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Analysis of Intact and Dissected Fungal Polyketide Synthase-Nonribosomal Peptide Synthetase in Vitro and in *Saccharomyces cerevisiae*

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Abstract: The widely found fungal iterative PKS-NRPS hybrid megasynthetases are highly programmed biosynthetic machines involved in the synthesis of 3-acyltetramic acids and related natural products. In vitro analysis of iterative PKS-NRPS has been hampered by the difficulties associated with obtaining pure and functional forms of these large enzymes (>400 kDa). We successfully expressed *Aspergillus nidulans* aspyridone synthetase (ApdA) from an engineered *Saccharomyces cerevisiae* strain. The complete functions of ApdA and its enoylreductase partner ApdC are reconstituted in vitro and in *S. cerevisiae* with the production of preaspyridone **7**. The programming rules of both the PKS and NRPS modules were then examined in vitro. The key interaction between the PKS and the NRPS was dissected and reconstituted *in trans* by using stand-alone modules. Analogs of **7** were synthesized through heterologous combinations of PKS and NRPS modules from different sources. Our results represent one of the largest, multidomain enzyme reconstituted to date and offer new opportunities for engineered biosynthesis of fungal natural products.

Filamentous fungi produce a diverse array of bioactive secondary metabolites. Among the small molecule natural products, polyketides and nonribosomal peptides are synthesized by multidomain enzymes such as polyketide synthases (PKSs) and nonribosomal peptide synthetases (NRPSs), respectively. The enzymology and biochemical properties of these megaenzymes are highly complex and are different from the well-studied bacterial PKSs and NRPSs.[1] In particular, the highly reducing PKSs (HR-PKSs), in which individual domains are programmed to function in different permutations during the iterative process of chain elongation, are particularly enigmatic as exemplified by the lovastatin nonaketide synthase LovB.[2] Notwithstanding the complexity of HR-PKSs, an even more impressive biosynthetic machinery that is found in nearly all filamentous fungi is the PKS−NRPS hybrid, in which a single module of NRPS is translationally fused to the *C*-terminus of an HR-PKS. A typical PKS−NRPS hybrid contains ∼10 catalytic domains; exceeds 400 kDa as a stand-alone protein; and synthesizes acyltetramic acid (3-acyl-pyrrolidine-2,4-dione) as the product. The tetramic acids are further tailored into complex natural products, such as fusarin C,[3] equisetin,[4] aspyridone A **1**,[5] pseurotin A,[6] cyclopiazonic acid **2**,[7] chaetoglobosin,[8] tenellin **3**,[9] etc. (Figure 1A). The widespread presence of fungal PKS-NRPS machineries,[10] along with their unique biosynthetic capabilities and highly evolved programming rules, makes them attractive targets for biochemical investigations in vitro and in genetically tractable hosts.

ApdA is the PKS−NRPS that is involved in the synthesis of **1** (Figure 1A). The role of ApdA was discovered via artificial transcription activation of the cryptic *apd* gene cluster in *Aspergillus nidulans*.[5] The HR-PKS module of the enzyme, which consists of ketosynthase (KS), malonyl-CoA:ACP transacylase (MAT), dehydratase (DH), methyltransferase (MT), ketoreductase (KR), and acyl carrier protein (ACP) domains, is proposed to synthesize the ACP-bound 4,6-dimethyl-3-oxooctanyl thioester **5**. A dissociated enoylreductase (ER) ApdC is proposed to function *in trans* with ApdA, analogous to the role played by LovC during LovB-catalyzed synthesis of dihydromonacolin L.[11] The downstream NRPS module contains the condensation (C), adenylation (A), and thiolation (T) domains and catalyzes the formation of the L-tyrosinyl-thioester **4** and the amide linkage between **4** and **5** to yield **6** tethered to the T domain. The bimodular assembly line is terminated with a putative reductase (R) domain that facilitates formation and release of the tetramic acid product. Combined with ApdC, ApdA is proposed to synthesize a precursor of **1** via ∼20 enzymatic steps. Although the PKS-NRPSs responsible for tenellin (TenS) and cyclopiazonic acid (CpaS) have both been reconstituted in the heterologous host

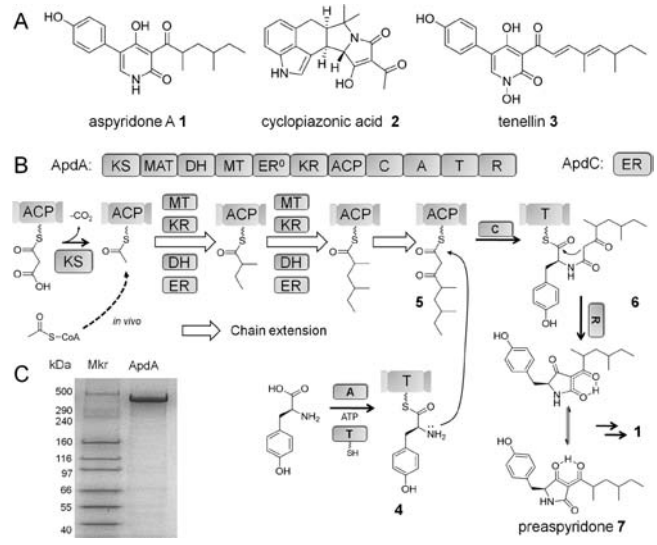

Figure 1. Tetramic acid synthesis by fungal PKS-NRPS. (A) Selected tetramic acid-derived natural products. (B) Proposed mechanism of preaspyridone **7** synthesis by ApdA (PKS-NRPS) and ApdC (ER). The phosphopantetheinyl arms of the ACP and T domains are shown as squiggle lines. Priming of the ACP by the acetyl group may be from the decarboxylation of malonyl-ACP by the KS domain (observed in vitro) or via the direct acetylation of KS by acetyl-CoA followed by transfer to the ACP domain (in vivo). The white arrow represents one round of chain elongation. **7** can be converted to the final natural product **1** after additional enzymatic oxidation and ring rearrangement.[5] (C) SDS-PAGE of purified ApdA (439 kDa, tags included). The enzyme is purified from *S. cerevisiae* BJ5464-NpgA by an anti-FLAG antibody affinity resin.

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Aspergillus oryzae,[12] in vitro reconstitution of fungal PKS−NRPS activities has not been accomplished. Reconstitution of purified biosynthetic enzymes in vitro enables the most direct analysis of the functions and products of the megasynthases.[13] Here we show that, using the *Saccharomyces cerevisiae* strain BJ5464-NpgA as the expression host,[2,14] the complete activities of the ApdA and ApdC can be reconstituted, which led to the identification of an acyltetramic acid **7** as the product.

The uninterrupted *apdA* and *apdC* genes were cloned from the sequenced *A. nidulans* strain FGSC A4 after reannotation of introns (Figures S1−S2). A gene encoding *N*-terminal FLAG, *C*-terminal hexahistidine tagged ApdA was placed under the control of the ADH2 promoter[15] in the 2-μm episomal vector pXW58. Full length ApdA (439 kDa) was then solubly expressed from BJ5464-NpgA transformed with pXW58 and purified to near homogeneity with an anti-FLAG antibody affinity resin to a final of yield of ∼1.2 mg/L (Figure 1C). ApdC was solubly expressed from *Escherichia coli* BL21(DE3) when coexpressed with chaperone proteins GroEL and GroES (Figure S3A). To test the activities of the two enzymes and identify possible tetramic acid products, equimolar amounts (25 μM) of purified ApdA and ApdC were incubated at room temperature for 12 h with all the cofactors and building blocks (2 mM NADPH, 1 mM SAM, 1 mM L-Tyr, 25 mM ATP, 10 mM $MgCl_2$, and 2 mM malonyl-CoA). LC-MS analysis of the organic extract showed the production of a predominant compound **7** with a UV absorption maximum (λ_{max}) at 279 nm with *m*/*z* $[M + H]^+ = 332$ (see Figure 2A, trace i; Figure S4). Exclusion of any of the cofactors abolished the synthesis of **7**. To assess the length of the polyketide portion of **7**, the in vitro assay was performed in the presence of [2-^{13}C] malonate (100 mM) and MatB (25 μM), which can generate [2-^{13}C] malonyl-CoA in the presence of ATP, Mg^{2+}, and CoA.[16] The assay yielded the same product profile and the mass of **7** was increased to *m*/*z* $[M + H]^+ = 336$ (Figure S4), suggesting the presence of a tetraketide chain which is consistent with the proposed function of AdpA as shown in Figure 1B.

To obtain sufficient amounts of **7** for structural elucidation, BJ5464-NpgA was cotransformed with expression plasmids (pXW58 and pXW51) for both ApdA and ApdC and cultured as previously described.[17,18] The RT, UV and mass spectra of **7** biosynthesized from yeast were identical to those of the in vitro sample (Figure 2B, trace ii). After 4 days, the culture was extracted and **7** was purified (final titer ∼4 mg/L) and fully characterized by ^{1}H, ^{13}C, and correlation NMR spectroscopy (Table S3; Figure S5−S8). Both proton and carbon NMR spectra in $CDCl_3$ showed two sets of signals with a relative ratio of 3:1 and these two sets collapsed into one single set when the NMR solvent was switched to CD_3OD (Figure S9), which is characteristic for 3-acyltetramic acids. The two sets of signals in $CDCl_3$ arise from the external tautomers, which slowly interconvert through the rotation around the acyltetramic acid linkage (Figure S10).[19] The collapse can be explained by the inclination of external tautomeric equilibrium to one side as a result of solvent effect.[20] The proton spectrum showed characteristics of a typical AA′BB′ spin system, which strongly indicated the presence of a *para*-substituted phenyl ring. COSY coupling between high field protons suggests a spin system of a branched aliphatic chain. Combining all the NMR information and in comparison to that of pretenellin-A[21] and **1**, **7** was determined as the 3-acyltetramic acid shown in Figure 1B and is named preaspyridone.

Synthesis of **7** using purified ApdA and ApdC confirms the complete reconstitution of the activities of the recombinant PKS-NRPS megasynthetase. Under in vitro conditions, the assembly of **7** can be initiated by loading of malonyl-CoA onto ApdA, followed by decarboxylation to yield the acetyl starter unit (Figure 1B). Under in vivo conditions, priming may also be initiated directly with an acetyl-CoA unit. The growing polyketide chain then elongates into the tetraketide **5** as shown in Figure 1B through differential tailoring of the α and β positions. None of the tailoring domains were used in the last PKS elongation step, which affords the β-keto thioester **5** required for formation of the acyltetramic acid. The acyl chain of **5** is then amidated with the α-NH_2 of **4** to afford **6**. No other tetramic acid or amidated polyketide product was observed in the in vitro reaction in detectable amounts, indicating the programming rules of AdpA are precise and only the correctly tailored polyketide acyl intermediates are transferred from the PKS to the NRPS. The in vitro synthesis of **7** in the absence of additional oxidative enzymes also confirms the release of **6** via an R-domain catalyzed Dieckmann cyclization instead of the originally proposed NADPH-dependent reductive release followed by cyclization and enzymatic oxidation.[5] The Dieckmann cyclase property of the ApdA R domain is therefore analogous to those of dissected R domains from the EqiS and CpaS fungal PKS-NRPSs.[22]

To probe the flexibility of the C and A domains toward different aromatic amino acids, we performed the in vitro reactions with various *para*-substituted phenylalanines and the synthesis of analogs

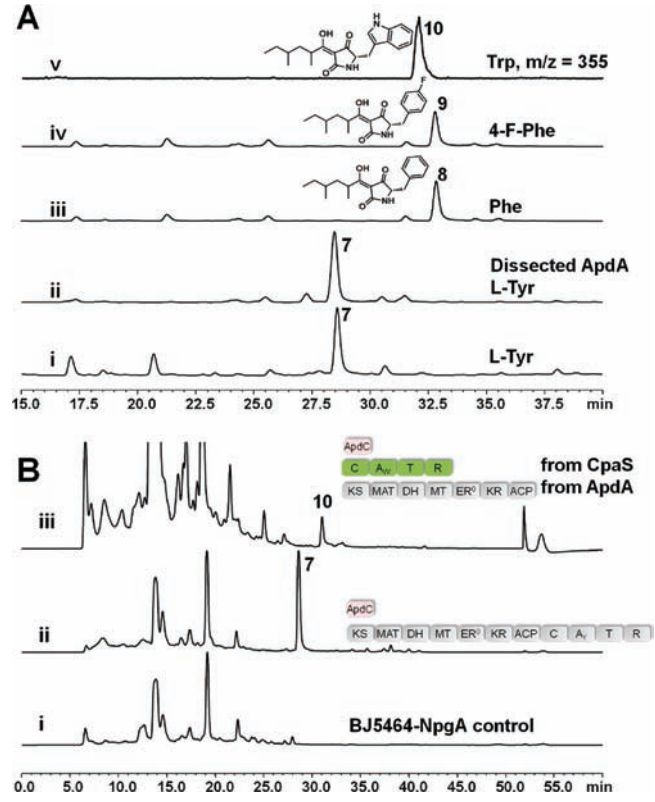
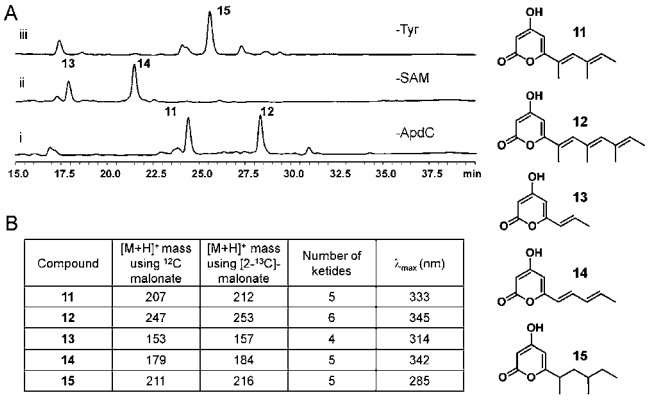


Figure 2. Reconstitution of ApdA in vitro and in *S. cerevisiae*. (A) In vitro reconstitution of ApdA using purified enzymes. In each trace, the supplied L-amino acid (1 mM) is shown. All experiments were performed with intact ApdA except trace ii in which dissected PKS and NRPS modules of ApdA were reassembled. Traces i−iv are HPLC traces monitored at UV $\lambda = 280$ nm; trace v is a selected ion monitoring trace $[M + H]^+ = 355$ of **10**, which is produced at lower titers. More detailed traces can be found in Figures S9, S11−S14. (B) HPLC traces (280 nm) that show the in vivo reconstitution of ApdA in BJ5464-NpgA. The cultures were extracted with ethyl acetate on day 4. Trace i: untransformed BJ5464 control; trace ii: BJ5464 transformed with ApdA. This culture was used to purify **7** for structural determination; trace iii: BJ5464 transformed with expression plasmids that separately encode the ApdA PKS and the CpaS NRPS modules. The synthesis of the expected analog **10** was observed in the extract (trace iii is scaled differently to clearly show **10**).

of **7** was monitored by LC-MS (Figure 2A, trace iii, iv and v; Figures S11−S13). ApdA was able to synthesize the corresponding analogs **8** and **9** in the presence of L-phenylalanine and L-4-fluorophenylalanine, respectively, at ∼50% efficiency compared to **7**. Increasing the sizes of the *para* substituent (−NH_2, −Cl, −Br) led to decreases in product turnover (Figures S14−S17). Surprisingly, adding L-tryptophan resulted in the synthesis of the indole-containing analog **10** (Figure 2A, trace v) as revealed by selected ion monitoring, albeit at significantly lower levels. Therefore, these results demonstrate that while the NRPS module clearly prefers L-tyrosine in the in vitro assay, both the activation (A) and the condensing (C) domains have some flexibility in utilizing other aromatic amino acids and lead to formation of analogs of **7**.

The in vitro assay developed for ApdA similarly allows probing of the programming rules of the HR-PKS portion of the megasynthetase. Toward this end, we first examined whether removal of SAM in the in vitro reaction can lead to unmethylated versions of **7**. While no tetramic acid was observed, two unsaturated α-pyrones (**13** and **14**) were produced (Figure 3A, trace ii; Figure S18). **13** was previously recovered as a derailment product of CalE8[23] and **14** was isolated from an *in vitro* LovB assay in the absence of SAM, in which the ER LovC failed to function without *C*-methylation of a tetraketide intermediate.[2] Therefore, the synthesis of **13** and **14** here indicates *C*-methylation is also a prerequisite modification for enoylreduction by the ER partner ApdC. The incorrectly tailored polyketide intermediates were then offloaded by ApdA as **13** and **14**, analogous to that observed for LovB upon derailment in the tailoring steps. These convergent observations between ApdA/ApdC and LovB/LovC thus point to a conserved programming rule, in which a subtle α-methylation can modulate the interaction between HR-PKSs and their dissociated ER partners.

Similarly, removal of ApdC from the assay produced two α-pyrones **11** and **12** (Figure 3A, trace i, Figure S19). Repeating the assay with 2-^{13}C malonate resulted in increase of 5 and 6 mu for **11** and **12**, respectively (Figure 3B). We also observed an increase in the λ_{max} of the α-pyrones, which is indicative of increased levels of conjugation. These observations, combined with the inference from biosynthetic logic and comparison to the LovB system,[2,11,24] led us to propose the structures of **11** and **12** as shown in Figure 3, of which the two compounds contain two and three α-methyl modifications, respectively. The failure of ApdA to synthesize tetramic acids containing unsaturated polyketide portions is evidence of the substrate specificity of the NRPS module toward incorrectly tailored polyketide acyl substrates. These stall products were instead offloaded as pyrones to afford **11** and **12**. Intriguingly, this result is different from that of TenS reconstituted in *A. oryzae* in which the conjugated prototenellins were synthesized in the absence of the ER TenC.[21] This is most likely due to the more relaxed substrate tolerance of the downstream C domain of TenS.



B

Compound	[M+H]$^+$ mass using ^{12}C malonate	[M+H]$^+$ mass using [2-^{13}C]-malonate	Number of ketides	λ_{max} (nm)
11	207	212	5	333
12	247	253	6	345
13	153	157	4	314
14	179	184	5	342
15	211	216	5	285

Figure 3. In vitro assay probing of the programming rules of the PKS-NRPS system. The putative structures of these polyketides are listed on the right. (A) HPLC UV traces that show the production of different polyketides through removal of the indicated components from the assay that synthesizes **7**. Traces i and ii are monitored at $\lambda = 330$ nm; trace iii is monitored at $\lambda = 280$ nm. The relative retention times of compounds are in agreement with their relative polarities based on the proposed structures. (B) The masses of the mentioned polyketides. The increment in the mass when fed with [2-^{13}C]-malonate indicates the number of ketides for the corresponding compound. Conjugation onto the pyrone ring will cause the λ_{max} to shift to a longer wavelength.

The formation of **11**, **12**, and **14** from the above assays points to an unexpected feature of ApdA, which is its ability to synthesize polyketide products longer than the tetraketide observed in **1** and **7**. To probe the chain length specificity of the HR-PKS, we sought to assay the PKS portion independent of the activities of the NRPS module. To do so, we (i) excluded L-tyrosine from the assay that produced **7** and (ii) cloned and expressed the PKS portion as a stand-alone enzyme (Figure S3B) from BJ5464-NpgA (5 mg/L) and assayed with ApdC in the presence of malonyl-CoA, SAM, and NADPH. In both assays, the same α-pyrone **15** was recovered from the reaction mixtures (Figure 3A, trace iii; Figure S20). The size of **15** was confirmed to be a pentaketide, and λ_{max} of 285 nm suggests that the linear portion of the molecule is not conjugated and is instead fully reduced, which is expected if all the tailoring domains functioned properly. Therefore the HRPKS module can indeed function correctly without the downstream NRPS module and synthesize a pentaketide. However, during the synthesis of **7**, in order to prevent continual elongation of the polyketide beyond the β-keto tetraketide and eventual offloading as **15**, the C domain of the NRPS must interact with the PKS accurately to capture **5** and condense with **4**.

To examine if this critical interaction between the HRPKS and NRPS can be established *in trans*, we expressed and purified the NRPS module as a *C*-terminus hexahistidine tagged stand-alone enzyme from *E. coli* BAP1[25] (5 mg/L) (Figure S3C). Incubation of the dissected ApdA PKS and NRPS modules in equimolar amounts (25 μM), along with ApdC, cofactors, and building blocks, produced **7** in a comparable yield as the intact ApdA (Figure 2A, trace ii). This is the first demonstration of physically separating a fungal PKS-NRPS megasynthetase into the two independent, functional modules. Encouraged by the *in trans* complementation findings, we explored whether heterologous PKS and NRPS modules can be functionally matched, which was previously engineered for some bacterial PKS−NRPS hybrids.[26] We cloned and expressed the NRPS module of *Aspergillus flavus* CpaS (Figure S3D), which incorporates L-tryptophan into the tetramic acid intermediate of **2**.[12b] When BJ5464-NpgA was cotransformed with expression plasmids that expressed ApdA PKS, CpaS NRPS, and ApdC, small amounts of **10** (∼0.1 mg/L) were observed from the extracts of the yeast culture (Figure 2B, trace iii; Figure S21), indicating successful communication between the two modules. The lower titer of **10** may be attributed to the substrate specificity of the CpaS C domain donor site[27] toward both the noncognate ApdA ACP and the bulkier **5** compared to the natural acetoacetyl thioester acyl donor. The lower turnover of **10** can also arise from the inability of the CpaS R domain to correctly process an unnatural substrate. Nevertheless, the result from the straightforward mix and match experiment shows the potential for using this dissociated platform to biosynthesize new tetramic acid compounds.

In conclusion, we reconstituted the complete activities of the PKS−NRPS ApdA through the synthesis of **7**. To the best of our knowledge, this represents the largest, single biosynthetic megasynthase reconstituted in vitro. Using both in vitro assays and the yeast

host, we were able to dissect the programming rules of ApdA, including the protein−protein interactions and the key acyl transfer step between the PKS and NRPS modules.

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Supporting Information Available: Experimental details and spectroscopic information. This material is available free of charge via the Internet at http://pubs.acs.org.

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