

# Cyclopiazonic Acid Biosynthesis in *Aspergillus* sp.: Characterization of a Reductase-like R\* Domain in Cyclopiazonate Synthetase That Forms and Releases *cyclo*-Acetoacetyl-L-tryptophan<sup>†</sup>

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**ABSTRACT:** The fungal neurotoxin  $\alpha$ -cyclopiazonic acid (CPA), a nanomolar inhibitor of  $\text{Ca}^{2+}$ -ATPase, has a pentacyclic indole tetramic acid scaffold that arises from one molecule of tryptophan, acetyl-CoA, malonyl-CoA, and dimethylallyl pyrophosphate by consecutive action of three enzymes, CpaS, CpaD, and CpaO. CpaS is a hybrid, two module polyketide synthase–nonribosomal peptide synthetase (PKS–NRPS) that makes and releases *cyclo*-acetoacetyl-L-tryptophan (*c*AATrp), the tetramic acid that serves as substrate for subsequent prenylation and oxidative cyclization to the five ring CPA scaffold. The NRPS module in CpaS has a predicted four-domain organization of condensation, adenylation, thiolation, and reductase\* (C–A–T–R\*), where R\* lacks the critical Ser–Tyr–Lys catalytic triad of the short chain dehydrogenase/reductase (SDR) superfamily. By heterologous overproduction in *Escherichia coli* of the 56 kDa *Aspergillus flavus* CpaS TR\* didomain and the single T and R\* domains, we demonstrate that CpaS catalyzes a Dieckmann-type cyclization on the *N*-acetoacetyl-Trp intermediate bound in thioester linkage to the phosphopantetheinyl arm of the T domain to form and release *c*AATrp. This occurs without any participation of NAD(P)H, so R\* does not function as a canonical SDR family member. Use of the T and R\* domains in *in trans* assays enabled multiple turnovers and evaluation of specific mutants. Mutation of the D3803 residue in the R\* domain, conserved in other fungal tetramate synthetases, abolished activity both in *in trans* and *in cis* (TR\*) activity assays. It is likely that cyclization of  $\beta$ -ketoacylaminoacyl-*S*-pantetheinyl intermediates to released tetramates represents a default cyclization/release route for redox-incompetent R\* domains embedded in NRPS assembly lines.

The heterocyclic pyrrolidine-2,4-dione ring systems commonly known as tetramic acids are found in many natural product scaffolds originating from a variety of terrestrial and marine species such as actinobacteria, fungi, sponges, and cyanobacteria (for selected examples, see Figure 1A) (1). Due to both the conformational constraint of the five-membered ring and the dicarbonyl functionality, tetramic acid moieties are specific pharmacophore elements for recognition by a range of biological targets. Such natural products thus exhibit a wide range of activities, including antibacterial, antiviral, and antitumor. The great majority of tetramic acids are 3-acyl substituted (**1a**) with the enol isomer (**1b**) as the predominating tautomer (Figure 1B), providing an additional group decorating the tetramic acid scaffold and interacting with cellular targets. Inspection of 3-acyl tetramate natural products suggests a polyketide origin for the 3-acyl chain, with a single amino acid moiety providing the nitrogen atom and the C5 side chain (Figure 1B). Several

biosynthetic gene clusters have been recently identified and sequenced that validate the polyketide synthase (PKS)<sup>1</sup>–nonribosomal peptide synthetase (NRPS) hybrid assembly prediction (2–9). In general, these assembly lines contain a series of polyketide synthase modules sufficient to build the full-length 3-ketoacyl chain and then a single nonribosomal peptide synthetase module as the chain-terminating catalyst. Therefore, the 3-acyl-2,4-pyrrolidinedione (tetramate) rings are thought to be formed in the release step of the PK–NRP hybrid chain from its enzymatic assembly line (Figure 1B).

This mode of NRPS-mediated release of thioester-linked chains, constituting pyrrolidine-2,4-dione formation, would join a variety of other known NRPS release mechanisms, including hydrolysis, macrolactonization, macrolactamization, and reduction (10–12). A typical chain termination NRPS module, both in pure NRPS assembly lines and in hybrid PKS–NRPS assembly lines, has a minimal domain composition of either C–A–T–TE, for hydrolysis or macrocyclization fates, or C–A–T–R, for reductive outcomes (C = condensation, A = adenylation, and T = thiolation). The chain-terminating TE and R domains mediate the different product outcomes. The TE (thioesterase) domains are 35 kDa  $\alpha\beta$ -hydrolase protein folds, while the R (reductase) domains belong to the short-chain dehydrogenase/reductase (SDR) superfamily with Rossmann-fold nucleotide binding motifs and use NAD(P)H to effect reductive release of the acyl chain as an aldehyde.

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Abbreviations: CPA, cyclopiazonic acid; *c*AATrp, *cyclo*-acetoacetyl-L-tryptophan; PKS, polyketide synthase; NRPS, nonribosomal peptide synthetase; SDR, short-chain dehydrogenase/reductase; C, condensation; KS, ketosynthase; AT, acyltransferase; A, adenylation; T, thiolation; R, reductase; TE, thioesterase; SNAC, *S*-(*N*-acetyl)cystamine; acac, acetoacetyl.

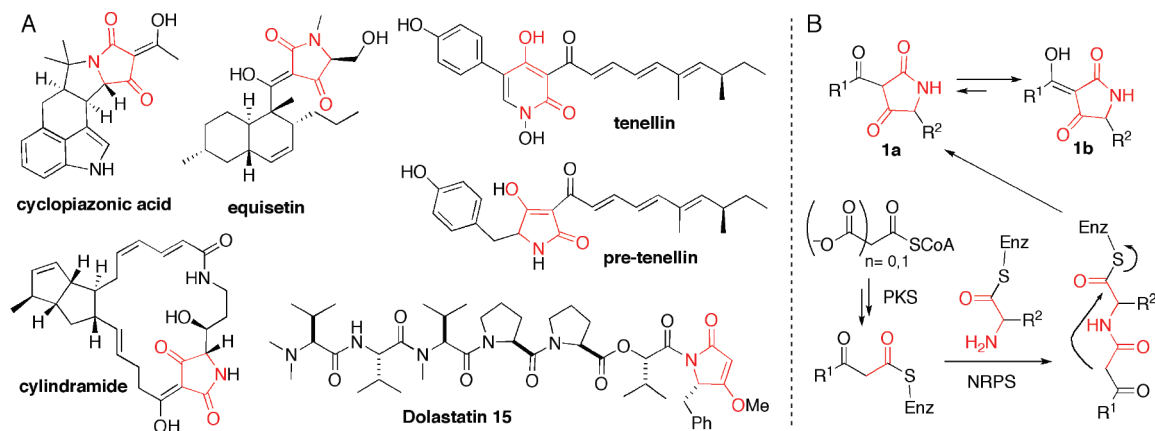
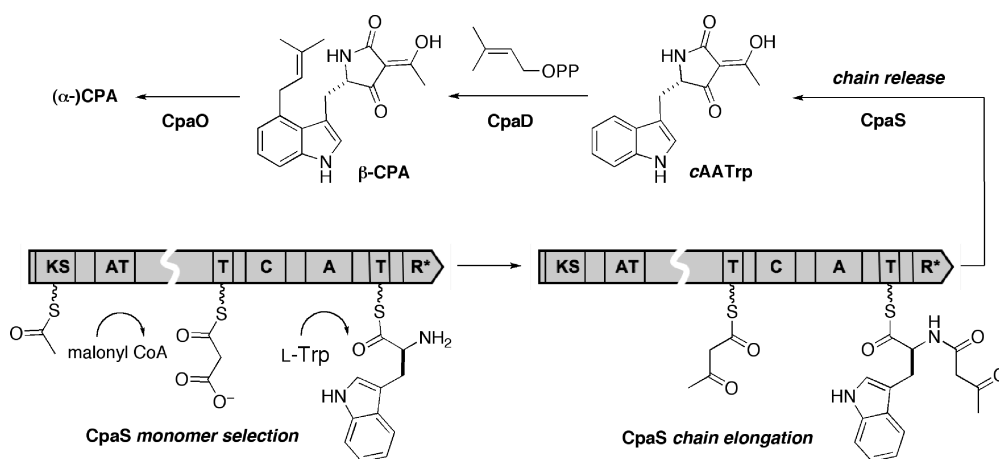


FIGURE 1: (A) Selected tetramic acid natural products. (B) Proposed biosynthetic route to 3-acyltetramic acid.

Scheme 1: CPA Biosynthetic Enzymes (CpaS, CpaD, and CpaO) and Their Roles To Generate and Tailor Intermediates *cyclo*-Acetoacetyl-L-tryptophan (cAATrp) and  $\beta$ -CPA To Afford  $\alpha$ -CPA



Identification of several fungal tetramate natural product biosynthetic gene clusters, including fusarin C (2), equisetin (3), tenellin (4), pseurotin A (5), aspyridone (6), and chaetoglobosin (7), highlighted conserved hybrid PKS-NRPS genes that encode a N-terminal iterative PKS module and a C-terminal NRPS module. The apparent function of the NRPS module would be two-fold: (a) selection and installation of the chain-terminating amino acid moiety into the polyketide scaffold generated by the iterative PKS and (b) carrying out the tetramic acid formation in the release step. Bioinformatic analysis of the chain-terminating NRPS modules in fungal tetramate synthetase indicates a C-A-T-R four-domain composition rather than a typical C-A-T-TE. An initial hypothesis was formulated (2–7) that the tetramate ring formation occurred via a reductive release to give an aldehyde intermediate that undergoes nonenzymatic ring closure to give a pyrrolidine-2-one, which was further oxidatively tailored by one or more P450 enzymes. However, a recent study by Cox and co-workers revealed that the heterologous overexpression of tenellin synthetase in *Aspergillus oryzae* led to *in vivo* production of a mature tetramic acid containing pretenellin, suggesting for the first time that the R domain in tenellin synthetase might function as a simple condensation catalyst, which we would label R\* to indicate a nonredox function (13). In addition, Schmidt and colleagues have examined the excised, purified TR\* didomain and R\* domain of equisetin synthetase with a model substrate *N*-acetoacetyl-L-alanyl-S-(*N*-acetyl)cysteamine (*N*-acac-L-Ala-SNAC) as a soluble mimic of

the complex acylpeptidyl chain that would normally be covalently tethered on the T domain (14). They observed the redox-independent generation of the corresponding tetramate moiety by Eqs<sub>TR</sub>\* and Eqs<sub>R</sub>\*, consistent with a Dieckmann-type formation of the 3-acyl tetramate ring, although they could not obtain saturation kinetics with the model SNAC substrate (14). These results raise the general question whether the reductase-like (R\*) domains in fungal tetramate synthetases are indeed not aldehyde generators, but instead act in a default nonredox mode to carry out different carbonyl chemistry.

The latest addition to the family of fungal tetramate biosynthetic gene clusters is for cyclopiazonic acid (CPA or  $\alpha$ -CPA; for structure, see Figure 1A), a potent neurotoxin with a rigid pentacyclic indole tetramate scaffold that acts as a nanomolar inhibitor of the sarcoplasmic Ca<sup>2+</sup> ATPase (15). Identification of CPA-related biosynthetic genes (16, 17) from two CPA-producing and one CPA-nonproducing *Aspergillus* species correlates (Figure 2) with the previous biosynthetic predictions that the cyclopiazone scaffold is derived from two biosynthetic intermediates (Scheme 1), *cyclo*-acetoacetyl-L-tryptophan (cAATrp) and  $\beta$ -CPA, which are assembled from tryptophan, two molecules of acetate, and dimethylallyl diphosphate in a three-enzyme pathway (18). The immediate precursor of  $\alpha$ -CPA is the tricyclic  $\beta$ -CPA, converted by the flavoprotein oxidocyclase (CpaO) in a redox step forming two rings (19–21); in turn,  $\beta$ -CPA should be derived from the regioselective prenylation of the indole moiety of cAATrp by a dimethylallyltransferase (CpaD) (22), the second

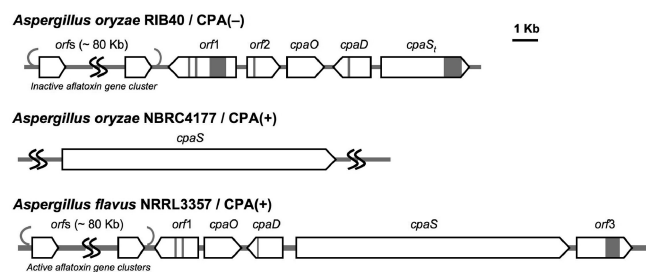


FIGURE 2: Summary of CPA biosynthetic genes identified in three *Aspergillus* sp. including two CPA producing/CPA(+) strains and one CPA nonproducing/CPA(-) strain (16, 17).

enzyme in this short metabolic pathway (Scheme 1). The first enzyme is a hybrid two-module PKS-NRPS (CpaS), homologous to other sequenced fungal tetramate synthetases, that should be responsible for elaborating one molecule of acetyl-CoA, malonyl-CoA, and tryptophan, to a tethered *N*-acetoacetyl-Trp-S-T domain intermediate on the way to the released tetramate cAATrp (Scheme 1). The occurrence of a truncated, rather than full-length, CpaS in *A. oryzae* RIB40 would thus account for its CPA nonproducing status (Figure 2) (17).

Our close inspection of the CpaS C-terminal R domain disclosed a mutation (Tyr → Leu) within the SDR Ser-Tyr-Lys catalytic triad that is of paramount importance for reductase activities (23–25). The absence of any redox ORFs, such as cytochrome P450s in the vicinities of CpaS, CpaD, and CpaO, further suggests that the tetramate moiety of cAATrp is likely derived from the direct action of CpaS in a nonredox fashion.

In this study we have overproduced the C-terminal T-R\* didomain of CpaS and establish that it does carry out a redox-independent Dieckmann cyclization, but only when the *N*-acacryptophanyl moiety is presented to the CpaS R\* domain on its normal pantetheinyl covalent tether on the immediately upstream T domain. The development of an *in trans* assay between the T and R\* domain of CpaS allowed us to carry out mutagenesis studies on CpaS\_R\* and identify unique amino acid residues within this fungal tetramate synthetase that are important for its Dieckmann cyclization activity.

## EXPERIMENTAL PROCEDURES

**General Methods and Materials.** Standard molecular biology procedures were performed as described (47). Oligonucleotide primers were synthesized by Integrated DNA Technologies (Coralville, IA). PCR was performed using either KAPA HiFi DNA polymerase (KAPA Biosystems) or Fusion high-fidelity DNA polymerase (New England Biolab) under suggested conditions on a Bio-Rad MyCycler thermocycler. For general cloning, *Escherichia coli* NovaBlue (DE3) cells (Novagen) were used. For protein overproduction, BL21 Star (DE3) cells (Invitrogen) were used. pET24b plasmid was obtained from Novagen. Conventional cloning was performed using DNA ligation kit version 2.1 (Takara). The pET151/D-Topo expression kit was obtained from Invitrogen. DNA sequencing was performed at the Molecular Biology Core Facilities of the Dana Farber Cancer Institute (Boston, MA). *Aspergillus flavus* NRRL3357 strain and genomic DNA were obtained from Dr. Jiujiang Yu (USGA). Dimethylallyl pyrophosphate (DMAPP) was obtained from Isoprenoids LC. [ $^{14}$ C]Acetyl-CoA (56 Ci/mol) was obtained from GE Healthcare. Phosphopantetheinyltransferase Sfp was heterologously overproduced and purified to apparent homogeneity as previously described (44). All other chemicals

were purchased from Sigma-Aldrich unless noted otherwise. HPLC was performed on a Beckman System Gold (Beckman Coulter) instrument on Phenomenex Luna C18 columns (250 × 4.5 mm, for analytical purpose; 250 × 21.6 mm, for preparative purpose). The eluent was monitored for absorption at 280 nm for all LC analysis of tryptophan derivatives. LC-MS analysis was carried out on Shimadzu LCMS-QP8000a (for low-resolution mass) and Agilent series 1100 LC/MSD-TOF (for high-resolution mass) machines.  $^1\text{H}$  NMR spectra were recorded on a Varian 600 MHz spectrometer. Chemical shifts are reported in parts per million with the solvent resonance resulting from incomplete deuteration as the internal standard ( $\text{CDCl}_3$   $\delta$  7.26,  $\text{CD}_3\text{OD}$   $\delta$  3.31). Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, br = broad, m = multiplet), coupling constants (hertz), and integration.

**Cloning of *cpaS\_TR\**, *cpaS\_T*, and *cpaS\_R\**.** The domain boundary of CpaS (3906 aa) was analyzed using the Pfam database (<http://pfam.sanger.ac.uk/>). *cpaS\_TR\**(3433–3906), *cpaS\_T*(3460–3543), and *cpaS\_R\**(3544–3906) were amplified directly from *A. flavus* NRRL 3357 genomic DNA using the following primers: *cpaS\_TR\** forward, CACCATGCGCCCTT-CCACAATCATC; *cpaS\_TR\** reverse, CTATTAATTACCA-AGTAGGGATATCG; *cpaS\_T* forward, CACCATGCCATG-GACGCGGCC; *cpaS\_T* reverse, CTATTATGATGGTGATT-CAGC; *cpaS\_R\** forward, GAGTCATATGGGCAATGCGC-CGTACGATATC; *cpaS\_R\** reverse, CTTAGCGGCCGCAT-TACCAAGTAGGGATATCG. Amplified genes were purified by agarose gel. *cpaS\_TR\** and *cpaS\_T* genes were cloned into the pET151/D-Topo vector according to the manufacturer's instruction to yield N-terminal His<sub>6</sub>-tagged expression plasmids. *cpaS\_R\** was ligated to the *Nde*I and *Not*I sites of the pET24b vector to give C-terminal His<sub>6</sub>-tagged expression plasmid. Correct clones were obtained by the subsequent restrictive digestions and sequencing of the isolated plasmids.

**Overproduction and Purification of *CpaS\_TR\**, *CpaS\_T*, and *CpaS\_R\**.** Plasmids containing the verified *cpaS\_TR\**, *cpaS\_T*, and *cpaS\_R\** genes were transferred into *E. coli* BL21 Star (DE3) competent cells for protein expression. Cells were cultured in Luria–Bertani medium containing appropriate antibiotics (100  $\mu\text{g}/\text{mL}$  ampicillin for pET151 vector and 50  $\mu\text{L}/\text{mL}$  kanamycin for pET24b vector). A fresh overnight culture from a single colony was used to inoculate 2 × 1 L medium. Cells were grown at 37 °C in a shaker at 200 rpm until midlog phase ( $A_{600} \approx 0.5$ –0.7). The cultures were then cooled to 16 °C and induced by isopropyl  $\beta$ -D-1-thiogalactopyranoside with a final concentration of 0.1 mM. The cultures were allowed to further grow at 16 °C for 12–16 h. The cells were harvested by centrifugation, and cell pellets from the 2 L culture were resuspended in a buffer (30 mL) containing 20 mM Tris-HCl, pH 8, 500 mM NaCl, 20 mM imidazole, 10 mM  $\text{MgCl}_2$ , and 1 mM DTT and homogenized. The homogenized cells were lysed by passing through a cell disruptor (Avestin EmulsiFlex-C5) three times at 5000–10000 psi, and the lysate was clarified by ultracentrifugation (35000rpm × 35 min). The supernatant was incubated with 2 mL of Ni-NTA-agarose resin (Qiagen) at 4 °C for 2 h and loaded to a column. The resin was then washed with a buffer (20 mL) containing 20 mM Tris-HCl, pH 8, 500 mM NaCl, and 20 mM imidazole. His<sub>6</sub>-tagged proteins were eluted with 200 mM imidazole in 20 mM Tris-HCl, pH 8, and 500 mM NaCl. The protein was further buffer-exchanged and purified by gel filtration chromatography using a HiLoad 26/60 Superdex 200 column



(GE Biosciences) with a buffer containing 25 mM HEPES, pH 7.0, 50 mM NaCl, 1 mM DTT, 5 mM MgCl<sub>2</sub>, and 5% glycerol. Fractions containing desired proteins were pooled and concentrated using an Amicon Ultra centrifugal filter device (Millipore). The protein concentration was determined by Bradford assay and the protein's absorbance at 280 nm with the predicted molar extinction coefficient. Proteins were aliquot-flash-frozen in liquid nitrogen and stored at -80 °C for further usages. The overproduction yield was ca. 12 mg/mL for CpaS\_TR\*, 7 mg/L for CpaS\_T, and 15 mg/L for CpaS\_R\*.

**Phosphopantetheinylation Assays of CpaS\_TR\* and CpaS\_T.** Phosphopantetheinylation assay was performed by measuring the incorporation of radiolabeled [1-<sup>14</sup>C]acetyl-SCoA onto heterologously overproduced CpaS\_TR\* and CpaS\_T, and the conditions are as follows: 75 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 0.5 mM TCEP, 100 μM [1-<sup>14</sup>C]acetyl-CoA (12.19 Ci/mol, final), and 10 μM CpaS\_TR\* (WT and mutants) or CpaS\_T. Reactions were started by adding 0.5 μM Sfp. Aliquots of the reaction mixtures were quenched at certain time intervals with 10% (w/v) TCA, followed by the addition of BSA (100 mg). The protein pellet was washed with 10% (w/v) TCA and resuspended in formic acid, and the amount of radioactive label was measured by liquid scintillation counting. Mass spectrometry analysis of overproduced CpaS\_TR\* and CpaS\_R\* was performed according to the described procedure at the Dana Farber Cancer Institute Core Facility.

**Preparation of cAATrp.** To a suspension of 4 Å molecular sieves (0.5 g) in THF (10 mL) was added L-Trp-OMe·HCl (254 mg, 1.0 mmol) and Et<sub>3</sub>N (280 μL, 2.2 mmol) at room temperature. After being stirred for 15 min, *s*-tert-butyl acetothioacetate (360 μL, 2 mmol) was added, followed by the dropwise addition of a solution of silver trifluoroacetate (660 mg, 3 mmol) in THF (5 mL). The mixture was stirred vigorously overnight. The precipitates and molecular sieves were filtered off, and the filtrate was evaporated to give the crude *N*-acac-L-Trp-OMe. Further purification by silica gel chromatography gave *N*-acac-L-Trp-OMe (156 mg, 52%). <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>): δ 8.36 (s, 1H), 7.52 (d, 1H, *J* = 7.8 Hz), 7.32 (d, 1H, *J* = 8.4 Hz), 7.18–7.14 (m, 1H), 7.11–7.07 (m, 1H), 7.03 (s, 1H), 4.93–4.89 (m, 1H), 3.68 (s, 3H), 3.33–3.30 (m, 1H), 3.27 (s, 2H), 2.11 (s, 3H). HRMS (ESI): Calcd for C<sub>16</sub>H<sub>18</sub>N<sub>2</sub>O<sub>4</sub> [M + Na]<sup>+</sup>: 325.1164. Found: 325.1163. To a solution of *N*-acac-L-Trp-OMe (61 mg, 0.2 mmol) in THF (5 mL) was added methanolic NaOMe (0.8 mL, 0.4 mmol, 0.5 M in MeOH) at 0 °C. The mixture was warmed to room temperature and further stirred for 1 h before being stopped by adding drops of acetic acid. The solvents were evaporated, and the residue was subjected to silica gel chromatography to give cAATrp (47 mg, 87%) as a pale yellow solid. [α]<sub>D</sub><sup>24</sup> = -117.8 (*c* = 0.62, MeOH). <sup>1</sup>H NMR (600 MHz, CD<sub>3</sub>OD): δ 7.56 (d, 1H, *J* = 8.4 Hz), 7.31 (d, 1H, *J* = 8.4 Hz), 7.11 (s, 1H), 7.08–7.06 (m, 1H), 7.01–6.98 (m, 1H), 4.76 (dd, 1H, *J* = 7.8, 5.4 Hz), 3.36 (dd, 1H, *J* = 14.4, 5.4 Hz), 3.19 (dd, 1H, *J* = 14.4, 7.8 Hz), 2.05 (s, 3H). HRMS (ESI): Calcd for C<sub>15</sub>H<sub>14</sub>N<sub>2</sub>O<sub>3</sub> [M - H]<sup>-</sup>: 269.0926. Found: 269.0923.

**Preparation of acac-Trp-SCoA and acac-Trp-SNAC.** acac-L-Trp was prepared according to the literature procedure (48). To a solution of acac-L-Trp (6.8 mg) in CH<sub>3</sub>CN (0.3 mL) and THF (0.3 mL) was added PyBOP (24.6 mg). After being stirred for 15 min at room temperature, coenzyme A trilithium salt (18.5 mg) in H<sub>2</sub>O (0.1 mL) was added. The resulting mixture was stirred vigorously for 1 h, and K<sub>2</sub>CO<sub>3</sub>

Table 1: Oligonucleotide Primers Used To Generate CpaS\_TR\* and CpaS\_R\* Mutants

CpaS_TR* and CpaS_R* mutant	primer sequence <sup>a</sup>
S3508A	5'-CTCCTCGGGGGAATGCG ACGCTGCTGGTCAGG
C3619A	5'-GTGGAAAGAGTGCATGCG ATCGCCGTGGAAAAGG
D3663A	5'-CTGCAAGACCGGATCGCG GTGGTGATCCACAAC
H3667A	5'-ATCGACGTGGTGATCGCG AACGGCTCGAATGGC
S3688A	5'-TCCTCCGCTCGACGGGGCG GACGGGCTCACAGCAAC
S3707A	5'-CCCCTGCACTATATCGCG TCCGGAAGGGTCATC
K3742A	5'-GGGCTCACAGCAACCGCG TGGGCCAGCGAAGTC
D3777A	5'-CAGGCTCCAGCACAGGCG GCACTGAACCTCGCTC
D3803A	5'-ATGGAAGGATACCTTGCG TTTCAGAAGGTAGAG
H3843A	5'-GGTTTCCTTTTCCACGCG TCGAGCAATGTCAAG

(6.5 mg) was added. The reaction was stirred further for 1 h before being quenched by the addition of AcOH (30 μL). The mixture was diluted with a THF/H<sub>2</sub>O mixture to provide a homogeneous solution and directly subjected to preparative HPLC purification. Fractions containing the target acac-Trp-SCoA were identified by LC-MS, combined, and lyophilized to give a white powder (15.6 mg, 62%). <sup>1</sup>H NMR (600 MHz, D<sub>2</sub>O): δ 8.45 (s, 1H), 8.02 (s, 1H), 7.32 (d, 1H, *J* = 7.8 Hz), 7.22 (d, 1H, *J* = 7.8 Hz), 7.00 (app t, 1H, *J* = 7.8 Hz), 6.98 (s, 1H), 6.90 (app t, 1H, *J* = 7.8 Hz), 5.99 (d, 1H, *J* = 6.0 Hz), 4.80–4.74 (m, 1H), 4.73–4.71 (m, 1H), 4.70–4.68 (m, 1H), 4.51–4.49 (m, 1H), 4.23–4.17 (m, 2H), 3.95 (s, 1H), 3.81 (dd, 1H, *J* = 9.6, 4.8 Hz), 3.56 (dd, 1H, *J* = 9.6, 4.8 Hz), 3.38–3.31 (m, 3H), 3.00–2.96 (dd, 1H, *J* = 15.0, 8.4 Hz), 2.88–2.84 (m, 2H), 2.29 (t, 1H, *J* = 6.0 Hz), 1.97 (s, 3H), 0.86 (s, 3H), 0.73 (s, 3H). HRMS (ESI): Calcd for C<sub>36</sub>H<sub>50</sub>N<sub>9</sub>O<sub>19</sub>P<sub>3</sub>S [M + H]<sup>+</sup>: 1038.2235. Found: 1038.2240. acac-Trp-SNAC was prepared in an identical procedure except replacing coenzyme A trilithium salt with *N*-Ac-cysteamine.

**Mutagenesis of CpaS\_TR\* and CpaS\_R\* and Mutant Protein Overproduction.** The mutants of *cpaS\_TR\** and *cpaS\_R\** were generated according to the protocol described in the Stratagene QuikChange multi-site-directed mutagenesis kit, using plasmids pET151::cpaS\_TR\* and pET24b::cpaS\_R\* as the templates and primers listed in Table 1. Primer oligonucleotides were *in situ* 5'-phosphorylated using T4 polynucleotide kinase (New England Biolabs). Correct mutant plasmids were verified by sequencing. The mutant proteins were overproduced and purified in the same manner as those described for the wild types.

**CpaS\_TR\* Activity Assay.** The single time point assay (Figure 4B) was carried out in a 100 μL scale at room temperature with CpaS\_TR\* (70 μM), acac-Trp-SCoA (50 μM), and buffer containing HEPES (50 mM, pH 7.0) and MgCl<sub>2</sub> (5 mM). The reaction was initiated by the addition of Sfp (30 μM) and terminated after 10 min with 0.2 mM TCA in MeOH (100 μL). Proteins were pelleted centrifugally (13000g) for 10 min at 4 °C, and the supernatants were subjected to HPLC and LC-MS analyses. To examine if NADH and/or NADPH is required for the activity of CpaS\_TR\*, NADH (10 mM) or NADPH

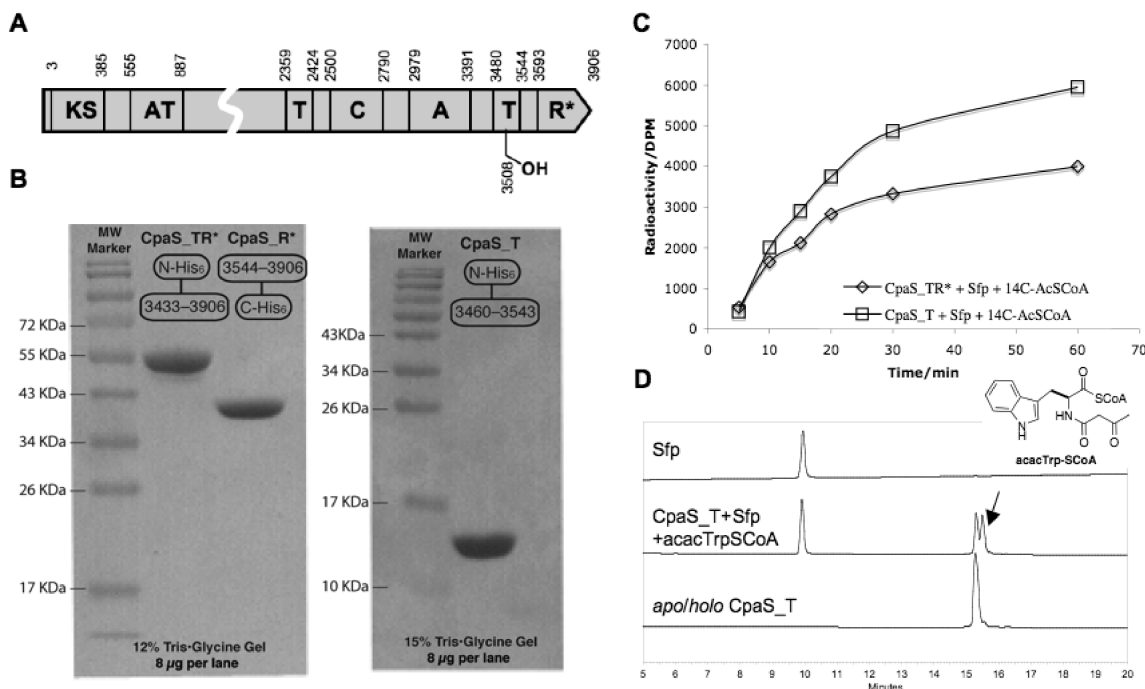


FIGURE 3: Overproduction of truncated CpaS proteins and their phosphopantetheinylation assays. (A) Domain architecture and boundaries of CpaS (NRRL3357). (B) SDS-PAGE purity of overproduced CpaS\_TR\*, CpaR\*, and CpaS\_T. (C) Phosphopantetheinylation of CpaS\_TR\* and CpaS\_T by Sfp. The time course was shown by analyzing the incorporation of the [1-<sup>14</sup>C]acetyl-S-phosphopantetheinyl group from [1-<sup>14</sup>C]acetyl-CoA. (D) HPLC analysis of overproduced apo/holo CpaS\_T and acac-Trp-SCoA modified CpaS\_T (the arrow indicates the *N*-acac-Trp-S-T intermediate; predicted mass, 14625.4 Da; observed mass, 14625.0 Da).

(10 mM) was included in the reaction buffer prior to the addition of Sfp. Control reactions that exclude CpaS\_TR\* and/or Sfp from the assay components were also carried out. Products were examined in the same fashion by HPLC.

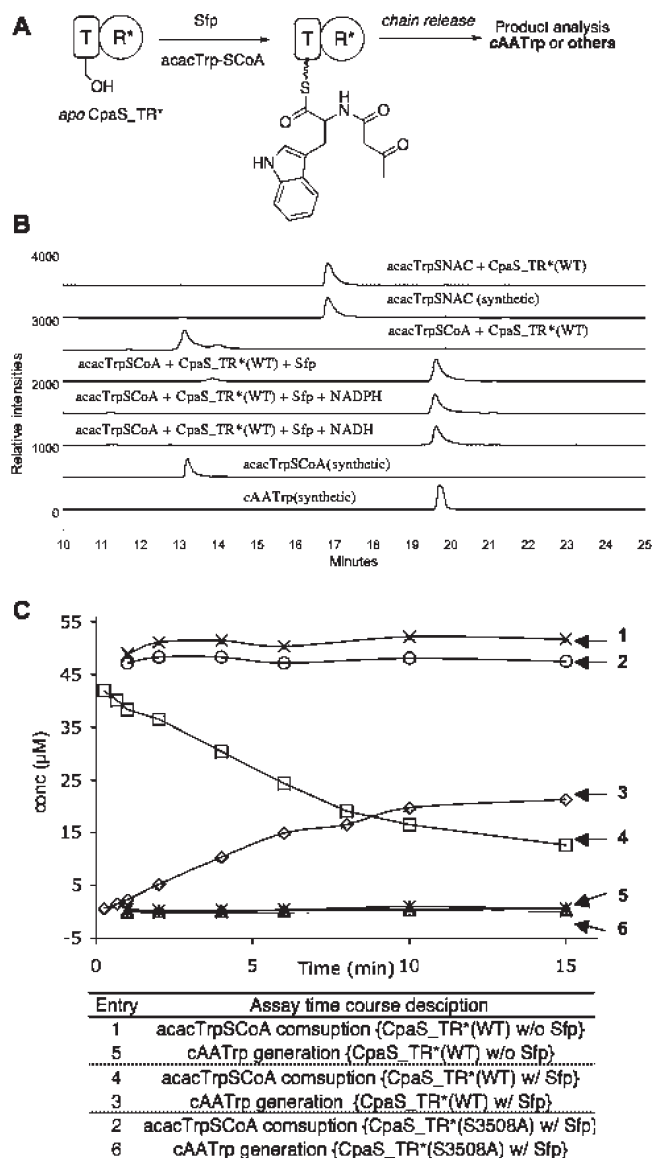
The time course analysis of CpaS\_TR\* (WT or mutants) mediated *c*AATrp generation and acac-Trp-SCoA consumption (Figures 4C and 8) was carried out in a similar fashion as the single point assay except in a 400–500  $\mu$ L scale. At each selected time point, 50  $\mu$ L of the reaction mixture was quenched with 0.2 mM TCA in MeOH (50  $\mu$ L), protein was pelleted, and supernatant was subjected to HPLC analysis. The areas of HPLC peaks corresponding to *c*AATrp and acac-Trp-SCoA were obtained after manually adjusting HPLC chromatograph baselines. The area was fitted into a standard curve (concentration vs area) of *c*AATrp and acac-Trp-SCoA generated using synthetic standards to calculate the corresponding concentration.

**CpaS\_T Assay with CpaS\_R\* in Trans.** The assay (Figure 5, entries 1 and 2) was carried out at room temperature with CpaS\_T (50  $\mu$ M), acac-Trp-SCoA (25  $\mu$ M), Sfp (5  $\mu$ M), and buffer containing HEPES (50 mM, pH 7.0) and MgCl<sub>2</sub> (5 mM). The reaction was initiated by the addition of CpaS\_R\* (2.5  $\mu$ M, 5 mol %). Analogous to CpaS\_TR\* time point assays, aliquots (50  $\mu$ L) of the reaction mixture were quenched at each selected time point with 0.2 mM TCA in MeOH (50  $\mu$ L). Protein was pelleted, and supernatant was subjected to HPLC analysis to determine the conversion rate based on HPLC area of *c*AATrp and acac-Trp-SCoA and the established standard curves. As control (Figure 5, entries 3 and 4), the assay was carried out without the addition of CpaS\_R\*, and the rates of *c*AATrp generation and acac-Trp-SCoA consumption were monitored in the same fashion. The *in trans* activities of nine CpaS\_R\* mutants with acac-Trp-SCoA primed CpaS\_T (Figure 7) were examined in the same fashion as CpaS\_R (WT), and the time courses of *c*AATrp generation were analyzed.

## RESULTS

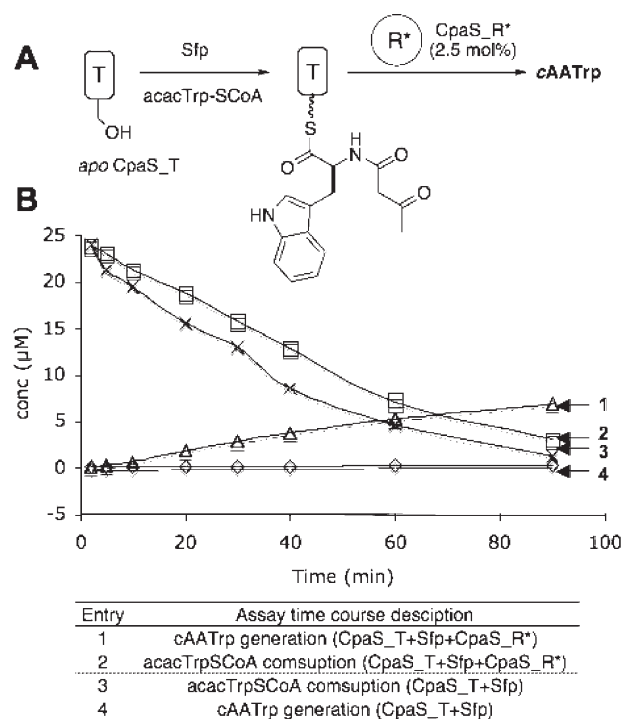
**Cloning and Overexpression of CpaS\_TR\*, CpaS\_R\*, and CpaS\_T.** CpaS is a 431 kDa hybrid PKS-NRPS that contains 3906 amino acids (Figure 3A). Two complete *cpaS* genes have been disclosed from *A. flavus* NRRL3357 and *A. oryzae* NBRC4177 (Figure 2), which are 93% identical (16, 17). *A. flavus* NRRL3357 *cpaS* was used in this study. To study the tetramate ring formation and chain release mechanism of CpaS, we undertook to clone and overproduce truncated forms of CpaS protein in *E. coli*, including the 56 kDa C-terminal TR\* didomain (CpaS\_TR\*), the 40 kDa R\* domain (CpaS\_R\*), and a 14 kDa stand-alone carrier protein/thiolation domain construct (CpaS\_T). The prediction of CpaS domain boundaries was based on bioinformatic comparisons with characterized NRPS modular enzymes. The genes were amplified directly from *A. flavus* NRRL3357 genomic DNA and cloned into p151ET/Topo and pET24b vectors to afford N-terminal His<sub>6</sub>-tagged CpaS\_TR\*, CpaS\_T, and C-terminal His<sub>6</sub>-tagged CpaS\_R\* expression constructs, respectively (see Experimental Procedures). Overproductions in *E. coli* gave soluble CpaS\_TR\*, CpaS\_T, and CpaS\_R\* proteins in good yields, which were further purified to apparent homogeneities (Figure 3B) by successive nickel affinity and gel filtration chromatographies. All truncated CpaS proteins appeared monomeric in solution, as estimated by size exclusion chromatography.

**Phosphopantetheinylation Assay of CpaS\_TR\* and CpaS\_T.** With ample amounts of protein in hand, we first sought to assess the apo/holo states of overproduced CpaS\_TR\* and CpaS\_T arising during heterologous expression. The time-dependent incorporation of [1-<sup>14</sup>C]acetylpanetheinyl groups onto the apo forms of the T domains in either construct from radiolabeled [1-<sup>14</sup>C]acetyl-S-CoA, catalyzed by the promiscuous phosphopantetheinyltransferase Sfp (5 mol %), was observed



**FIGURE 4:** CpaS\_TR\* catalyzed formation of cAATrp requires the modification of apo CpaS\_TR\* by phosphopantetheinyltransferase Sfp with acac-Trp-SCoA, independent of NADH and NADPH. (A) Schematized CpaS\_TR\* assay procedure. (B) HPLC traces of cAATrp formation from acac-Trp-SCoA primed CpaS\_TR\* with or without NADH and NADPH (lines 3–5), in comparison with synthetic standards of acac-Trp-SCoA and cAATrp (lines 1 and 2). CpaS\_TR\* did not take acac-Trp-SCoA and acac-Trp-SNAC as substrates (lines 2 vs 6 and lines 7 vs 8). (C) Time course analysis of cAATrp generation and acac-Trp-SCoA consumptions, where CpaS\_TR\* was primed with acac-Trp-SCoA with Sfp (entries 3 and 4); CpaS\_TR\* was incubated with acac-Trp-SCoA only (entries 1 and 5); CpaS\_TR\* (S3508A) mutant was incubated with acac-Trp-SCoA and Sfp (entries 2 and 6).

within 60 min (Figure 3C). By increasing the amount of Sfp to 30 mol %, the posttranslational phosphopantetheinylation process can be expedited to reach saturation within 10 min. The measured total incorporation of  $^{14}\text{C}$ -radiolabel corresponds to ca. 50–60% of the calculated value, suggesting the presence of 40–50% holo forms of the T domains in the overproduced CpaS\_TR\* and CpaS\_T that are posttranslationally primed by endogenous phosphopantetheinyltransferase activity of *E. coli* cells. This was further confirmed by mass spectrometry analysis of the intact proteins (data not shown). We further demonstrated that apo CpaS\_T can be primed with synthetic acac-Trp-S-CoA



**FIGURE 5:** acac-Trp-S-CpaS\_T and CpaS\_R\* activities *in trans*. (A) Schematized CpaS\_T and CpaS\_R\* *in trans* assay procedure. (B) Time course analysis of cAATrp generation and acac-Trp-SCoA consumption from acac-Trp-SCoA primed CpaS\_T with or without CpaS\_R\* present *in trans*.

(see Experimental Procedures for preparation) using Sfp, as shown by the generation of the *N*-acac-L-Trp-S-T covalent adduct (Figure 3D). HPLC analysis showed baseline separation between apo/holo forms of CpaS\_T and *N*-acac-L-Trp-S-T species, whose identity was confirmed by Q-TOF MS analysis (Figure 3D).

**Stoichiometric Generation of cAATrp by Priming Apo-CpaS\_TR\* with acac-Trp-S-CoA Using Sfp.** To address the role of the C-terminal R\* domain of CpaS in cAATrp generation, we first sought to prime the apo form of the T domain in CpaS\_TR\* with synthetic acac-Trp-S-CoA using Sfp to generate a transient *N*-acac-L-Trp-S-T tethered thioester (Figure 4A). This covalent intermediate should be presentable *in cis* to the downstream R\* domain. If the R\* domain acts as a Dieckmann cyclization catalyst, the cAATrp product should be released and be detectable. As shown in line 5 of Figure 4B, incubation of an equimolar amount of apo CpaS\_TR\* with acac-Trp-SCoA in the presence of Sfp at pH 7, room temperature for 10 min, led to the generation of a new peak that corresponds to cAATrp, as verified by high-resolution MS analysis ( $[M - H]^-$  calcd for  $\text{C}_{15}\text{H}_{13}\text{N}_2\text{O}_3$ , 269.0926; obsd, 269.0924) and HPLC coelution with synthetic cAATrp. The generation of cAATrp from acac-Trp-SCoA at pH 7 was independent of and did not require NAD(P)H cofactor (Figure 4B, lines 3 and 4), reminiscent of the recent observation with the Eqs\_TR\* didomain from equisetin synthetase (14). However, in distinction to the Eqs-TR\*, which accepts soluble aminoacyl SNACs, the production of cAATrp by CpaS\_TR\* strictly depends on the presence of both CpaS\_TR\* and the priming phosphopantetheinyltransferase Sfp, as schematized in Figure 4A. We did not observe the conversions of acac-Trp-SCoA or acac-Trp-SNAC to cAATrp by either CpaS\_TR\* or CpaS\_R\* alone (for CpaS\_TR\* assay, see Figure 4B, lines 1 and 6–8; for CpaS\_R\* assay, data not shown),



CpaS3357R	-LTGSTSFLGRVLVQVL--LQVPEVERVHCIAVEKEQEHLVLP-----TSDKVSLYYGSLLDPNLGLSTAEWASLQDRIDVVTHNGSNG	3672
CpaS4177R	-LTGSTSFLGRVLVQVL--LQVPEVERVHCIAVEKEQEHLVLP-----TSDKVSLYYGSLLDPNLGLSTAEWASLQDRIDVVTHNGSNG	3672
ApdA_Red	-LTGSTSFLGKNIQLSL--LHNPLVERVHCIAVPAEDIPRLP-----ASEKISITYGSLTTPSLGLTKTEIAVLQSSLDVIIHAGSTG	3697
CheA_Red	-LTGATSFGLGAILRAELTGSRIKRVHCIAVLDPDEKLIP-----ASQSVAYYNGSLMSRRLGLTVPEYEHLSQNSVDLIHAGSNG	3766
TenS_Red	-LTGATFGLGQILLERL--VQSPRVSTVHCIAVLDPDEKLIP-----ASQSVAYYNGSLMSRRLGLTVPEYEHLSQNSVDLIHAGSNG	3987
FusS_Red	-LTGATFGLGVSIETL--LEDKRVSKVHCIAVVPDEQSLLEPFLQ-----QQADGTRRKVRCYIGNLAAPALGLTAADQALSAQVDIRIHNAGDV	3511
PsoA_Red	-LTGATFGLGGHILRQL--VQLPSVEHVHCIAVRS--SNTSDP-----VFSSSRVACYPGLDSLRLGLSQQFDQLANSIDVIVHNGAEV	3763
EqiS_Red	-LTGATCYLGRHLVPAL--VQSAQIEKVFCLVRGKSDDEARRLEATFDTPLLRKHFNELATERLQVVAGDKGQPNLGLDEQWQRLAEDSDIILHSGANR	3718
MxcG_Red	-LTGATGFVGAHLLDQLLRQT--QAKVVCVAIRPNKVDVRRQL-----SVESPKIIRYSGDLALPNMGMSESEFSDLFKECKMILHNAAVV	1190
LtxA_Red	-LTGATGFLGAVLLYELLKQT--DANVFLVRARDEAHAMERLREAMTSQRLSTASL--SERVLALPADLGPWPLGLSSARFHLAALIDAIYHNGAQV	2204
MxaA_Red	-LTGATGFLGAFLEELCRRT--DARIYCLVRSNHSEAAQRIHSTLKFYQLWSESW--RSRIIPVCGDLSQPSLGLSAEEFSKLTTEEIDAIYHNGALV	1246
SacC_Red	-LTGATCYLGAFLLEQLLKRT--RATVYCLVRSKTEQEGMNRIRKNLESYSLWNEAL--APRIVPVRGIDGQPLGLSEKEFQRLSEELSDIYHNGALV	2319
Lys2_Red	-LTGNGVGLGVILRLDQLQRNN--NVRVYVLRRAADPAQAMDRVRATMHQYLWDEAY--AERIRPLVGLDGKPLRGLSREWERLGLTVDTIHNAGLV	1100
Mps2_Red	-LTGATGFLGRYLLLEWLRLQRRVDKVICLVRAKSEEHLQRLQDSCIAYGWEDAW--KDKITVTVTGLAKPQWGMDDATWKHVAESVDLIVDSAAVF	2255
1Y1P	-VTGANGFVASHVLEQLLEHG--YKVRCLVRHSEVDVALRK-----ASEKIIITYGDLGEVDLGLSKDEFQSLSRGAAGVAHIASVV	81
1SB8GlcN	-ITVAGFVIGSNVLETLKLDQKVVGLDNFATGHORNLDSESVSEK-----QWSNFKFIQGDIR-----NLDNCNACGAGVYVHLHQAALG	113
AgmE	-VTGAGFVIGSNVLETLKLDQKVVGLDNFATGHORNLDSESVSEK-----QWSNFKFIQGDIR-----NLDNCNACGAGVYVHLHQAALG	79
CpaS3357R	-HCLNTYNSLKGPNLGS--THRLAEFALQSQ--IPLHYIS-----SGRVILQSGQT-----ALGPTSVSFHPPPLDGSQDITATKWASEVFLERLAE--	3754
CpaS4177R	-HCLNTYNSLKGPNLGS--THRLAEFALQSQ--VPLHYIS-----SGRVILQSGQT-----ALGPTSVSFHPPPLDGSQDITATKWASEVFLERLAE--	3754
ApdA_Red	-HCLNNYSSLRASNVDSTKFLAAIALLCR--IPIHFIIS-----SNRVTLSSGST--SLPPASVSSSLPNTDGESEFTASKWASERLLESVAN--	3779
CheA_Red	-HCLNNFHLRMANLNS--THELIMALPRS--TPLYLYS-----SNRVTLSSGST--SYPPASISRSPPPKDGESEFTASKWASEVFLERLAE--	3846
TenS_Red	-HCLNTYATLAAPNFASTRHLCSLALSRSPPIPLAFAS-----SNRVALLTGST--APPPASAAAFPPPP--GAQC--TASKWASEAFLEKLAASIM	4073
FusS_Red	-SFLKTYQSLQSRNSVSSSRELAARMAITRR--IPMHFVS-----TGGVVQLTGQD--GLDEVSVIDSTPPNDGSLGYVASKWASEAILEKYAS--	3586
PsoA_Red	-SHMKNYRSLRAANFSTVGLARAASVSG--IPIHFIIS-----TGGVARSLSVAD--EQPEASLAHFPPIDGSDGYVASKWASEVFLERLAE--	3847
EqiS_Red	-SFWDDEYVLRPANTNSIKELARLALPRR--IPFFHFIIS-----SGSVRIYDDEEGPKIYNIDQSLPISPTPSSDGSQDGYVASKWAAEFKLRVTAQSQ--	3777
MxcG_Red	-SVVREYGLSQATNVRGCTRELRLAASVRP--KPLHYVS-----TLAVAPQANLS--PEVP--EAFVP--AHPGLRDCYQGSKWAERLVEQASER--	1282
LtxA_Red	-SAIEPPTYLTKPTNVLCSTSELDFAACRV--KPLHFVS-----TAAVAVSSKGN--PDIIYENFRLGADSVLPSSGVSSKWAELVWVASDR--	2288
MxaA_Red	-NFLYPYPSMAANVLGCTREILRLATRTRI--KPLHYVS-----TVSVLPLGRKA--PIREDEPLEG--PSSLVGGAQSKWAEKLVREASRR--	1328
SacC_Red	-NFVYPSYALRGPNVHCTQEVRLRLGCQHRL--KAVHYVS-----TIDVLLATHMP--RPFMEDDAPLRNP IEPVPGGYTGSKWAEKLVVNIARAR--	2403
Lys2_Red	-HWVYPYTKLRANFVICTLTAIINLCSIGKP--KSLSFVS--[10]--NLSADIMSKGG--NGVPESDDLEGSAGVLQAGGQGSKWAEEYLRQAGAR--	1196
Mps2_Red	-NSVLPYSELFGPNVVGTAELIRFALTSKL--KPFNFVS--[5]--RQIEPSRFTQEQ--ADIRLVSAATRKIEVGYANGYGNKSWAGEVLLREAHDC--	2351
1Y1P	-SFSNKYDEVVTPAIGGLTNALRAAAATPSVKRFLVS--[11]--VEGIYLDSEKSN--LESIDKAKTLPESDPQKSLWVYAAASKTEAELAANKFMDEN--	194
1SB8GlcN	-SVPRSINDPITSNRPSSITGD--DAKVFQS-----FTYAASSSTYGDHP-----GLPKVEDTIGKPLSPYAVTYVHLYAGVDFVSRCY--	195
AgmE	STTEWDGKYMMDNNYQYSKELLHYCLEREI--PFLYAS-----SAATYGGRT-----SDFIESREYKPLNVVYGSKFLFDEYVRQILPEAN	159
CpaS3357R	-[2]--DISISIHPRCTPIGD--QAPAQDALNSLLRYSVNLGATP-[4]--MEGYLDPQKVEIIAQEIATLVTSRFTKRSNTSSFTTPGVSFHHSSN	3844
CpaS4177R	-[2]--DISISIHPRCTPIGD--QAPAQDALNSLLRYSVNLGATP-[4]--MEGYLDPQKVEIIAQEIATLVTSRFTKRSNTSSFTTPGVSFHHSSN	3844
ApdA_Red	-[4]--LPVTIHRPCAVFGE--EAPNEDALNALIKYSKLRCPV-[4]--FEGYLPFEDVHRVAATIAADALSAES--RESKS--AARVRHHSSG	3864
CheA_Red	-[5]--QLPIQISRPCVVGGA--EAPNSDALNKLKYSRLMQTVP-[4]--VEGYLPFKNVQVEVAQVVDALALD--LSTDSS--AIRFRHSSG	3934
TenS_Red	-[16]--PWRVSIHRPCALISD--RAPNSDALNAILRYSTSMRCVP-[6]--AEGYLDPGQVQDKVEEMVGDILGLADERQQEGP--AVVYRHHSSG	4170
FusS_Red	-[2]--NLPVWIHRPSNITGP--NAPKADLMQNIPIHYSAKTASLP-[5]--SGCFPFVFPVDVVAAGIASSIYETQET--VAYKHHCCT	3665
PsoA_Red	-[2]--QQQVWIHRPSSITGD--DVPNDIAHSLKFSRELGAVP-[4]--SGGFPFIPNVTVSNNIAASVVRSSSEKS--GGGLIYLHSGE	3929
EqiS_Red	-----LQLPVTIHKPMMPVPGV-[7]--EPESDHMVNELVIDIYKRLQVRP-[4]--LQGWADILDQSVVNDILDSILSEQG--VELQEVLITAV	3863
MxcG_Red	-----GLPVTYVRLGRVSGA-[4]--IVNPQDLVWRILLAGIPAGALP--QLDVGEWTPVDYVARALVRLSLVPR-----PGTVFNLTIP-A	1359
LtxA_Red	-----GLPVTVTHRPGRISGD-[4]--IGNTDDTFWQIVRAIVVLGVVP-[4]--QDDAGIDLMPVDRVASAIVHLSRHKQS-----ISKVHLLTC-P	2370
MxaA_Red	-----GLPVTILRPGRVTGH-[4]--AWNTDDLVCRTLKGCVRMGVAP--SVDALLDLTPVDYVSSAIVDLMSRPE--IGQTYHLN-P	1406
SacC_Red	-----GIPVCYIRPGLILSH-[4]--ATQNTDYLVAFRGYVPMGIIP--DYPRIFDIPVDYAAKAIHVHISTQREA--LGRFFHLLN-P	2481
Lys2_Red	-----GLRGVIVRPPIYFGD-[4]--AINTDDFLVIMTKSTQLGLSP--DINNTVMNVPDTHVARCATAAFAHPPK--SGVCVAQVTS	1275
Mps2_Red	-----GLPVAVFRSGMIMVD-[5]--QLNVDTVSRMVLISIVATGVAP-[11]--RQRAHFGCLPVDFVAQAITKLGWQVARSVTDSTA--SGFETYHVMH	2451
1Y1P	-----KPHFTLNAVLPNYTIGT-[7]--SGSTSQWMMSLFNGEVSPALP--MPPQYVSAVDIGLHLGLCLVLPQIE-----RRRVYGT	274
1SB8GlcN	-----GFSTIGLRYFNVPGR-[1]--QDPNGAYAAVPIKFSSTMQGD-[6]--DGETSRDPCYIENTVQANLLAATAGLD--ARNQVYNTAVG	277
AgmE	-----SQIVGFYRYFNVYGP-[1]--EGHKGSMAVAFHLNQLNNGE-[7]--SENFKRDPVYVGDVADVNWLWLENGVS-----GTFNLGTG	238
CpaS3357R	-VKVPVKSFKKEYMEK-VHGRPFQELGLREWSSSLA-----EQGIEPLIPSFLEAVDDNEETLRYPYLGN-----	3906
CpaS4177R	-IKVPVKSFKKEYMEK-VHGRPFQELGLREWSSSLA-----EQGIEPLIPSFLEAVDDNEETLRYPYLGN-----	3906
ApdA_Red	-HKVSMKDFKGRMET-LFACPFKEVSMMAEWTERAL-----QAGIDPLITGYLEAMTKGEIIRFPYMGAGSL-----	3931
CheA_Red	-VRTSLGEFRQRMET-IYEVPFDECSLDQWIDRAL-----QASIDPLITAYLEGMVERGEVIRFPFMGEVVSQGEVSTGNDEA-----	4013
TenS_Red	-VKVPVKSFKKEYMEK-VYGGRFESVELQGWIVRAV-----DAGMDPLISAYLETFLGEGDMSVFPYMGQAV-----	4239
FusS_Red	-EKISVEDLPSYLA-KHGK--IERSVEEWLERSK-----AAGLDEVTV--AVLVEKTLRSGGIVPWLRRAN-----	3733
PsoA_Red	-QVIVPGDLQKYVEE-LEGRPLQVLPLKEWVDLSI-----RKGLDEVLSGYMLASKGVIR--APLLQRGPHVE-----	3991
EqiS_Red	-LRNWRQFIHELHNSPCLSPKDTLLWIGQAK-----RSYGYSLMPAHLRVVVDNDDDEMVSRR-----	3951
MxcG_Red	-PEVRLSEVFGWQD-YGYPVA-LCPVPEWRTRVA-[35]--VLQALSDTGIS-CPRTDRPLLHRYLDYCVGQGLLRQPPHRR-----	1456
LtxA_Red	-TIVKLDVVFNELSK-LGYQLT-TVSYSEWVKQLE-[35]--SNTLTGLAEAPFK-FPCIDRHLRGYLYTFINSKFFPQITYTGK-----	2480
MxaA_Red	-QFVRADFMWNYMRA-FGYGLR-VLPYDQWLSELG-[36]--GDTLKLGGGTGS-CPVSDASLISTYSSSLVHRGFLKAPEVR-----	1515
SacC_Red	-APVSLRRFCDDWIRS-YGYAFD-IVPFDEARRQAL-[40]--RSAVEVLGASDIR-CPMTTELAHRCLQYLVDIGFLQRPVLRARQOK	2605
Lys2_Red	-PRLRFSQFLGTLTPV-FGFNVK-AVEYQLQWRVALE-[39]--LKQDTAWTGVDVSGAAVLEPQMGLYLAYLVKTFGLKKPEGEKPLPKV	1414
Mps2_Red	-DGIGIDTYIDWLIE-AGYPIERIEDFGELQRF-[42]--DRFQAARVDANIGVEGEPHPTREVIVKYVTDLQLLGLL-----	2559
1Y1P	-GTFDWNVTALTFRRKLYPSKTFPADFPDQGDLSK-[3]--APSLIELKSLGRPGWRSIEESIKDLVGSETA-----	342
1SB8GlcN	-GRTSLNQLFFALRDGLAENGVSYPHREPVYRDFRE-[4]--HSLADISKAALKGYAPKYDVSAGVALAMPWYIMFLK-----	352
AgmE	-RAESFQAVADATLAYHKKGQIEYIPFPDKLGKRY-[4]--QADLTNLRAAGYDKPFKTVAGVTEYMAWLNRA-----	310

FIGURE 6: Sequence alignment of the C-terminal reductase domains of CpaS from *A. flavus* NRRL3357 and *C. oryzae* NBRC4177 with (1) C-terminal reductase domains of all other known fungal tetramate synthases, (2) known redox-active C-terminal reductase domains from selected nonribosomal peptide synthetases, and (3) selected structurally characterized short-chain reductase/dehydrogenases. Sequences were aligned using ClustalW2 (<http://www.ebi.ac.uk/Tools/clustalw2>), starting from the conserved nucleotide binding domain. Sequence numbers represent the actual position number of the amino acid in the full-length synthetase. The red dot indicates the conserved NADH/NADPH binding domain; the blue square highlights the positions of the SDR Ser-Tyr-Lys catalytic triad; the black arrow points to the conserved polar amino acid residues in the R\* domain of fungal tetramate synthase that distinguish from either other NRPS redox-active R domains and/or structural characterized SDRs. R or Red represents the abbreviation of reductase. Protein name abbreviations: aspyridone synthetase (ApdA), chaetoglobosin synthetase (CheA), tenellin synthetase (TenS), fusarin synthetase (FusS), pseurotin synthetase (PsoA), equisetin synthetase (EqiS), myxochelin NRPS module (MxcG), lyngbyatoxin synthetase (LtxA), myxalamid NRPS module (MxaA), safracin NRPS (SacC),  $\alpha$ -aminoadipate reductase (Lys2), mycobacterial glycopeptidolipid synthetase (Mps2), aldehyde reductase (1Y1P), UDP-N-acetylglucosamine 4-epimase (1SB8), and ADP-L-glyceromannoheptose 6-epimerase (AgmE).

indicating the R\* domain in CpaS recognized the *N*-acac-L-tryptophanyl group *only* when it is tethered to the phosphopantetheinyl arm of the upstream CpaS\_T domain and presented in its native context.

We next carried out a time course analysis of *c*AATrp generation versus acac-Trp-SCoA consumption in the presence of CpaS\_TR\* and Sfp. As shown in Figure 4C (entries 3 and 4), the rate of *c*AATrp generation correlates closely with the consumption of acac-Trp-SCoA, indicating the priming of the carrier protein/thiolation domain of CpaS\_TR\* with Sfp is likely a rate-limiting step; any generated *N*-acac-L-Trp-S-T intermediate is rapidly acted on by the intramolecular R\* domain to release the *c*AATrp. To validate the requirement for display of the *N*-acac-Trp moiety on the pantetheinyl arm of the T domain, the CH<sub>2</sub>OH side chain of Ser3508 was mutated to an alanine, blocking posttranslational priming. In this CpaS\_TR\*(S3508A) T domain active site mutant, the generation of *c*AATrp dropped to the baseline level (Figure 4C, entry 6), identical to what is observed for the assay using wild-type CpaS\_TR\* without Sfp (Figure 4C, entry 5). In both cases, there is minimal consumption of acac-Trp-SCoA (Figure 4C, entries 1 and 2). These studies thus consolidate the evidence that the CpaS R\* domain is a nicotinamide-independent condensation catalyst that releases the *N*-acac-L-tryptophanyl group tethered to the upstream T domain. In the didomain TR\* construct, where the *N*-acac-Trp-pantetheinyl arm is delivered by Sfp, only single turnovers occur, and the kinetics of interdomain chain transfer and cyclization cannot be readily measured.

**acac-Trp-S-CpaS\_T and CpaS\_R\* Activities in Trans.** Given the above findings that the CpaS\_R\* domain was active in the T\_R\* didomain construct, the question arises why the CpaS R\* domain and the EqiS R\* domain, and by extension the reductase-like domains of other PKS-NRPS hybrid tetramate synthetases, are different from the traditional redox-active SDR enzyme family members. Our approach was to make mutants in the R\* domain of CpaS and analyze effects on activity. We have noted above the major obstacle that when the T and R\* domains are *in cis* within the same protein, as they are in native CpaS, and the substrate is introduced onto the apo form of the T domain by the phosphopantetheinyltransferase Sfp, only single turnovers can occur. Further, since the apo/holo levels of T domains in T-R\* didomains varied from preparation to preparation for both wild-type and mutant forms of CpaS fragments, quantitation of differences in substoichiometric yields of product was not viable. Another limitation to kinetics is that the R\* domain would not accept soluble thioester substrates (*N*-acac-Trp-SNAC or -SCoA). To overcome this limitation, we sought to excise CpaS\_T and R\* as separate, folded domains and establish a CpaS\_T/CpaS\_R\* assay *in trans*.

To this end, an N-His-tagged CpaS\_T and a C-His-tagged CpaS\*\_R were generated (Figure 3B) and used for assays. Now the acac-Trp-pantetheinyl-primed CpaS\_T domain could be used as substrate in excess over the CpaS\_R\* domain as catalyst, and multiple turnovers were assessed between the *in trans* pair of domains (Figure 5A). CpaS\_R\* did mediate release of *c*AATrp. Using the same HPLC conditions as for CpaS\_TR\*, the *in trans* generation of *c*AATrp and the consumption of acac-Trp-S-CoA were monitored over a 90 min time course (Figure 5B). While the incorporation rate of acac-Trp-S-pantetheinyl from acac-Trp-SCoA into CpaS\_T appeared similar with or without the presence of CpaS\_R\* (2.5 mol %) (Figure 5B, entries 1 and 3), the generation of *c*AATrp from acac-Trp-SCoA-primed CpaS\_T

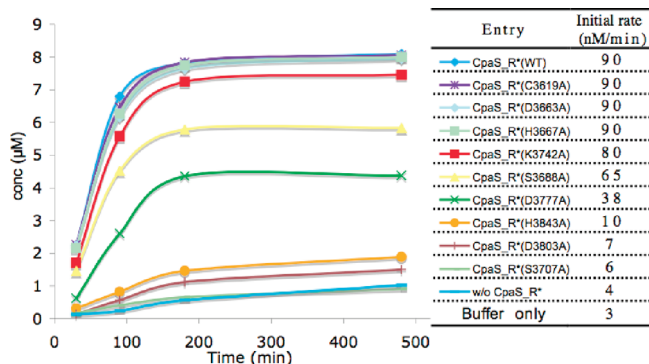


FIGURE 7: Time course and rate comparison of *c*AATrp generation from *N*-acac-Trp-S-T<sub>CpaS</sub> with CpaS\_R\* (WT and mutants) *in trans*.

was clearly accelerated (Figure 5B, entries 2 and 4). The relatively low turnover rate ( $0.03 \pm 0.004 \text{ min}^{-1}$ ) is likely due to the disrupted CpaS\_T and CpaS\_R\* interaction (now *in trans* rather than in the native *in cis* array) and constitutes three turnovers in the 90 min assay. This gave a 30-fold signal-to-noise ratio which allowed us to proceed with initial evaluation of mutants in the R\* domain.

**Identification of Residues of the CpaS R\* Domain Related to Its Unusual Nonredox Dieckmann Cyclase Activity.** To identify residues within CpaS\_R\* that contribute to its unusual redox-independent cyclase activity, a sequence comparison of the C-terminal R\* domains of known fungal tetramate synthetases were mapped onto two comparator lists. One involved selected NRPS C-terminal reductase domains that are known to be *bona fide* redox-active, enzyme domains including those involved in myxochelin (26, 27), lyngbyatoxin (28), myxalamid (29), safracin (30), lysine (yeast) (31), and mycobacterial glycopeptidolipid biosynthesis (32). The second comparator set was three well-characterized SDR enzymes, an aldehyde reductase (33), UDP-*N*-acetylglucosamine 4-epimerase (34), and ADP-L-glycero-mannoheptose 6-epimerase (35). Multiple sequence alignments using ClustalW2 (Figure 6) showed the presence of the well-conserved nucleotide binding domain GXX-(G/S)XXG within all 17 sequences. However, the SDR catalytic triad Ser-Tyr-Lys (24) is not well conserved in the R\* domains. Notably, Tyr → Phe, Tyr → Leu mutations are present in the R\* domains of tenellin, aspyridone, and cyclopiazonic acid synthetases. The critical roles of tyrosine residues in the SDR Ser-Tyr-Lys catalytic triad have been examined and discussed in many cases, where the mutation Tyr → Phe would abolish the reductase activities (23–25).

Further detailed examination of the aligned sequences highlighted seven other polar amino acid residues (C3619, D3663, H3667, S3688, D3772, D3803, H3843) that are exclusively conserved within all R\* domains of known fungal tetramate synthetases but not for redox-active NRPS R domains or the selected SDRs. We therefore chose these residues, along with S3707 and K3742 that are present in the Ser-Tyr-Lys triad, to carry out focused mutagenesis studies. CpaS\_R\* carrying the alanine mutations of these targeted nine polar residues were generated, and the single mutant proteins were overproduced as for the wild type. Most of the CpaS\_R\* mutant proteins were obtained in good yields (5–15 mg/L), comparable to the wild type, except for CpaS\_R\*(S3707A) and CpaS\_R\*(H3843A) that overexpressed poorly in *E. coli* with yields of ca. 0.1 mg/L. All CpaS\_R\* mutant proteins were purified to homogeneity by successive nickel affinity and gel filtration chromatographies.



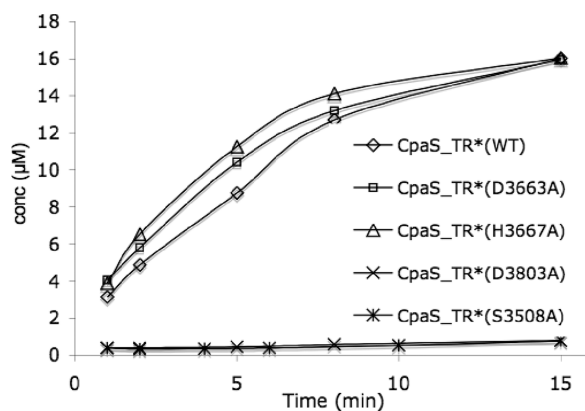


FIGURE 8: Time course comparison of *cAATrp* generation from *acac-Trp-SCoA* primed *CpaS\_TR\** (WT) with selected *CpaS\_TR\** mutants (S3508A, D3663A, H3667A, and D3803A).

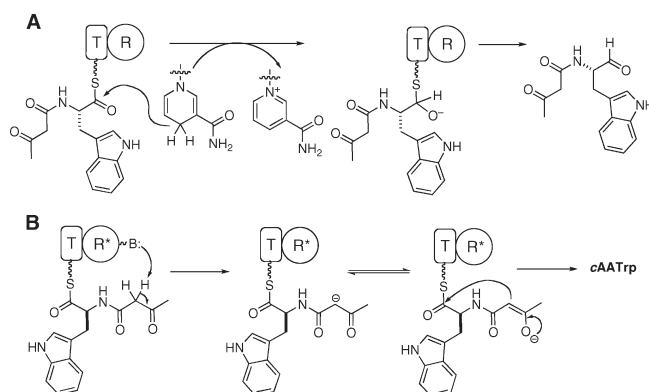
They were subjected to *in trans* assay with *acac-Trp-SCoA*-primed *CpaS\_T*, in a manner identical to that of the wild-type *CpaS\_R\**, and the generation of *cAATrp* was monitored by HPLC over a time course of 6 h. The results are summarized in Figure 7 and clearly indicate the activities of three *CpaS\_R* mutants, H3843A, D3803A, and S3707A, dropped dramatically to the background level, close to the background rate of non-enzymatic generation of *cAATrp* by *N-acac-Trp-CpaS\_T* alone or by buffer (pH 7.0) from *acac-Trp-S-CoA*.

We further attempted to correlate the observed *CpaS\_R\** mutant activities *in trans* to *N-acac-Trp-CpaS\_T* to the native context *in cis* by generating three corresponding *CpaS\_TR\** didomain mutants and assaying *cAATrp* release. One mutant, D3803A, had markedly diminished activity to generate *cAATrp* from *N-acac-Trp-CpaS\_T* *in trans*, while two mutants, H3667A and D3663A, had activity comparable to that of the wild type and could serve as positive controls. The three *TR\** didomain mutant proteins were overproduced and purified, and activities were compared to both the wild type and the anticipated catalytically dead mutant (S3508A) carrying the Ser-Ala mutation in the *CpaS\_T* active site. As observed in Figure 8, *CpaS\_TR\** (H3667A and D3663A) still had comparable activities as the wild type; however, *CpaS\_TR* (D3803A) was as inactive for *cAATrp* generation as that of *CpaS\_TR* (S3508A). This result further validated that the D3803 residue in the *R\** domain of *CpaS* is catalytically consequential.

## DISCUSSION

Pyrrolidinedione ring systems are found in a variety of polyketide and nonribosomal natural products and are pharmacophoric elements in medicinally active members of these natural product classes. The 2,5-pyrrolidinedione ring system is a succinamide ring, and this regioisomer is found in andrimid and moiramide (36–38), but the 2,4-pyrrolidinedione, known commonly as the tetramate ring system, is more widespread (1). The tetramate ring can be a terminal structural element as in the anti-HIV molecule equisetin and the related trichosetin or in the antitumor peptide dolastatin 15, or it can be an embedded ring system as found in the antitumor metabolite cylindramide and in the fused pentacyclic scaffold of  $\alpha$ -cyclopiasonate (for structures, see Figure 1A). The nitrogen atoms of the tetramate scaffolds arise from an amino acid monomer that is incorporated as part of the chain termination process, and the tetramate side chain at C<sub>5</sub> derives from the side chain of that amino acid building block. Most of the naturally occurring tetramate rings have a polyke-

Scheme 2: Comparison of the NRPS C-Terminal R vs *R\** Domain Using *CpaS* as an Example<sup>a</sup>



<sup>a</sup>(A) Genuine *R* domain generates aldehyde product via hydride transfer from NAD(P)H cofactor and a thioacetal intermediate. (B) Redox-incompetent *R\** domain subtracts C2 hydrogen of  $\beta$ -ketoamide to form a resonance-stabilized carbanion intermediate that undergoes intermolecular Claisen condensation to give *cAATrp*.

tide-derived substituent at C<sub>3</sub>, reflecting the hybrid PKS-NRPS assembly lines that generate them. The 2,5-dicarbonyl groups offer H-bond interactions with protein targets, as validated by the X-ray structures of streptolydigin complexed with RNA polymerase (39) and of  $\alpha$ -cyclopiasonate with sarcoplasmic reticulum Ca<sup>2+</sup>-ATPase (40, 41).

Because of the combined structural constraints and hydrogen-bonding potential that 2,4-pyrrolidinedione rings impart to the hybrid PK-NRP natural products, there is of interest to decipher the molecular logic by which these ring systems are installed during biosynthesis. Fungal genomics and bioinformatic analyses for the biosynthetic assembly lines for the hybrid PK-NRP molecules show one or more PKS modules with an NRPS module that acts as the most downstream, chain-terminating module. It is therefore likely that tetramate ring formation is the last step in all such PKS-NRPS hybrid assembly lines, raising the question of how cyclization is coupled to disconnection of the full-length acyl/peptidyl chain from the enzymatic assembly lines.

The cyclopiasonate biosynthetic pathway in the *Aspergillus* strains has the virtue of a short three-enzyme pathway. The first enzyme is the single two-module protein, *CpaS*, with a PKS module upstream of the NRPS module. The single PKS module converts an acetyl-CoA and a malonyl-CoA to an acetoacetyl-S-pantetheinyl intermediate (Scheme 1). The NRPS module selects and activates Trp, installing it on its thiolation domain. Action by the NRPS condensation domain makes the amide linkage and presumably generates the *N*-acetoacetyl-Trp-S-pantetheinyl intermediate in the NRPS module (Scheme 1) that is the last tethered intermediate. Action of the *R\** domain converts the *acac-Trp-S-pantetheinyl-T* into *cyclo-acetoacetyl-Trp* product. The indole side chain of Trp becomes the C<sub>5</sub> substituent of the released tetramate ring, and the acetoacetyl moiety has functioned as an intramolecular nucleophile, leaving an acetyl group as the C<sub>3</sub> substituent, which exists predominantly in the enol form. The next two enzymes in the pathway *CpaD* and *CpaO* are post-assembly line tailoring catalysts, with *CpaD* effecting a regio-specific prenylation at the indole C<sub>4</sub> position of *cAATrp* to yield  $\beta$ -CPA (Scheme 1). *CpaO* then carries out the deceptively simple dehydrogenation of  $\beta$ -CPA to yield an unstable enimine

product, which is captured by intramolecular cyclization to create the pentacyclic fused scaffold of  $\alpha$ -cyclopiasonate (19). In this double cyclization step the nitrogen of the tetramate moiety can be viewed as the initiating nucleophile for the cascade.

Inspection of the NRPS tetramate-forming modules shows that the typical four-domain termination module (C-A-T-TE) is replaced with a C-A-T-R\* module, in which the terminal thioesterase domain is replaced with a domain of the short-chain reductase/dehydrogenase superfamily. The thioesterase (TE) domains of NRPS termination modules have been shown to be the catalysts for hydrolytic and/or macrolactamizing/macrolactonizing release of full-length peptidyl chains from the covalent thioester linkage to the pantetheinyl arm of the neighboring T (thiolation) domain. It therefore followed that replacement of a TE domain by an R domain in a module would indicate reductive release by delivery of a hydride from NAD(P)H to the thioester to yield the tethered thiohemiacetal which unravels nonenzymatically to the free aldehyde and the HS-pantetheinyl arm on the T domain (10–12). Indeed, in earlier efforts we proved this NAD(P)H-dependent reductive release mechanism by purified yeast Lys5 in conversion of aminoadipate to the cyclic aldimine in lysine biosynthesis (31). We have also described a tandem reduction of *N*-Me-Val-Trp-S-LtxA by two molecules of NADPH to yield first the dipeptidyl aldehyde and then the four-electron-reduced alcohol in the lyngbyatoxin biosynthetic pathway (28).

While a reduction/reoxidation pathway is conceivable for action of terminal R\* domains in tetramate assembly (2–7), studies on the T and R\* domains from the fungal equisetin synthetase showed conclusively that the EqsS\_R\* domain was redox incompetent (14). Instead, it acted on soluble *N*-acac-L-Ala-SNAC substrate mimics to produce the corresponding tetramate by an intramolecular Dieckmann cyclization.

In this study we made a similar T\_R\* didomain construct excised from the *A. flavus* CpaS bimodular enzyme and confirmed that this R\* did not carry out *reductive* release of the *N*-acac-Trp thioester intermediate. Unlike EqsS\_R\*, the CpaS\_R\* domain did not accept low molecular weight *N*-acac-Trp thioester mimics (*N*-acac-Trp-SNAC and -SCoA) but only recognized the authentic *N*-acac-Trp thioester intermediate present natively on the pantetheinyl prosthetic group of the upstream thiolation domain. The tethered *N*-acac-Trp thioester intermediate was generated using the methodology we have discovered and developed in previous NRPS assembly line studies (42–44), by combining the partially apo form of the CpaS\_TR\* construct heterologously overproduced in *E. coli*, synthetic acac-Trp-S-CoA, and the promiscuous phosphopantetheinyltransferase Sfp. The acac-Trp chain was thus installed regiospecifically on the T domain of CpaS\_TR\* and then rapidly acted on *in situ* by the R\* domain to release authentic cAATrp.

We likewise formulate this tetramate-forming chain release step as a Dieckmann cyclization with the R\* domain recognizing the *N*-acac-Trp-S-pantetheinyl arm for capture of the thioester carbonyl by the C<sub>2</sub> carbanion of the acac moiety (Scheme 2B). As in the equisetin case, this transformation was NAD(P)H independent (14). While it is on the one hand satisfying to study the native tethered acac-Trp-S-pantetheinyl thioester as the Dieckmann cyclization substrate for the action of the adjacent downstream R\* in the purified CpaS T-R\* didomain, it is very difficult to study kinetics and mechanism when the substrate (*N*-acac-Trp-S-T domain) and enzyme (R\* domain) are present in stoichiometric amounts and coupled intramolecularly. To that

end we were able to overexpress, purify, and assay separated CpaS T and R\* domain protein fragments, allowing initiation of kinetic studies with substrate concentrations in large excess over enzyme. While rates were sluggish, they sufficed for some initial examinations of the residues of R\* domains that are different from canonical R domains.

Given that both EqsS\_R\* and CpaS\_R\* domains are not redox active but still are members of the nicotinamide-dependent short-chain reductase (SDR) superfamily, we assume that R\* domains may have evolved from R domains that were functional redox catalysts as in Lys5 and LtxA and other *bona fide* NRPS R domain reductases. The proposed default pathway of redox-dead R\* domains on *N*-acylaminoacyl-S-pantetheinyl-T domain substrates to be a Dieckmann cyclization/chain release requires the *N*-acyl substituents to be 3-ketoacyl groups. These can yield the resonance-stabilized C<sub>2</sub> carbanions, from dissociation of an acidic C<sub>2</sub>-H, as kinetically accessible carbon nucleophiles (Scheme 2B).

We looked for clues as to how R domains might evolve/decay to R\* domains by sequence comparisons, first around the conserved R domain catalytic triad of Ser-Tyr-Lys and then for residues conserved in R but not R\*, or vice versa. In canonical SDR enzymes, such as androsterone 17-keto reductase, mutation of either the active site Ser to Ala or Tyr to Phe residue completely abolishes measurable activity (25). This catalytic Tyr is indeed mutated to Leu in the CpaS\_R\* domain and to Phe in the corresponding tenellin synthetase R\*. However, this cannot be the only mechanism for loss of redox activity in R\* domains since other tetramate synthetases, including the equisetin R\*, maintain the catalytic triad. As noted in the results and Figure 8, we then mutagenized a series of polar/charged residues that are conserved in the R\* domains but not in the known redox-active R domains. One aspartate residue, D3803, when mutated to Ala brought Dieckmann condensation levels back to baseline. It remains to be seen if this result is generalizable across R\* domains and will probably require an X-ray structure with bound substrates or substrate surrogates to determine the role of this aspartate carboxylate side chain; for example, it could function as a base to generate the C<sub>2</sub> carbanion of the acac moiety of tethered acac-Trp substrate to initiate the tetramate ring cyclization. Also, we will need to achieve heterologous expression and isolation of the full-length two-module eight-domain 3906 residue (431 kDa) CpaS to study the mechanism of chain release in the intact PKS-NRPS assembly line *in vitro* and any residual role of NAD(P)H in modulating the nonredox tetramate ring formation pathways; it is not yet clear if this will be achievable in bacterial or fungal systems (45).

One distinction between NRPS chain termination modules with composition C-A-T-TE vs C-A-T-R/R\* involves the distinction between thioester chemistry and oxoester chemistry in the release step. For all modules with a thioesterase domain, the full-length NRP or PK-NRP chain is transferred covalently from the pantetheinyl arm of the most downstream T domain to the CH<sub>2</sub>OH side chain of the active site Ser of the TE, prior to product release by hydrolysis or macrocyclization. Thus, the assembly line switches from thioester chemistry to oxoester chemistry at the chain dismount step in the TE active site. In contrast, it is more likely that the R domains do not transfer the acyl/peptidyl chains from those pantetheinyl thiol tethers but deliver a hydride ion from NAD(P)H to yield the thiohemiacetal still tethered to the pantetheinyl arm of the immediately upstream T domain. The hemithioacetal unravels to the released peptide

aldehyde and regenerates the HS-pantetheinyl arm on the T domain so the assembly line can run through another cycle (Scheme 2A).

As the R domains may act on thioester not oxoester substrates, the thioester linkage may be consequential for the R\* domains to effect tetramate ring cyclization. As a nonredox default pathway the R\* domain performs a Dieckmann reaction on thioester not oxoester substrate. The R\* domains may not need to be very efficient since background cyclization of acac-aminoacyl thioesters can proceed nonenzymatically because the S-pantetheinyl arm is a good leaving group. This may be of relevance in consideration of how  $\alpha$ -lipomycin (8) and kirromycin (46) assembly lines form tetramate and pyridone rings in their chain release steps, as those NRPS modules have only C-A-T domains: they lack both TE and R or R\* release catalytic domains. Finally, it is likely that nature's strategies for disconnection of assembled covalent chains by Dieckmann cyclization routes include additional variants to explain tetramate ring formation in the fungal signaling molecule HSAF and cyclindramide where the tetramate lies between polyketide regions (9).

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