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Functional expression of the *Aspergillus flavus* PKS–NRPS hybrid CpaA involved in the biosynthesis of cyclopiazonic acid

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ABSTRACT

α-Cyclopiazonic acid (CPA) is an indole tetramic acid mycotoxin. Based on our identification of the polyketide synthase–nonribosomal peptide synthase (PKS–NRPS) hybrid gene *cpaA* involved in cyclopiazonic acid biosynthesis in *Aspergillus* fungi, we carried out heterologous expression of *Aspergillus flavus cpaA* under α-amylase promoter in *Aspergillus oryzae* and identified its sole product to be the CPA biosynthetic intermediate *cyclo*-acetoacetyl-L-tryptophan (cAATrp). This result rationalized that the PKS–NRPS hybrid enzyme CpaA catalyzes condensation of the diketide acetoacetyl-ACP formed by the PKS module and L-Trp activated by the NRPS module. This CpaA expression system provides us an ideal platform for PKS–NRPS functional analysis, such as adenylation domain selectivity and product releasing mechanism.

α-Cyclopiazonic acid (**1**, CPA) is an indole tetramic acid that was first isolated from *Penicillium cyclopium* as a toxic metabolite in 1968.[1] CPA (**1**) inhibits sarcoplasmic reticulum Ca^{2+}-ATPases,[2] and it is known that inhibition of Ca^{2+}-ATPases results in cell death through apoptotic pathways within the endoplasmic reticulum and mitochondria.[3] In addition to *Penicillium* species several other fungi including the *Aspergillus* species have been reported to produce CPA (**1**),[4,5] whose contamination of agricultural products such as maize, corn and peanut, is a serious economic and health problem.[6–8] It is also noteworthy that some strains of *Aspergillus oryzae* that are used in fermentation industry are capable of producing CPA (**1**).[9]

Feeding experiments with labeled tryptophan, mevalonic acid and acetate in *P. cyclopium* suggested that CPA (**1**) could be biosynthesized from these precursors via the formation of *cyclo*-acetoacetyl-L-tryptophan (**2**, cAATrp) and then β-cyclopiazonic acid (**3**, β-CPA).[10] In fact, the accumulation of cAATrp (**2**)[11] and detection of dimethylallyl diphosphate: cAATrp dimethylallyltransferase activity[12] confirmed the biosynthetic pathway scheme. β-CPA (**3**) is further converted to CPA (**1**) by oxidation and cyclization catalyzed by β-CPA oxidocyclase, which was purified and characterized.[13] (Fig. 1). Since cAATrp (**2**) was considered to be the first stable intermediate in CPA biosynthesis starting from Trp, its tetramic acid moiety could be formed from diketide acetoacetate and Trp by a polyketide synthase–nonribosomal peptide synthase (PKS–NRPS) hybrid enzyme.

Recently, the identification and cloning of a putative dimethylallyl cycloacetoacetyl trypophan synthase (DCAT-S) gene was reported from *A. oryzae* IFO0417, a CPA producing strain. (US Patent 6828137) Its gene disruptant appeared CPA non-producing. Furthermore, we identified PKS–NRPS gene in *A. oryzae* RIB40, NBRC 4177 and its close relative *Aspergillus flavus* NRRL 3357. *A. oryzae* RIB40 is CPA non-producing and its PKS–NRPS gene possesses only the sequence encoding the β-ketoacyl synthase (KS) and acyltransferase (AT) domains of the PKS–NRPS in NBRC 4177 which is capable to produce CPA (**1**). Its gene disruption made NBRC 4177 to be CPA non-producing. Thus, we identified the PKS–NRPS gene, named *cpaA*, in *A. oryzae* and *A. flavus*.[14] Although the product of PKS–NRPS hybrid enzyme CpaA was assumed to be cAATrp (**2**), no direct confirmation has been carried out. Since *A. flavus* genome sequence was available before we carried out *A. oryzae cpaA* sequencing and its gene disruption, we first set

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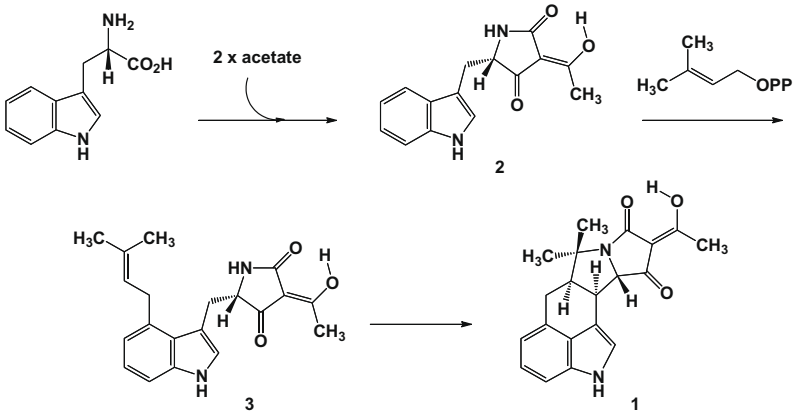


Figure 1. Biosynthesis of α-cyclopiazonic acid.

out the heterologous expression of *A. flavus* CpaA PKS–NRPS in *A. oryzae*. Later, it was confirmed that CpaAs from *A. flavus* NRRL 3357 and *A. oryzae* NBRC 4177 are quite identical with each other with 92% amino acid sequence identity.

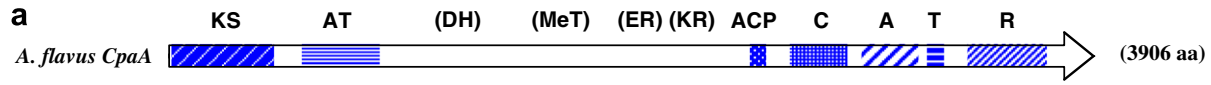
The *cpaA* gene from *A. flavus* NRRL 3357 (Accession No. AB493824) is 11,721bp-long and encodes a PKS–NRPS hybrid enzyme of 3906 amino acids with a calculated molecular mass of 431 kDa. Dot matrix comparison of the PKS module in CpaA N-terminal half with LDKS which is a diketide synthase involved in lovastatin biosynthesis[15] indicated that they are similar in amino acid lengths and show fairly high homology from the N-terminus to the acyl carrier protein (ACP) domain. Detailed comparison showed that CpaA PKS module possesses, in addition to KS, AT, and ACP domains, C-methyltransferase (MeT), dehydratase (DH), enoyl reductase (ER) and ketoreductase (KR) domain regions as LDKS does though these domains seem to be non-functional (Fig. 2). While the C-terminus half of CpaA contains a NRPS module with condensation (C), adenylation (A), thiolation (T or peptidyl carrier protein (PCP)) and putative releasing (R) domains. In order to verify whether CpaA is responsible for the formation of cAATrp (**2**) or any other biosynthetic intermediate, we carried out the heterologous expression of CpaA PKS–NRPS.

The *cpaA* gene was PCR-amplified from the genomic DNA of *A. flavus* NRRL 3357 strain with the specific forward primer (TCAAA ATTACCCCACGGCCATG) and reverse primer (TGACGATGGTTAAT TACCCAAGTAG) designed according to the genome database (http://www.aspergillusflavus.org/genomics/). The start and stop codon are underlined. The PCR amplification was carried out using Phusion HF DNA polymerase (New England Biolabs). The amplified product was cloned into the *Sma* I digested expression vector pTAex3 that contained the *amyB* promoter and terminator for inducible gene expression.[16] The expression plasmid pTA-cpaA thus constructed was transformed into *A. oryzae* M-2-3[17] by protoplast-PEG method.[18] The *A. oryzae* M-2-3 transformant with pTAex3 served as a control. Although it has not been confirmed whether *A. oryzae* M-2-3 possesses the intact *cpaA* ortholog gene, cyclopiazonic acid-related metabolites including cAATrp (**2**) have not been detected in our inductive PKS expression experiments using *A. oryzae* M-2-3 as a host fungus. Transformants selected on minimal medium were first cultured in DPY medium[18] and then transferred to the media containing maltose to induce the expression of *cpaA*. After culturing in the induction media for 3 days, ethyl acetate extract of the culture media under acidic condition was subjected to HPLC analyses on Shimadzu Prominence HPLC system (Shimadzu, Japan) with an Inertsil ODS-SP reverse-phase column (2.1 × 100 mm, GL Science, Japan). In the pTA-cpaA transformant extract, a single major peak with the absorption maxima at 217 nm and 275 nm was detected at retention time of 7.8 min, which was completely absent in the extract of control transformant (Fig. 3).

Since this compound was considered to be a specific product synthesized due to the overexpression of *cpaA*, isolation of the compound was carried out as follows. The pTA-cpaA transformant was initially cultured in DPY medium at 30 °C for 2 days with shaking, and then in induction medium at 30 °C for 3 days with shaking at 160 rpm. After removal of mycelia by filtration, combined induction culture media were applied onto a reverse phase silica gel column (Cosmosil 75C_{18}-OPN, Nakalai Tesque, Japan). Elution was carried out with 50% aq methanol containing 0.1% formic acid. The CpaA product was then applied onto an Inertsil ODS-3 JET HPLC column (10 × 50 mm, GL Science, Japan), and eluted with 50% aq acetonitrile containing 0.1% formic acid at a flow rate of 1.0 ml/min. From 1600 ml induction culture medium, 4.7 mg of the CpaA product was isolated, which was subjected to physicochemical analysis.

The molecular formula of the compound was determined to be $C_{15}H_{14}N_2O_3$ (*m/z* 271.1083 $[M+H]^+$, calculated for $C_{15}H_{15}N_2O_3$, 271.1083) by LC–ESI-TOFMS analysis (LC–MS-IT-TOF mass spectrometer, Shimadzu, Japan). Its chemical structure was identified to be cAATrp (**2**) by ^1H and ^{13}C NMR, and hetero-nuclear multiple-bond correlations (HMBC). The presence of two sets of ^{13}C signals for C-5, C-6 and C-7, respectively, indicated that the tetramic acid moiety of cAATrp (**2**) exits as two external enolic tautomer pairs in non-polar solvent.[19] NMR data are shown in Supplementary data. Identification of cAATrp (**2**) as the product of CpaA PKS–NRPS hybrid rationalized the reactions catalyzed by CpaA as shown in Figure 4. Essentially, the diketide acetoacetyl-ACP formed from condensation of the acetyl-CoA and malonyl-CoA by the PKS module, and L-Trp activated by A domain of the NRPS module is loaded on the PCP in T domain, and then condensation is catalyzed by C domain. Thus the formed intermediate is released from T domain catalyzed by R domain with the formation of tetramic acid moiety.

Presence of PKS–NRPS hybrid in fungi was first reported in *Fusarium moniliforme* and *Fusarium venenatum* involved in fusarin C biosynthesis.[20] Since then, fungal tetramic acids and derivatives, including tenellin,[21] pseurotin A,[22] and chaetoglobosin A,[23] have been revealed to be biosynthesized by PKS–NRPSs. Their deduced enzyme architecture is quite similar with each other including CpaA. Our fungal PKS expression system under inducible α-amylase promoter in *A. oryzae* was recently applied to fungal PKS–NRPS functional analysis, *Beuveria bassiana* TenS expression, by Cox and co-workers.[24] Considering the relatively simple reactions involved, and specific and high production of cAATrp (**2**) by CpaA, our pres-



b

DH domain

```
                 *   *     *
 LDKS     WLRDHVVGSHIVFPGA
 PKSN     WIREHKVQGDILYPGA
 FUM1     WLRDHQVLNDVVFPCA
 EqiS     WMQGHKLQGQIIFPAT
 LNKS     WLDGHALQGQTVFPAA
(CpaA)    WAEGYKEDGRVVLSAA
```

MeT domain SAM binding

```
                 *  *   *
 LDKS     ARILEIGGGTGGCTQLVVDSL
 PKSN     LRILEIGAGTGGTTYHVLERL
 FUM1     LRVLEIGAGTGGGAQVILEGL
 EqiS     MKILEIGAGTGGTTQATLPSL
 LNKS     MDILEIGAGTGGATKYVLATP
(CpaA)    MNVLELDAGTSVVTHQILEVV
```

ER domain NADPH binding

```
                   *  *   *
 LDKS     GETVLIHAGAGGVGQAAIILAQ
 PKSN     GEKVLIHAAAGGVGQAAIMIAQ
 FUM1     GQSILIHSACGGIGIAALNLCR
(EqiS)    GRMALVTTAAHLVADNLVHNIP
(LNKS)    AETVISTAKCLGVTDSILVLNP
(CpaA)    SGPILLYEPDELLAAAVEQARE
```

KR domain NADPH binding

```
                   *  *   *
 LDKS     VSYLVAGGLGGIGRRICEWLVDRGARYLIILSRTAR
 PKSN     ATYMIA-GLGGITREIARWLAEKGARYLVFLSRSAA
 FUM1     ASYLLVGGLGGLGRAAATWMVESGARYLIFFSRSAG
 EqiS     RTYLLIGLSGEVGQSICQWMVSHGARHVVLTSRKPG
 LNKS     KTYLLVGLTGDLGRSLGRWMVQHGACHIVLTSRNPQ
(CpaA)    GTYWMIDMATPLGLSILKWMATNGARTFVLAGRNPR
```

Figure 2. Domain architecture of CpaA PKS–NRPS and its non-functional PKS domains. (a) *A. flavus* CpaA architecture KS: β-ketoacyl synthase, AT: acyltransferase, DH: dehydratase, MeT: C-methyltransferase, ER: enoylreductase, KR: β-ketoreductase, ACP: acyl carrier protein, C: condensation domain, A: adenylation domain, T: thiolation domain, R: releasing domain. (b) Comparison of putative non-functional PKS domains of CpaA with fungal PKSs; *: conserved amino acid. Non-functional domains due to apparent lacks of conserved amino acids are shown in parentheses. LDKS (Accession number: AAD34559), PKSN (BAD83684), FUM1 (AAD43562), LNKS (AAD39830), EqiS (AAV66106).

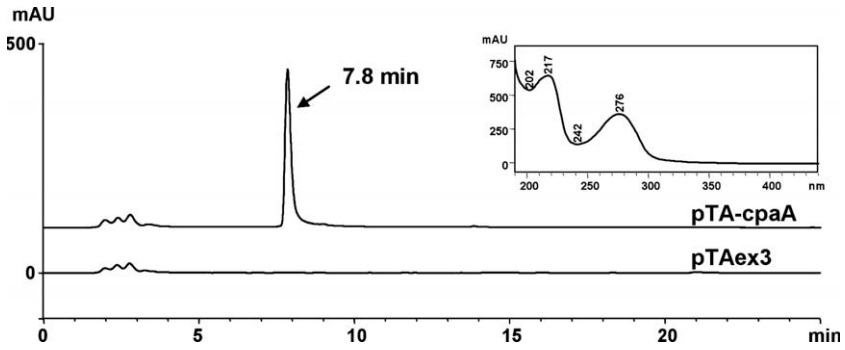


Figure 3. HPLC analysis of *Aspergillus flavus* CpaA product expressed in *Aspergillus oryzae*. HPLC was run with a solvent system of acetonitrile containing 0.1% formic acid (solvent B) and H_2O containing 0.1% formic acid (solvent A) at flow rate 0.2 ml/min (25% solvent B for 10 min, 25–100% B for 10 min, then 100% B for 10 min) at 40 °C. UV absorbance was monitored at 280 nm.

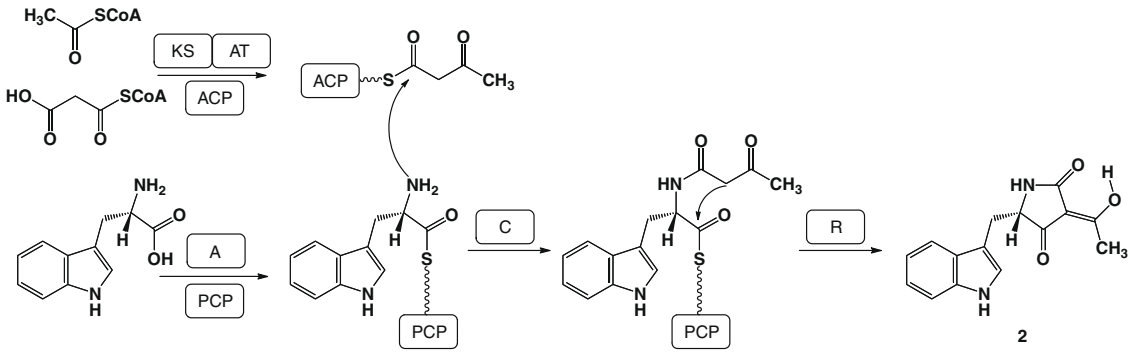


Figure 4. Reactions catalyzed by CpaA to form *cyclo*-acetoacetyl-L-tryptophan (cAATrp) from acetyl-CoA, malonyl-CoA, and L-tryptophan.

Table 1
Fungal PKS–NRPS signature residues for amino acid selection by A domains

Sequence	Amino acid	Signature residue position									
		235	**236**	**239**	**278**	**299**	**301**	**322**	**330**	**331**	**517**
GrsA A1	Phe	D	A	W	T	I	A	A	I	C	K
CpaA	Trp	D	M	A	L	C	G	S	A	C	K
CheA	Trp	D	M	I	I	C	G	C	A	A	K
TenS	Tyr	D	M	V	I	C	G	C	A	A	K
ApdA	Tyr	D	M	V	I	C	G	C	A	A	K
PsoA	Phe	D	A	Y	T	M	A	A	I	C	K
FUSS	Hse	D	M	T	F	S	A	G	I	I	K
EqiS	Ser	D	F	E	S	F	G	N	I	A	–

–: Not present. Sequence numbering from gramicidin S synthase (GrsA) A domain 1.[29] NRPSpredictor was used to assign signature residues.[28] GrsA (Accession number: P0C061); CpaA (AB493824); CheA (CAO91861); TenS (CAL69597); ApdA (EAA67034); PsoA (ABS87601); FUSS (AAT28740); EqiS (AAV66106).

ent result not only adds to successful example of fungal PKS–NRPS expression, but also provides an ideal platform for PKS–NRPS functional analysis.

Alignment of the CpaA NRPS module with those of TenS[24] clearly indicated the presence of C, A, T, and R domains in this order (Fig. 2). Amino acid selection in NRPS is performed by A domain and bacterial signature sequences for prediction on amino acid selection has been proposed based on structure and sequence analysis that amino acid binding pocket is composed of ten key residues.[25,26] However, understanding fungal A domain selectivity is still in infancy due to relatively little data available on fungal A domains with their known substrates. There have been only four reports on fungal A domains of which aromatic amino acid selectivity was experimentally confirmed or deduced before our CpaA expression. Those are A domains of TenS for Tyr,[24] ApdA for Tyr in aspiridone biosynthesis,[27] and PsoA for Phe in pseurotin A biosynthesis.[22] The A domain of CheA in chaetoglobosin biosynthesis[23] is for Trp along with that of CpaA. NRPSpredictor is a bioinformatic tools available for prediction of A domain selectivity.[28] Although it could not correctly predict these fungal PKS–NRPS A domain selectivity mostly due to limited data so far available, it could align fungal PKS–NRPS A domains with gramicidin PheA[29] and indicate signature residues for amino acid selection by A domains as shown in Table 1. Since a number of PKS–NRPS gene sequence have been

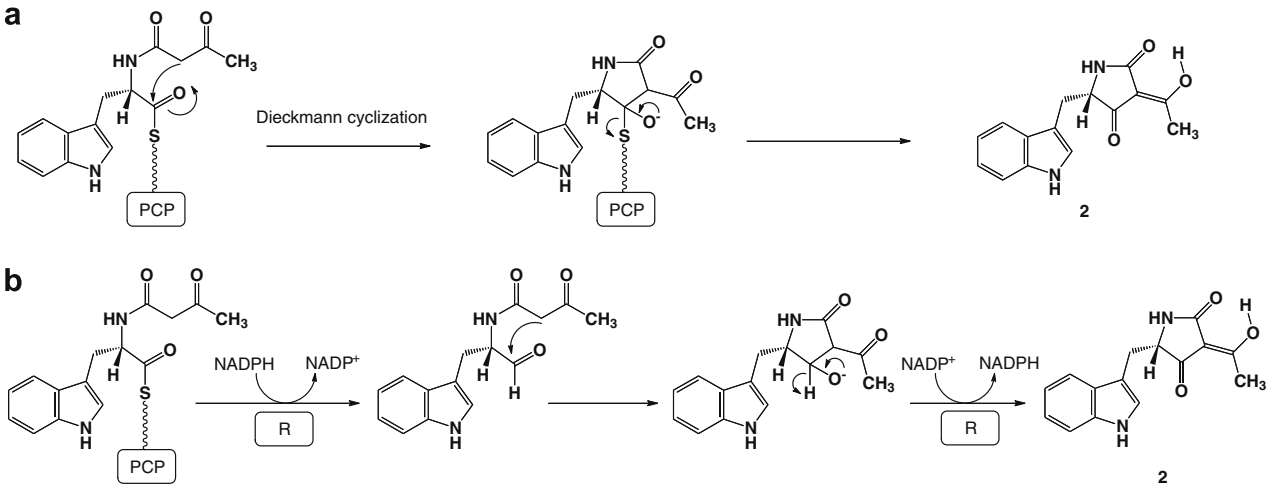


Figure 5. Possible roles of CpaA releasing (R) domain in *cyclo*-acetoacetyl-L-tryptophan (cAATrp) formation (a) Dieckman cyclization/releasing mechanism; (b) reductive releasing/cyclization/reoxidation mechanism.

deposited on database, our fungal expression system will be utilized for their functional analyses including identification of A domain selection mechanism.

The C-terminus of CpaA is a putative releasing (R) domain as are other fungal PKS–NRPSs. In bacteria, some NRPSs terminate in R domains instead of commonly found thioesterase (TE) domains. Function of NRPS R domain was reported to release the mature peptide product reductively with aid of a NAD(P)H cofactor. The first such example was an enzyme α-amino adipate reductase (Lys2) for Lys biosynthesis.[30] The NRPS modules of fungal PKS–NRPS show end-to-end homology to Lys2. Thus, it had been believed that fungal PKS–NRPS R domain could act on acyl amide thioester intermediate to release it as an aldehyde by NAD(P)H aided reduction. However, products of TenS expression in *A. oryzae* were identified to be teramic acid/hydroxypyrolinone intermediates that are considered to be directly formed by TenS PKS–NRPS. From these results, Cox and co-workers proposed that the putative R domain catalyzes a Dieckmann cyclization of the bound *N*-β-ketoacyl α-aminothioester intermediate and releasing the cyclized product.[24] In our experiments of CpaA expression, cAATrp (**2**) with tetramic acid structure was identified to be a PKS–NRPS product as was reported for TenS expression. In NRPSs, it was reported that peptide aldehydes held in the R domain active site undergo second round of reduction[31] or intramolecular cyclization.[32] Read and Walsh reported the reductive peptide aldehyde release and reoxidation to peptide carboxylic acid by transfer of a hydride back to the enzyme-bound $NADP^+$ in in vitro analysis.[31] Recently, Sims and Schmidt carried out in vitro functional analysis of equisetin synthase (EquiS) R domain to determine the enzymatic basis on tetramic acid formation. Their results using synthetic substrate analogs strongly supported that EqiS R domain catalyzes a Dieckmann condensation.[33] This mechanism will further be verified by functional analysis of CpaA R domain using the expression system here we reported (Fig. 5).

In summary, we succeeded in the expression of the PKS–NRPS hybrid CpaA from *A. flavus* in *A. oryzae* to produce the CPA biosynthetic intermediate cAATrp (**2**) as a specific PKS–NRPS product.

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Supplementary data

Supplementary data (NMR data of CpaA product *cyclo*-acetoacetyl-L-tryptophan (cAATrp)) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.04.073.

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