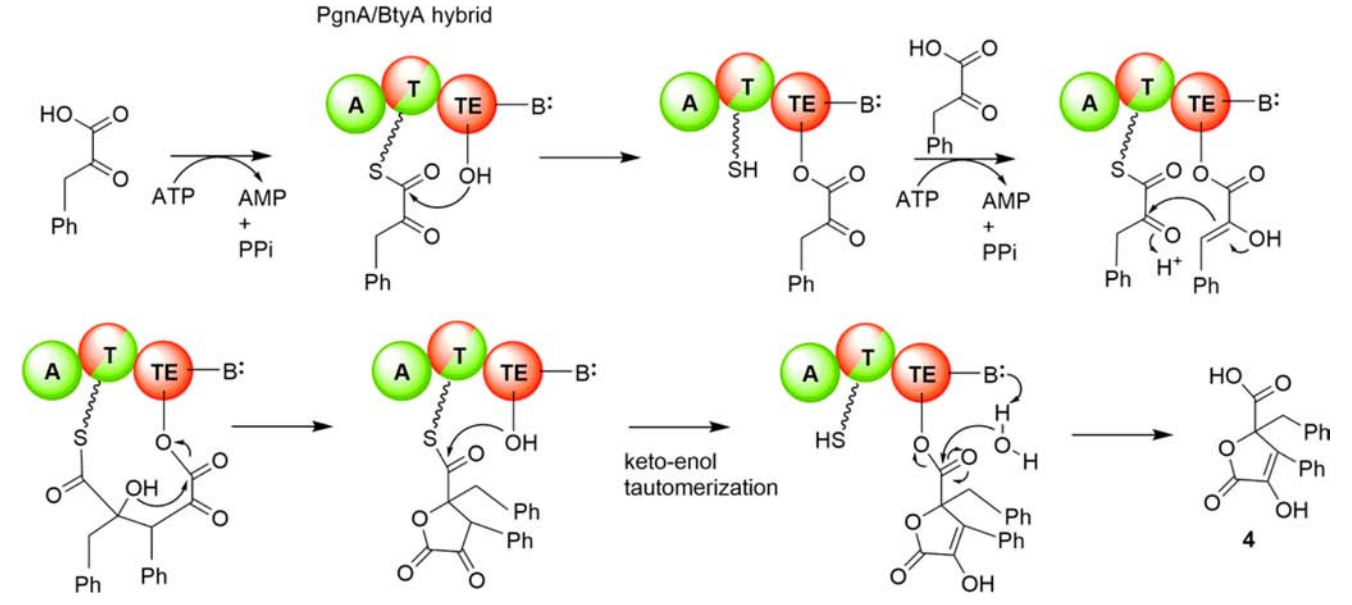

Figure 4. Proposed biosynthesis pathway for phenylbutyrolactone IIa **4**. The cyclization mechanism is the same as that proposed for butyrolactone II but the changed A domain recognizes a different pyruvic acid moiety to generate compound **4**. The mechanism works for T domains from either parent enzyme.

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MS. The first ApvA/BtyA hybrid construct produced compound **2** (Figure 3B). ApvA normally produces aspulvinone E (**1**), but when the original TE domain of ApyA is replaced by the TE domain of BtyA, butyrolactone IIa was produced instead. This indicated that the TE domain is the main determining factor for cyclization. The second PngA/BtyA hybrid produced again compound **4** confirming this hypothesis (Figure 3D).

The results presented in this study show that it is possible to engineer new functional NRPS-like enzymes by recombining domains of natural ones. This effort led to the production of a new natural product phenylbutyrolactone IIa (**4**) (Figure 4). The fact that A domains can be exchanged means they can function independently of their T and TE domains. Engineering A domain substitutions in NRPSs has been proven successful in the past to generate novel NRPs.[14−16] Classic NRPSs catalyze peptide bond formation between two monomers from distinct A domains, while these NRPS-like enzymes only have a single A domain and catalyze a different reaction, so up until now it was not clear whether the same strategy would work here. The function of the TE domain seems similarly independent as the A domain, since both TE swapped hybrids function in the same fashion as their A domain swapped hybrid counterparts. Previous studies concluded that T and TE domains are dependent on each other and need to be preserved in recombinant NRPSs. Whether NRPS-like enzymes form an exception to this rule will become clear as more hybrids are generated. The future directions for this project are twofold. One direction is to expand further domain combinations to gain access to more novel structures. For example, the A domain of ApvA combined with the T and TE domain of PgnA should produce 4-hydroxyphenguignardic acid. The other is to take the functional PgnA/BtyA hybrid enzyme and coexpress additional enzymes from NRPS-like gene pathways, such as SAM-dependent methyltransferases or prenyltransferases to further diversify the structures that can be produced using this approach. This may also stabilize the produced compound, making purification possible. Using the *Aspergillus nidulans* heterologous expression system, we are pursuing both avenues, and the results will be reported in due course.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications Web site. Supporting_information_vandijk-wang.pdf The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b02821.

A. nidulans strains and primers used in this study, diagnostic PCR results, sequencing data, compound characterization with spectral data, high resolution mass spectroscopy, and 2-D NMR (PDF)

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Notes

The authors declare no competing financial interest.

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