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Physically Discrete β-Lactamase-Type Thioesterase Catalyzes Product Release in Atrochrysone Synthesis by Iterative Type I Polyketide Synthase

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SUMMARY

ATEG_08451 in *Aspergillus terreus*, here named atrochrysone carboxylic acid synthase (ACAS), is a nonreducing, iterative type I polyketide synthase that contains no thioesterase domain. In vitro, reactions of ACAS with malonyl-CoA yielded a polyketide intermediate, probably attached to its acyl carrier protein (ACP). The addition of ATEG_08450, here named atrochrysone carboxyl ACP thioesterase (ACTE), to the reaction resulted in the release of products derived from atrochrysone carboxylic acid, such as atrochrysone and endocrocin. ACTE, belonging to the β-lactamase superfamily, thus appears to be a novel type of thioesterase responsible for product release in polyketide biosynthesis. These findings show that ACAS synthesizes the scaffold of atrochrysone carboxylic acid from malonyl-CoA, and that ACTE hydrolyzes the thioester bond between the ACP of ACAS and the intermediate to release atrochrysone carboxylic acid as the reaction product.

INTRODUCTION

Various fungal polyketides, including aflatoxin B_1 (Minto and Townsend, 1997), melanin, tetrahydroxynaphthalene (Takano et al., 1995; Tsai et al., 1998), and lovastatin (Kennedy et al., 1999), illustrate the diversity of chemical structures and the diverse biosynthetic potential of fungi. These polyketides are synthesized by iterative type I PKSs, which have a single modular architecture with individual functional domains and catalyze a series of polyketide-forming reactions iteratively in a similar way to mammalian fatty acid synthases (FASs).

The typical reaction catalyzed by a fungal PKS, consisting of a β-ketoacyl synthase (KS), acyltransferase (AT), and acyl carrier protein (ACP) domains, is as follows. The starter unit is transferred to the KS domain, and an extender unit, such as malonyl thioester, is transferred to the ACP by AT domain. Then, the KS domain catalyzes a decarboxylative Claisen condensation between an acyl thioester and an extender unit, resulting in the formation of a new carbon-carbon bond. The decarboxylative Claisen condensation occurs iteratively, catalyzed by a single module, and the polyketide chain is thus extended. Additionally, β-ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) domains are sometimes included in the system to synthesize reduced compounds.

Among the iterative type I PKSs, those consisting of KS, AT, and ACP domains and possessing no reducing domains are classified as nonreducing PKSs (NR-PKSs). In addition to KS, AT, and ACP domains, NR-PKSs have some unique domains, such as a starter unit ACP transacylase (SAT; Crawford et al., 2006), product template (PT; Crawford et al., 2008), and thioesterase (TE) domains (Fujii et al., 2001). A general pattern of domain organization of NR-PKSs is SAT, KS, AT, PT, ACP, and TE domains, from the N- to C-terminal end (Cox, 2007). The TE domain, usually located at the C-terminal end of NR-PKSs, catalyzes the last step to release products from the enzymes by a Claisen cyclization of the polyketide intermediates, leading to C-C bond formation, accompanied by cleavage of the thioester bond between ACP and the polyketide intermediate (Fujii et al., 2001). The TE domains of NR-PKSs contain a conserved Ser, His, Asp catalytic triad, which is also common among the TE domains of bacterial modular type I PKSs (Tsai et al., 2001) and mammalian type I FASs (Chakravarty et al., 2004). The TE domain of WAS in *Aspergillus nidulans* catalyzes a Claisen cyclization of the second ring of a naphthopyrone, YWA (Fujii et al., 2001). Site-directed mutagenesis of the conserved Ser and His of the TE domain of WAS resulted in a switch from synthesis of a naphthopyrone to synthesis of a citreoisocoumarin, a product derived from a lactone. Site-directed mutagenesis abolished the Claisen cyclization catalysis by this TE domain and resulted in lactonization, a *nonenzymatic* C-O bond-forming reaction. Such lactone production is a well-known PKS shunt reaction.

In some NR-PKSs, the TE domain is absent or substituted by other domains. The TE domain of PksCT in *Monascus purpureus* is replaced by methyltransferase and thioester reductase domains (Shimizu et al., 2005). The thioester reductase domain of PksCT appears to be responsible for product release, due to its similarity in amino acid sequence to the reductase domain of nonribosomal peptide synthetases (NRPSs), which catalyzes product release from the NRPSs. Several known NR-PKSs do not contain TE or apparently any other domain responsible for product release. How such TE-less NR-PKSs release their

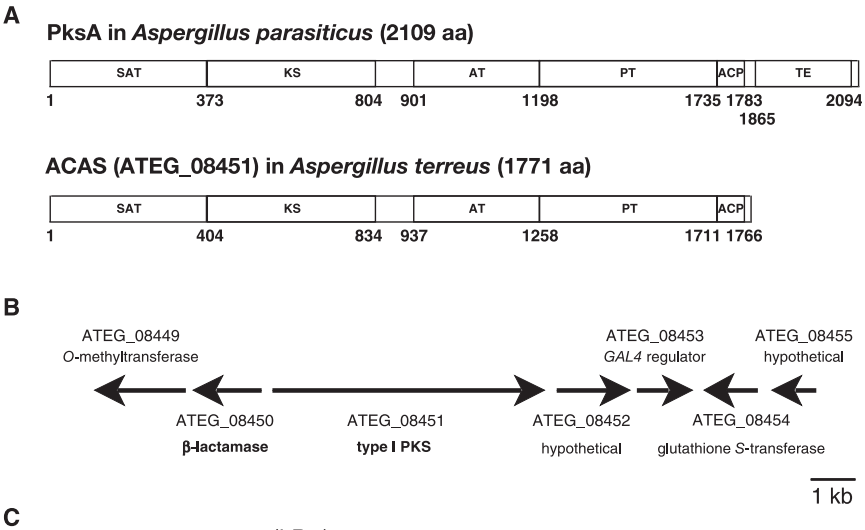

Figure 1. Domain Organization of ACAS (ATEG_08451) and ORFs near ATEG_08451

(A) Domain organizations of PksA in *Aspergillus parasiticus* (AAS66004) and ACAS (ATEG_08451) in *A. terreus*.

(B) ORFs in the vicinity of ATEG_08451.

(C) Coomassie brilliant blue-stained SDS-PAGE analysis of purified ACAS (lane 1) and ACTE (lane 2), together with molecular size markers.

product is not well understood. Recently, Szewczyk et al. (2008) showed, by gene disruption experiments in the asperthecin biosynthesis gene cluster in *A. nidulans,* that a TE-less NR-PKS member of this gene cluster required a β-lactamase-like protein to synthesize asperthecin. They suggested that the β-lactamase-like protein was responsible for releasing the product from the TE-less type I PKS. However, the role of this β-lactamase-like protein in product release from a TE-less NR-PKS needs further investigation.

Anthraquinones are a large family of compounds isolated from plants, fungi, lichens, and insects. Laxative effects are a well-known bioactivity of some natural anthraquinones. Recently, anthraquinones have been reported to possess some anticancer properties, such as inhibiting cellular proliferation, inducing apoptosis, and preventing metastasis (Huang et al., 2007). Emodin is a representative anthraquinone, present in the roots and barks of many plants (Huang et al., 2007) and in several fungi, including *Dermocybe*, *Cladosporium*, *Penicillium*, and *Aspergillus* (Wells et al., 1975). The PKS (emodin anthrone PKS) that is involved in the biosynthesis of emodin anthrone in *A. terreus* RED1 was identified during gene disruption experiments to eliminate the production of sulochrin, an undesirable cometabolite in the lovastatin fermentation by this fungus (Couch and Gaucher, 2004).

We studied ATEG_08451, a homolog of the emodin anthrone PKS in *A. terreus* NIH2624. Interestingly, a domain search showed that ATEG_08451 was a NR-PKS containing no TE domain. Thus, it was a mystery as to how the TE-less enzyme, ATEG_08451, released its products.

In this report, we show that the TE-less NR-PKS, ATEG_08451, produces several anthraquinones in collaboration with a physically discrete thioesterase, ATEG_08450, by in vitro reconstitution of the two enzymes and their heterologous expression in *A. oryzae*. ATEG_08450, sharing no apparent homology with the classical TE domain of type I PKSs, belongs to the β-lactamase superfamily (Daiyasu et al., 2001). ATEG_08451 catalyzes the formation of the scaffold of atrochrysone carboxylic acid, and ATEG_08450 catalyzes hydrolysis of the thioester between the ACP domain and the polyketide intermediate. Thus, we named ATEG_08451 "atrochrysone carboxylic acid synthase" (ACAS) and ATEG_08450 "atrochrysone carboxyl ACP thioesterase" (ACTE). This is the first in vitro demonstration that a β-lactamase-like thioesterase releases the product from a TE-less type I PKS.

RESULTS

Domain Organization of ACAS

On the basis of the partial nucleotide sequences for the KS and AT domains of the emodin anthrone PKS in *A. terreus* RED1 (Couch and Gaucher, 2004), we searched the genomic sequence of *A. terreus* NIH2624 for candidate emodin anthrone PKS genes in this strain using a BLAST search (Altschul et al., 1990). ATEG_08451, sharing 97% identity in amino acid sequence with the emodin anthrone PKS in strain RED1, was found. As described below, ATEG_08451 was confirmed to be responsible for the formation of atrochrysone carboxylic acid and was named ACAS.

The domain search program (National Institute of Immunology) predicted that ACAS consisted of SAT, KS, AT, PT, and ACP domains, from the N to C terminus (Figure 1A). Interestingly, ACAS lacked a TE domain.

A PKS whose TE domain is inactivated or removed cannot catalyze a Claisen cyclization and thus yields a product with a lactone ring (Fujii et al., 2001); nevertheless, a plausible emodin biosynthetic pathway should include aldol cyclization of the C ring, *not* lactonization, in addition to formation of the A and B rings by an aldol cyclization and hydrolysis of the thioester bond between the ACP and the product (Figure 2B). Comparison of this pathway with that for norsolorinic acid anthrone catalyzed by PksA (Minto and Townsend, 1997; Figure 2A), a well-studied NR-PKS having a domain organization of NR-PKS, SAT, KS, AT, PT, ACP, and

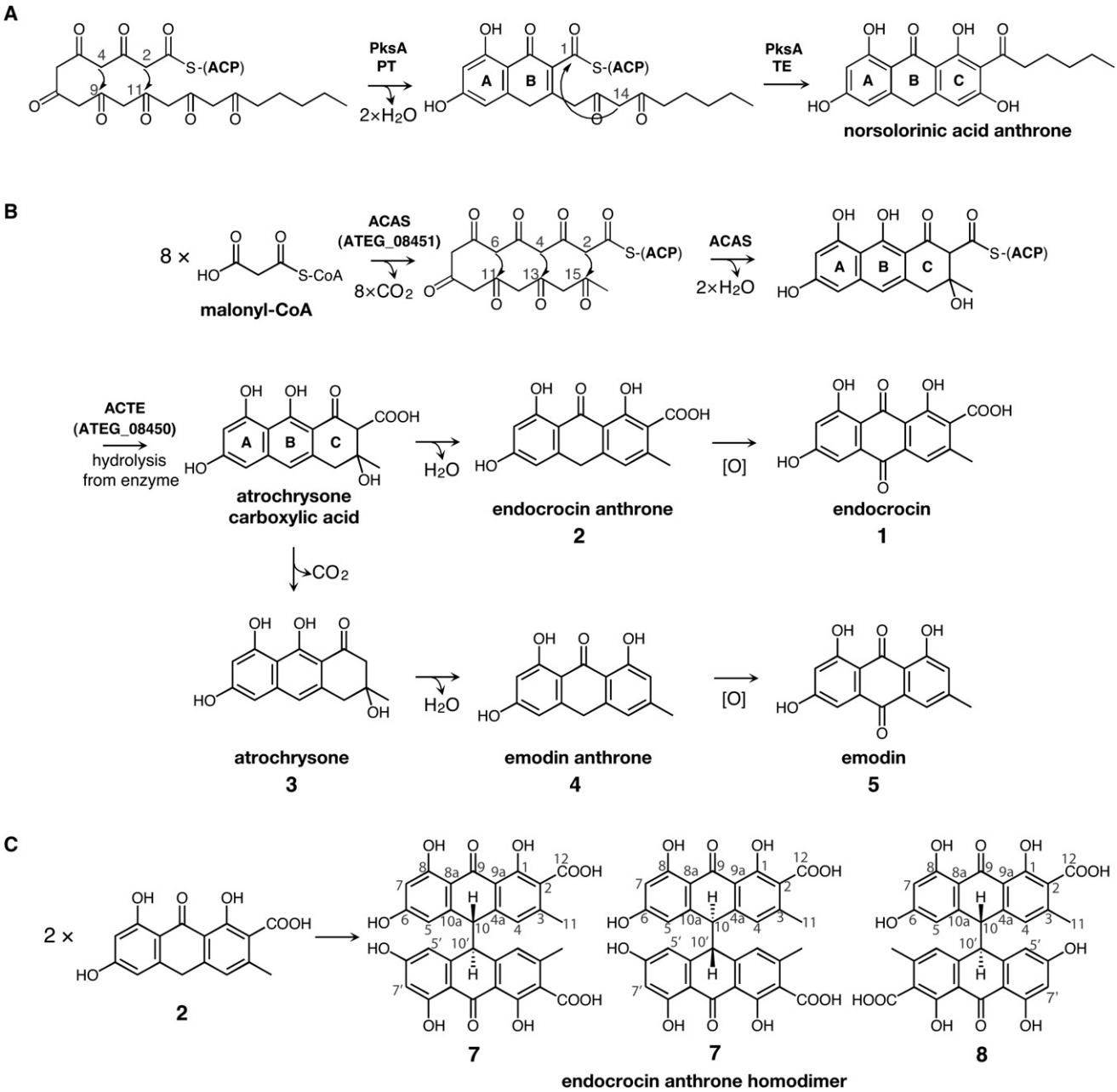

Figure 2. The Route Proposed for Biosynthesis of Anthraquinones by the Actions of ACAS and ACTE

(A) Ring closure by PksA. The PT domain of PksA catalyzed the closure of the A and B rings, and the TE domain catalyzed the closure of the C ring by a Claisen cyclization, resulting in the formation of norsolorinic acid anthrone.

(B) In vitro reactions proposed for ACAS and ACTE. ACAS catalyzes the closure of the A and B rings of atrochrysone carboxylic acid, and the C ring was presumably cyclized non-enzymatically. ACTE catalyzed the hydrolysis of the thioester bond in the atrochrysone carboxyl ACP, resulting in product release. Consequently, ACAS and ACTE produce compounds **1** to **6**, in vitro. Endocrocin anthrone (**2**) and emodin anthrone (**4**) auto-oxidize in vitro to form endocrocin (**1**) and emodin (**5**), respectively. Although **6** (*m/z* 257 $[M-H]^-$) was predicted to be derived from a heptaketide intermediate judging from their *m/z* values, the structure of **6** remained unidentified (C). Dimerization of endocrocin anthrone (**2**) in *A. oryzae*. Compound **7** is probably a mixture of enantiomers (*10R, 10′R*) and (*10S, 10′S*)-endocrocin anthrone homodimer. Compound **8** is a meso form (*10R, 10′S*)-endocrocin anthrone homodimer. Dimerization of **2** is probably catalyzed by an endogenous enzyme in *A. oryzae*.

TE, suggested that the PT domain of ACAS was involved in the closure of the A and B rings of emodin because the PT domain of PksA catalyzes the formation of the A and B rings of this anthrone (Crawford et al., 2008). The C ring closure of norsolorinic acid anthrone is catalyzed by the TE domain via a Claisen condensation. However, how C ring formation in emodin occurs is unclear due to the absence of a TE domain. Additionally, how thioester bond cleavage occurs in emodin synthesis remains unknown.

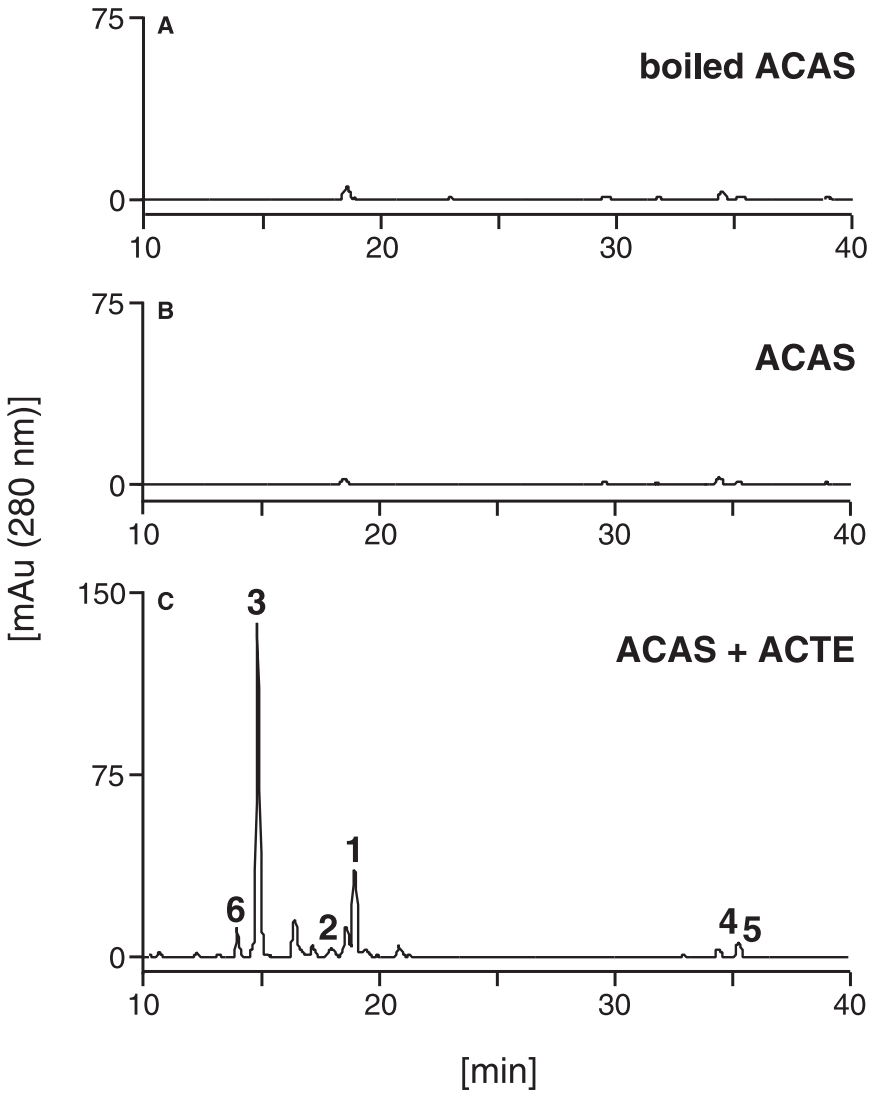

Figure 3. HPLC Analysis of products from in vitro reactions catalyzed by ACAS and ACTE

All reactions contained malonyl-CoA. Boiled ACAS (A) and ACAS (B) produced no product. Coincubation of ACAS and ACTE in the presence of malonyl-CoA yielded products **1** to **6** (C).

In Vitro Reactions of ACAS

We first checked whether ACAS synthesized the emodin scaffold in vitro. Our attempts to produce an active ACAS enzyme using various expression systems in *Escherichia coli* failed for unknown reasons. We then used an *A. oryzae* host-vector system, in which the ACAS cDNA was inserted between the *amyB* promoter and terminator in plasmid pTAex3, which was then integrated (several copies) into the chromosome. The plasmid pTAex3-ACAS-Nhis could direct the synthesis of the ACAS enzyme as Met-Glu-His_6-ACAS. The ACAS protein with a His-tag was thus produced in *A. oryzae* and purified by nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography. Purified ACAS gave a single major protein band of 195 kDa on SDS-PAGE (Figure 1C). Incubation of the purified ACAS in the presence of malonyl-CoA alone gave no product (Figures 3A and 3B).

The presence of [2-^{14}C]malonyl-CoA in the reaction mixture led to ^{14}C-labeling of ACAS, showing that the AT domain of the ACAS protein was active and that the ACP domain was in the holo form (data not shown). We assumed that the polyketide intermediate remained attached to the ACP domain due to the absence of any TE domain in ACAS. Alkaline hydrolysis of the probable ACAS-polyketide complex released product **1** and a trace amount of a putative nonaketide (*m/z* 355 $[M-H]^-$), as determined by liquid-chromatography atmospheric pressure chemical ionization mass spectrometry (LC-APCIMS) analysis (see Figure S1 available online). Product **1** was identified as endocrocin by comparing its retention time (RT), ultraviolet irradiation (UV), mass spectrometry (MS), and MS/MS spectra with those of the chemically synthesized authentic samples (Figure S2).

Because nonenzymatic cyclization or dehydration of the intermediate can occur during hydrolysis at an alkaline pH, it was unclear whether endocrocin itself was attached to ACAS. Our attempts to release and detect the product produced by ACAS by incubating the ACAS-polyketide complex in the presence of hydroxylamine at physiological pH (pH 7.5) or in ethanol failed, giving no product, presumably because the amount was below the detection limit.

C ring formation by ACAS was thus not confirmed by these experiments. However, the octaketide, from which endocrocin

A Claisen cyclization, catalyzed by a TE domain, includes the cleavage of the thioester bond between ACP and the polyketide intermediate in addition to C ring formation, which occurs via the attack of the carbanion on the carbon atom adjacent to ACP. However, the aldol cyclization that is required for the C ring formation in emodin is not accompanied by the cleavage of the thioester bond. Thus, to complete emodin formation by ACAS after C ring closure, hydrolysis of the thioester bond is still required. These observations suggested that ACAS cannot release its products alone and that an additional enzyme might be responsible for the hydrolysis of the thioester bond in these fungi.

Apart from the TE domain, the SAT domain of ACAS also deserves mention. The SAT domain transfers acyl moieties, as starter substrates, to ACP. The SAT domain of ACAS does not contain the conserved GX*C*XG (X = any amino acid) active motif (Crawford et al., 2006); instead, it is replaced by a GX*G*XG sequence. The SAT domain might thus be inactive, and the AT domain could be responsible for both starter and extender unit loading. In this study, however, we did not characterize the SAT domain further.

was derived, and the nonaketide detected were likely produced by ACAS. These findings suggested that recombinant ACAS itself could catalyze loading and condensation of several malonyl-CoAs but could not release its product. We thus assumed that ACAS required an additional factor or enzyme to cleave the thioester bond between ACP and its intermediates.

In Vitro Reactions of ACAS and ACTE

We focused on ATEG_08450, which is located upstream of *ACAS,* in the opposite orientation on the chromosome (Figure 1B), as a candidate for the thioesterase able to release the product by cleaving the thioester bond between the ACP of ACAS and the polyketide intermediate. ATEG_08450 contains a metal-binding domain (THXHXDH; X = any amino acid) that is conserved in the family of metallo-β-lactamases (Crowder et al., 1996; Concha et al., 1996) and has 22% identity in amino acid sequence to human glyoxalase II, as determined by three-dimensional position-specific scoring matrix (3D-PSSM) analysis. Glyoxalase II is responsible for the detoxification of methylglyoxal by hydrolyzing a thioester bond of *S*-D-lactoylglutathione to form D-lactic acid and regenerate glutathione (Cameron et al., 1999). As described below, ATEG_08450 turned out to be the thioesterase, as expected, and was named ACTE.

We produced ACTE as a His-tagged protein with the structure Met-Asn-His-Lys-Val-His_6-ACTE in *E. coli,* and purified it by affinity chromatography with the TALON metal affinity resin. The SDS-PAGE pattern of the purified ACTE is shown in Figure 1C. The addition of ACTE to the reaction mixture, consisting of ACAS and malonyl-CoA, led to production of six compounds (**1**–**6**; Figure 3C), as determined by high-performance liquid chromatography (HPLC) and LC-APCIMS analysis, whereas (as described above) no product was detected in the absence of ACTE. Products **1**, **4**, and **5** were identified as endocrocin (**1**), emodin anthrone (**4**), and emodin (**5**), by comparing their RT, UV, MS, and MS/MS spectra with authentic samples (Figures S2 and S3). Products **2** (*m/z* 299 $[M-H]^-$) and **3** (*m/z* 273 $[M-H]^-$) were predicted to be derived from an octaketide, and **6** (*m/z* 257 $[M-H]^-$) was perhaps derived from a heptaketide, judging from their *m/z* values. Thus, ACAS apparently required ACTE to produce the emodin scaffold in vitro, perhaps with product release as a result of the thioesterase activity of ACTE. The chemical structure of the major product (**3**), atrochrysone, is described below. Interestingly, the yields and the molar ratios of the products formed in vitro remained invariable in reactions containing larger amounts of ACTE than ACAS. Furthermore, the yields of the products decreased dramatically in a reaction containing a lower concentration of ACTE than ACAS.

The presence of [1-^{14}C]acetyl-CoA as a starter substrate and nonlabeled malonyl-CoA as an extender substrate in the reaction mixture containing both ACAS and ACTE gave no radiolabeled product, indicating that ACAS did not accept acetyl-CoA as a starter. Such an inability to use acetyl-CoA as a starter was also observed for PKS1 in *Colletotrichum lagenarium* (Fujii et al., 2000) and in PKS4 in *Gibberella fujikuroi* (Ma et al., 2007).

Analysis of ACAS and ACTE Reactions by Heterologous Expression in *A. oryzae*

To prepare product **3** in large amounts for structural elucidation, both *ACAS* and *ACTE* were expressed in *A. oryzae*. *ACAS* was in pTAex3 and *ACTE* in pPTRTA; in both plasmids, the cDNAs were under the control of the *amyB* promoter, and they integrated (several copies) in the chromosome in the same cell. HPLC and LC-APCIMS analyses of an ethyl acetate extract of the culture broth prepared from the *A. oryzae* transformant carrying both cDNAs revealed the production of several products, **1**–**10**, whereas the transformants carrying either cDNAs gave almost no new product (Figures 4A–4D). Products **1** to **6** were also detected in the above-described in vitro reaction (Figure 3C); they were identified as endocrocin (**1**); compounds (**2** and **3**), derived from an octaketide; emodin anthrone (**4**); emodin (**5**); and compound (**6**), derived from a heptaketide. Products **7**–**10** were newly detected in *A. oryzae*.

Product **3**, which was prepared in large amounts, was identified as atrochrysone by comparing its ^{1}H nuclear magnetic resonance (NMR) spectrum with reported data (Gill and Morgan, 2001). Products **7** (*m/z* 597 $[M-H]^-$) and **8** (*m/z* 597 $[M-H]^-$) were identified as diastereomers of homodimer of endocrocin anthrone (**2**; Figure 2C) by ^{1}H- and ^{13}C-NMR, heteronuclear multiple quantum correlation (HMQC), heteronuclear multiple bond correlation (HMBC), and nuclear Overhauser effect correlated spectroscopy (NOESY) analyses. The details of the NMR data are described in Figure S4. HMBC analysis of **7** and **8** revealed a ^{1}H-^{13}C coupling between H10 and C10′, indicating the presence of a C10-C10′ bond joining two monomers of endocrocin anthrone. Nuclear Overhauser effect (NOE) couplings between H11 and H7′ and between H4 and H5′ were detected for **8**, although no such coupling was detected for **7**. This difference in NOE couplings reflects the difference in the dimerization manner between **7** and **8**. Presumably, **7** is a mixture of enantiomers, (*10R, 10′R*) and (*10S, 10′S*)-endocrocin anthrone homodimer. The NMR data of (*10R, 10′R*) and (*10S, 10′S*)-endocrocin anthrone homodimer were identical. These two enantiomers (**7**) could not be separated by our HPLC method. Product **8** was probably a meso form, (*10R, 10′S*)-endocrocin anthrone homodimer. We assume that endocrocin anthrone (**2**) was dimerized by an endogenous enzyme of *A. oryzae*. Production of **7** and **8** supported the idea that endocrocin (**1**) was synthesized through endocrocin anthrone (**2**). Products **9** (*m/z* 553 $[M-H]^-$) and **10** (*m/z* 553 $[M-H]^-$) were likely diastereomers of the heterodimer consisting of emodin anthrone (**4**) and endocrocin anthrone (**2**). The homodimer of endocrocin anthrone (**7**+**8**) was produced in the highest yield among the products in *A. oryzae*, followed by endocrocin (**1**), atrochrysone (**3**), and emodin (**5**).

The main product resulting from the action of ACAS and ACTE in vitro was thus atrochrysone (**3**) (molar ratio **1**:**3**:**5** = 1:8:0.1), whereas the main product in vivo in *A. oryzae* was a homodimer of endocrocin anthrone (**7** + **8**; molar ratio **1**:**3**:**5**: **7** + **8** = 1:1:0.6:8; Table 1). These findings suggest that ACAS and ACTE produce endocrocin anthrone as the major product in *A. oryzae* (discussed below). Detection of atrochrysone (**3**) and endocrocin (**1**) suggested that atrochrysone carboxylic acid was a direct product of ACAS and ACTE (Figure 2B). Atrochrysone carboxylic acid was unstable and converted readily to atrochrysone (**3**) or endocrocin anthrone (**2**), as described in the Discussion.

The introduction of *ACAS* alone into *A. oryzae* gave endocrocin (**1**) and emodin (**5**) in trace amounts (data not shown). Because the coexistence of *ACTE* and *ACAS* in *A. oryzae* induced a 100-fold increase in the yields of the products, we

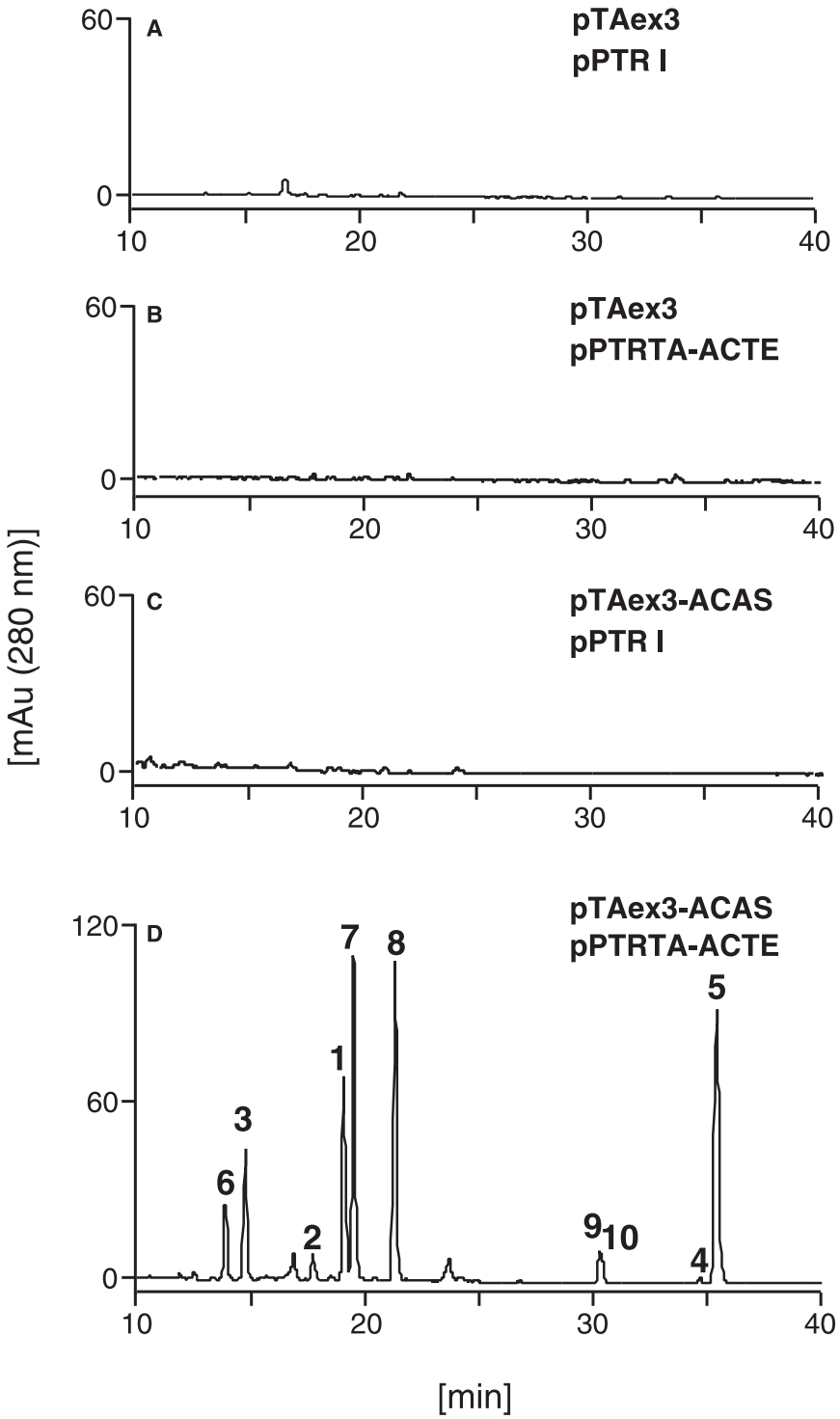

Figure 4. HPLC Analysis of Ethyl Acetate Extracts Prepared from *A. oryzae* Transformants

The transformants harboring pPTR I and pTAex3 (A), pPTRTA-ACTE, and pTAex3 (B) produced no detectable compound. The transformants harboring pPTR I and pTAex3-ACAS (C) produced trace amounts of **1** and **5** (data not shown). The transformant harboring pPTRTA-ACTE and pTAex3-ACAS (D) produced compounds **1** to **10**. Compounds **9** and **10** were not separated in the HPLC analysis, but were clearly separated in LC-APCIMS analysis (data not shown).

ACTE as a Thioesterase Responsible for Product Release from ACAS

All the above findings suggested the involvement of ACTE in the release of atrochrysone carboxylic acid as the product from ACAS after cleavage of the thioester bond between the ACP of ACAS and the octaketide intermediate (Figure 2B). The expected thioesterase activity of ACTE was tested using a synthetic analog of the ACP-tricyclic octaketide intermediate anthraquinone-2-carboxylic acid-*N*-acetylcysteamine (Ac-NAC; **11**) as a substrate (Figure 5A). NAC is a mimic of the terminal portion of phosphopantetheine, and NAC thioesters have been widely used as ACP thioester analogs. HPLC analysis of the reaction mixture consisting of ACTE and Ac-NAC revealed the production of anthraquinone-2-carboxylic acid (**12**; Figures 5B and 5C), indicating that ACTE actually catalyzed hydrolysis of the thioester bond of Ac-NAC. Kinetic analysis showed that ACTE gave **12** with a k_{cat} of 24.5 ± 0.9 s^{-1} and a K_m of 63.2 ± 3.3 μM (Figure 5D). These data suggested that ACTE catalyzed hydrolysis of the thioester bond between the ACP of ACAS and the octaketide intermediate, releasing the corresponding acid as the product.

We tested the ability of ACTE to hydrolyze an oxygen ester using 2-acetamidoethyl-anthraquinone-2-carboxylate as a substrate. This compound, having an oxygen ester, is structurally analogous to Ac-NAC. Incubation of ACTE with the compound under various conditions, however, did not hydrolyze the oxygen ester. Thus, ACTE is unlikely to generally cleave an oxygen ester.

We next examined the possible metal ion requirement of ACTE for activity, because it belongs to the metallo-β-lactamase family. Metallo-β-lactamases are inactivated by chelating agents

assumed that an endogenous ACTE homolog in this host cell served as a thioesterase to release atrochrysone carboxylic acid from ACAS. In fact, *A. oryzae* contains one ACTE homolog (AO090701000530). Because the yields of the products of the in vitro reaction containing ACTE at a lower concentration than ACAS were dramatically decreased (as described above), we assumed that ACTE was expressed at a higher level than ACAS.

Table 1. The Yields of the Compounds Produced by ACAS and ACTE In Vitro and by Heterologous Expression in *A. oryzae*

		In *A. oryzae*	
	In Vitro (nmol/120 μg)[a]	(nmol/ml)[b]	(mg/l)[c]
Endocrocin (**1**)	1.00 ± 0.06	17.3 ± 3.5	5.43 ± 1.10
Atrochrysone (**3**)	7.66 ± 0.13	15.8 ± 1.3	4.33 ± 0.36
Emodin (**5**)	0.135 ± 0.01	9.83 ± 1.51	2.65 ± 0.41
Endocrocin anthrone homodimer (**7**)	ND	58.1 ± 9.4	34.7 ± 5.6
Endocrocin anthrone homodimer (**8**)	ND	67.5 ± 11.0	40.4 ± 6.6

Results are mean ± standard error (n = 3). ND, not detected.
[a] The yields of the compounds produced by ACAS and ACTE in vitro, per 120 μg ACAS.
[b] The yields of the compounds produced by ACAS and ACTE in *A. oryzae*, per 1 ml broth.
[c] The yields of the compounds produced by ACAS and ACTE in *A. oryzae*, per 1 l broth.

such as EDTA (Wang and Benkovic, 1998). Preparation of the apoenzyme by vigorously dialyzing purified ACTE against a buffer containing 10 mM EDTA resulted in precipitation of 65% of the enzyme, as determined by measuring the protein concentration in the soluble fraction. The initial velocity of the Ac-NAC hydrolysis by the soluble ACTE apoenzyme dropped by 96%. These data clearly showed that ACTE required a metal ion for catalytic activity and that ACTE was a metalloenzyme, like other β-lactamases. We are currently studying the metal in ACTE.

DISCUSSION

Because endocrocin and atrochrysone were detected by LC-APCIMS and HPLC analyses as major products of ACAS and ACTE both in the in vitro reaction of malonyl-CoA and in the in vivo expression in *A. oryzae*, we assumed that the direct product was atrochrysone carboxylic acid (Figure 2B). Because of the instability of atrochrysone carboxylic acid and its tendency to readily convert to atrochrysone or endocrocin anthrone, atrochrysone carboxylic acid was not detected throughout our study. β-Keto acids are generally decarboxylated because they have a low energy path for decarboxylation by way of the enol of the product ketone (Guthrie, 2002). Because of this instability of atrochrysone carboxylic acid, having a ketone group at the β-position relative to the carboxyl group, decarboxylation occurs readily, yielding atrochrysone. When dehydration of atrochrysone carboxylic acid occurs, instead of decarboxylation, the observed product is endocrocin anthrone, which is unlikely to be decarboxylated due to the absence of a ketone at the β-position (Figure 2B). Emodin anthrone and emodin, both of which were detected as products of ACAS and ACTE, were probably derived from atrochrysone, not endocrocin, because the carboxyl group of endocrocin is stable and acid treatment of atrochrysone yielded emodin anthrone and emodin (data not shown). This is consistent with the previous observation that radiolabeled endocrocin was not incorporated into emodin in *Dermocybe,* a fungus belonging to Basidiomycota (Steglich et al., 1972). Homodimers of endocrocin anthrone were the major products in *A. oryzae* carrying *ACAS* and *ACTE*, suggesting that endocrocin anthrone is primarily produced in this heterologous host. We suppose that a dehydratase, forming endocrocin anthrone, is present in *A. oryzae*. In *A. terreus*, it is unknown whether atrochrysone carboxylic acid is converted to atrochrysone or to endocrocin anthrone. Although we attempted to detect atrochrysone and endocrocin anthrone in the culture of *A. terreus* NIH2624, no such compounds were detected. Because these compounds are metabolites at an early step in the biosynthesis of geodin and asterric acid, atrochrysone might

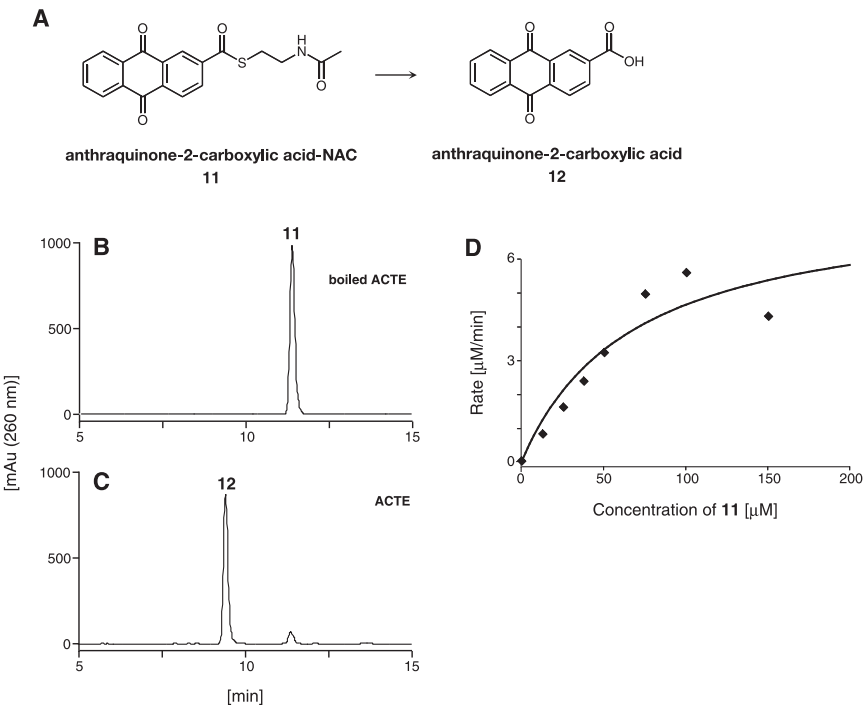

Figure 5. Hydrolysis of a Thioester Bond by ACTE
When anthraquinone-2-carboxylic acid-NAC (**11**) was incubated with ACTE, anthraquinone-2-carboxylic acid (**12**) was obtained (C) as a result of the cleavage of the thioester bond as in the scheme in (A). Boiled ACTE, as a negative control, gave no product (B). Kinetic analysis of the synthesis of **12** was performed in the presence of **11** at varying concentrations (D).

be rapidly modified by tailoring modification enzymes (Couch and Gaucher, 2004).

In the norsolorinic acid anthrone-producing reaction catalyzed by PksA, the PT domain catalyzed the closure of the A and B rings, followed by the Claisen cyclization of the C ring by the TE domain, resulting in product release (Crawford et al., 2008; Figure 2A). Although the alkaline hydrolysis of ACAS after incubation in the presence of malonyl-CoA gave only endocrocin and a putative nonaketide product, the PT domain of ACAS is likely to catalyze the closure of at least the A and B rings, like the PT domain of PksA. If ACAS does not catalyze the closure of the A and B rings of the intermediate, various cyclization patterns of the intermediate might yield many byproducts derived from the octaketide intermediate in addition to endocrocin. However, closure of the C ring might occur nonenzymatically at an alkaline pH, because aldol cyclization occurs readily under such conditions. At physiological pH, however, it is unclear whether the C ring of endocrocin is formed enzymatically or nonenzymatically. In the actinorhodin biosynthesis in *Streptomyces coelicolor* A3(2), lactonization of a bicyclic intermediate is led by ActVI-ORF1. In the absence of ActVI-ORF1, nonenzymatic aldol cyclization of the C ring occurs, resulting in the formation of 3,8-dihydroxyl-1-methylanthraquinone-2-carboxylic acid, having the C ring of anthraquinone (Ichinose et al., 1999). Thus, it is likely that the C ring of atrochrysone carboxylic acid is also closed by aldol cyclization nonenzymatically. We propose that the A and B rings of atrochrysone carboxylic acid are cyclized by the PT domain of ACAS and that the C ring is cyclized nonenzymatically. The PT domain of PksA catalyzes C2–C11 and C4–C9 cyclizations of the octaketide intermediate (Figure 2A), whereas that of ACAS catalyzes C4–C13 and C6–C11 cyclizations (Figure 2B). This regiospecificity of cyclization might reflect their structural differences.

The TE domains of modular type I PKSs and type I FASs belong to the α/β hydrolase superfamily (Chakravarty et al., 2004; Tsai et al., 2001). The core of the α/β hydrolase superfamily forms an α/β sheet consisting of eight β sheets connected by α helices (Ollis et al., 1992). The members contain a characteristic Ser, His, Asp catalytic triad. The TE domains of iterative type I PKSs, with homology to those of FASs and containing this conserved catalytic triad, appear to be members of the α/β hydrolase superfamily. Another type of TE includes SgcE10 and NcsE10 that serve as physically discrete TEs for bacterial iterative type I PKSs SgcE, responsible for the biosynthesis of C-1027 in *Streptomyces globisporus,* and NcsE, responsible for biosynthesis of neocarzinostatin in *Streptomyces carzinostaticus,* both of which consist of KS, AT, ACP, KR, DH, and phosphopantetheinyl transferase domains and yield an enediyne core (Zhang et al., 2008). These two thioesterases are similar to 4-hydroxybenzoyl-CoA thioesterase, which catalyzes the hydrolysis of the thioester of 4-hydroxybenzoyl-CoA in the degradation of 4-chlorobenzoate. A homotetramer of the 4-hydroxybenzoyl-CoA thioesterase in *Pseudomonas* sp. strain CBS2 forms a "hotdog-fold" thioesterase structure, the monomer of which consists of a long α helix wrapped by a five-stranded β sheet (Benning et al., 1998; Dillon and Bateman, 2004). ACTE is also a discrete thioesterase that is responsible for the product release from ACAS, an NR-PKS. ACTE shows no significant homology with any TE domain of type I PKSs, type I FASs, or the hotdog-fold thioesterase. Our 3D-PSSM analysis revealed that ACTE has some structural similarity with human glyoxalase II, which consists of a domain folding into a four-layered β sandwich and a domain that is predominantly α-helical. Glyoxalase II is a thioesterase in the glyoxalase system, which is responsible for detoxification of methylglyoxal, a byproduct of metabolism, by hydrolyzing *S*-D-lactoylglutathione to form D-lactic acid and regenerating glutathione (Cameron et al., 1999). It is a member of the metallo-β-lactamase superfamily and has a conserved metal-binding site, characteristic of this superfamily, as the active site. Alignment of ACTE with members of glyoxalase II showed that ACTE has a metal-binding site (TH*X*H*X*DH; *X* = any amino acid) of the metallo-β-lactamase superfamily (Crowder et al., 1996; Concha et al., 1996; Figure S5). These bioinformatics analyses suggested that ACTE belongs to the metallo-β-lactamase superfamily and has a structure different from α/β hydrolase-fold and hotdog-fold thioesterases. Reflecting these structural differences, ACTE is likely to catalyze the hydrolysis of the thioester between the ACP domain and the octaketide intermediate, not cyclization, whereas the TE domains of iterative type I PKSs catalyze the Claisen cyclization to release the products. This is consistent with the biosynthetic pathway proposed for asperthecin by Szewczyk et al. (2008). Furthermore, our study using a chelating agent, EDTA, suggested that ACTE requires a metal ion(s) for its activity. ACTE, belonging to the β-lactamase superfamily, is apparently a metalloenzyme. Taken together, ACTE is a novel type of thioesterase that is responsible for product release in the reaction of a type I PKS.

The presence of ACTE homologs is predicted in the clusters, including an open reading frame (ORF) for a TE-less NR-PKS in several filamentous fungi: five ORFs in *Neosartorya fischeri*, three ORFs in *Aspergillus fumigatus* and *A. nidulans*, and one ORF in *Pyrenophora tritici-repentis, Aspergillus niger,* and *A. oryzae* (Figure S6). These ACTE homologs probably catalyze the cleavage of the thioester bond between the TE-less type I PKSs and resulting polyketides to release the products. In addition to iterative type I PKSs, some type II PKS clusters and modular type I PKS clusters also have ACTE homologs. In the actinorhodin biosynthesis cluster, for example, ActIV shows similarity with β-lactamases, including a similar metal-binding domain (Fernández-Moreno et al., 1992). The bacillaene biosynthesis gene cluster in *Bacillus subtilis* also includes a β-lactamase homolog, PksB (Butcher et al., 2007). We predict that these homologs are involved in the polyketide biosynthesis as a thioesterase for release of the product as ACTE in atrochrysone carboxylic acid biosynthesis.

SIGNIFICANCE

Fungal polyketides, most of which are synthesized by iterative type I PKSs, possess structural diversity and a variety of biological activities. However, only a few fungal PKS reactions have been characterized biochemically. The present study showed that atrochrysone carboxylic acid synthase (ACAS) in *A. terreus* produces atrochrysone carboxylic acid as a direct product in collaboration with a physically discrete thioesterase, atrochrysone carboxyl ACP thioesterase (ACTE). ACTE has a conserved active site common to the β-lactamase superfamily and shows no similarity to the general TE domain of NR-PKSs. In vitro reconstitution of

the reaction revealed the roles of ACAS and ACTE in the synthesis of atrochrysone carboxylic acid. To our knowledge, this is the first demonstration that a β-lactamase-like thioesterase (ACTE) releases the product from a TE-less NR-PKS (ACAS) in vitro. The present discovery can be applied to ACTE homologs that are encoded in the vicinity of the putative TE-less NR-PKS genes on chromosomes in several filamentous fungi. These ACTE homologs presumably release a product from the TE-less NR-PKSs by cleaving the thioester bond between the ACP of the PKSs and the polyketide intermediate. Furthermore, ACTE homolog genes are also found in type II and modular type I PKS gene clusters in bacteria. Thus, we presume that an ACTE-type thioesterase playing a role similar to ACTE in polyketide biosynthesis is widely distributed in nature.

EXPERIMENTAL PROCEDURES

Materials

E. coli strains JM109, BL21(DE3) and plasmids pUC19, pT7blue, and pCold II, pPTR I, restriction enzymes, T4 DNA ligase, Klenow enzyme, and PrimeSTAR HS DNA Polymerase were purchased from Takara Biochemicals (Shiga, Japan). The *A. oryzae* host-vector system pTAex3 and strain M-2-3 were obtained from K. Gomi (Tohoku University). *A. terreus* NIH2624 was purchased from the Fungal Genetic Stock Center (University of Kansas Medical Center, Kansas City, KS). *N*-Acetylcysteamine was purchased from Aldrich, and malonyl-CoA and emodin were from Sigma. Anthraquinone-2-carboxylic acid was purchased from TCI (Tokyo, Japan). [1-^{14}C]Acetyl-CoA was purchased from PerkinElmer Life Sciences (Boston, MA), and [2-^{14}C]malonyl-CoA was purchased from American Radiolabeled Chemicals (St. Louis, MO).

Construction of Plasmids

Please refer to Supplemental Experimental Procedures.

Production and Purification of Histidine-Tagged ACAS

The expression plasmid pTAex3-ACASNhis was linearized with PvuI and introduced in the fungal host *A. oryzae* M-2-3 by the protoplast-polyethylene glycol method (Gomi et al., 1987). Transformants were grown in Czapek-Dox medium (0.3% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% KCl, 1% polypeptone) with 2% glucose as a carbon source for 3 days and then transferred to Czapek-Dox medium with 2% starch to induce the expression of *ACAS* under the control of the *amyB* promoter. After 2 days of cultivation, mycelia were ground in liquid nitrogen and suspended in buffer A (50 mM KH_2PO_4 [pH 7.5], 150 mM NaCl, 10% glycerol, 1 mM Tris[2-carboxyethyl]phosphine) and Complete EDTA-free protease inhibitor cocktail (Roche). The mixture was stirred at 4°C for 30 min and subsequently centrifuged (13,000 *g*, 20 min). The supernatant was filtered through Miracloth (Calbiochem). Ni-NTA superflow was added to the supernatant, and the solution was stirred at 4°C for 1 hr. The protein/resin mixture was loaded into a gravity flow column, and proteins were purified with a gradient of imidazole in buffer A. The purified histidine-tagged protein was dialyzed against buffer A. The ACAS protein gave a single protein band at a position of ca. 195 kDa on SDS-PAGE (Figure 1C).

Production and Purification of Histidine-Tagged ACTE

E. coli BL21 (DE3) harboring pCold II-ACTE was precultured overnight in Luria-Bertani (LB) medium containing 100 μg/ml ampicillin. The preculture was transferred into LB medium containing 100 μg/ml ampicillin and incubated at 37°C to an OD_{600} of 0.6. The cells were cultured further at 15°C for 30 min and then induced with 1 mM isopropyl thio-β-D-galactoside at 15°C for 24 hr. The cells were harvested by centrifugation, resuspended in buffer containing 50 mM KH_2PO_4 (pH 7.5), 150 mM NaCl, and 10% glycerol, and disrupted by sonication. A crude cell-lysate was prepared by removal of cell debris by centrifugation (10,000 *g*, 20 min). ACTE was purified using TALON Metal Affinity Resin (Clontech), according to the manufacturer's protocol. The purified histidine-tagged protein was dialyzed against 50 mM KH_2PO_4 (pH 7.5), 150 mM NaCl, and 10% glycerol. The ACTE protein gave a single protein band at a position of ca. 37 kDa on SDS-PAGE (Figure 1C).

PKS Assay

The standard reaction mixture contained 0.13 mM malonyl-CoA, 50 mM KH_2PO_4 (pH 7.5), 150 mM NaCl, 10% glycerol, 1 mM Tris[2-carboxyethyl] phosphine, and 120 μg ACAS in a total volume of 760 μl. After incubation at 37°C for 120 min, the reactions were quenched by the addition of 760 μl ice-cold 20% (wt/vol) trichloroacetic acid. The pellet was resuspended in 500 μl 350 mM KOH and incubated at 65°C for 30 min to release covalently bound products. After acidification with 50 μl 6 M HCl, the products were extracted twice with 500 μl ethyl acetate, and the organic layer was evaporated to dryness. The residual material was dissolved in 20 μl methanol for LC-APCIMS analysis. LC-APCIMS analysis in a negative mode was performed using the Agilent 1100 series (Agilent) with an ODS-80Ts reversed-phase HPLC column (2.0 × 150 mm; TOSOH) and the Esquire high-capacity trap plus system (Bluker Daltonics), and the sample was eluted with a linear gradient of 20%–60% CH_3CN in water (each containing 1% acetic acid) at a flow rate of 1 ml/min.

ACTE (21.8 μg) was additionally used in the standard reaction. The reaction mixture was incubated at 37°C for 120 min before being quenched with 76 μl 6 M HCl. The products were extracted with ethyl acetate and the organic layer was evaporated to dryness. The residual material was dissolved in 20 μl methanol for HPLC and LC-APCIMS analyses. LC-APCIMS analysis was carried out as described above. HPLC analysis with an ODS-80Ts reverse-phase HPLC column (2.0 × 150 mm; TOSOH) was carried out on a Hitachi LaChrom ELITE system, and the sample was eluted with a linear gradient of 20%–60% CH_3CN in water (each containing 1% trifluoroacetic acid) at a flow rate of 1 ml/min. UV spectra were detected on a Hitachi L-2450 diode array detector.

Syntheses of Emodin Anthrone and Endocrocin

Emodin anthrone was synthesized from emodin, as described by Falk et al. (1993). The UV chromatogram, UV spectrum, MS/MS fragment pattern of emodin anthrone are shown in Figure S3 with those of emodin as references. Endocrocin was synthesized as described by Waser et al. (2005). The NMR data, UV chromatogram, UV spectrum, and MS/MS fragment pattern of endocrocin are shown in Figure S2.

Production and HPLC Analysis of Products of ACAS and ACTE in *A. Oryzae*

The expression plasmid pTAex3-ACAS, linearized by PvuI, was used to transform *A. oryzae* M-2-3 by the protoplast-polyethylene glycol method. pPTRTA-ACTE, linearized by MunI, was then introduced in the transformant harboring pTAex3-ACAS. Cotransformants were selected on Czapek-Dox pyrithiamine (0.1 μg/ml) medium. Cotransformants were grown in Czapek-Dox medium with 2% glucose as a carbon source for 3 days and then transferred to Czapek-Dox medium containing 2% starch to induce the *amyB* promoter. Cultivation was continued for 3 days. The culture broth and cells were separated through Miracloth. The broth was acidified to pH 3 with HCl and extracted with ethyl acetate. The organic layer was evaporated to dryness. The residual material was dissolved in 20 μl methanol for HPLC and LC-APCIMS analyses. HPLC and LC-APCIMS analyses were carried out as described above.

Large Scale Preparation and Characterization of Products of ACAS and ACTE

The culture conditions of *A. oryzae* carrying both *ACAS* and *ACTE* were the same as those for the analytical scale, except that the culture was scaled up to 2.4 l. After cultivation, broth and cells were separated through Miracloth. The broth was acidified to pH 3 with HCl and extracted three times with ethyl acetate. The organic layer was dried with Na_2SO_4 and evaporated to dryness. The ethyl acetate extract was passed through a Sep-Pak Vac 20 cc (5 g) C18 cartridge (Waters) to remove lipid compounds. The crude material was dissolved in a small amount of chloroform/methanol (5:1, v/v) and flash-chromatographed on silica gel using chloroform/methanol/acetic acid (98:2:1, v/v) as the eluent. The eluates were evaporated and dissolved in methanol for reverse-phase preparative HPLC, equipped with a DOCOSIL-B column (10 × 250 mm; Senshu Scientific, Tokyo). The structure of **1** was determined by proton NMR spectroscopy and HR-MS: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.40 (s, 3H, C11H), 2.77 (dd, *J* = 17 Hz, 1H, C2H), 2.90 (d, *J* = 17 Hz, 1H, axial H of C2), 2.99

(d, J = 16 Hz, 1H, equatorial H of C4), 3.08 (d, J = 16 Hz, 1H, axial H of C4), 3.30 (s, 1H, C3-OH), 6.38 (d, J = 2.5 Hz, 1H, C7H), 6.59 (d, J = 2.5 Hz, 1H, C5H), 6.83 (s, 1H, C10H), 9.84 (s, 1H, C8-OH). HR-MS (ESI): found for $[C_{15}H_{14}O_5\text{-}H]^-$, 273.07419. The structures of **7** and **8** were determined by proton and carbon NMR spectroscopy with the aid of HMQC, HMBC, and NOESY analyses. The NMR data of **7** and **8** are shown in Figure S4.

Synthesis of Anthraquinone-2-carbonyl-N-acetylcysteamine

Anthraquinone-2-carboxylic acid (252 mg, 1.0 mmol), water soluble carbodiimide (384 mg, 2.0 mmol) and *N*,*N*-dimethyl-4-aminopyridine (19.5 mg, 0.16 mmol) in CH_2Cl_2 (15 ml) were added to *N*-acetylcysteamine (110 μl, 1.0 mmol). The mixture was stirred at room temperature for 16 hr. The reaction was quenched by the addition of H_2O and extracted three times with chloroform. The organic layer was dried, and anthraquinone-2-carbonyl-*N*- acetylcysteamine (Ac-NAC) was purified by silica gel chromatography to give 320 mg solid powder (90% yield).

Hydrolytic Activity Assay of ACTE

A 100-μl-scale reaction contained 50 mM KH_2PO_4 (pH 7.5), 50 μM Ac-NAC (1 μl, 5 mM in dimethyl sulfoxide [DMSO]), and 12 μg ACTE. The reaction was incubated at 37°C for 60 min before being quenched with 10 μl 6 M HCl. The products were extracted with ethyl acetate, and the organic layer was evaporated to dryness. The residual material was dissolved in 20 μl DMSO for HPLC analysis. HPLC analysis was carried out using a LaChrom ELITE system with an ODS-80Ts reverse-phase HPLC column. The products were eluted with a linear gradient of 40%–55% CH_3CN in water (each containing 0.1% trifluoroacetic acid) at a flow rate of 1 ml/min.

Apo-ACTE was prepared by dialyzing 1.3 mg purified ACTE against three changes of a 100-fold volume excess of 50 mM potassium phosphate buffer (pH 8.0), 500 mM NaCl, 10% glycerol, 10 mM EDTA, at 4°C with a duration of 12 hr for each dialysis. The chelating agent was then removed by extensive dialysis against eight changes of a 100-fold volume excess of metal-free, 50 mM potassium phosphate buffer (pH 8.0), 500 mM NaCl, 10% glycerol at 4°C. Enzyme that did not precipitate was prepared as apo-ACTE by removal of the precipitate by centrifugation (6000 *g*, 5 min). The enzymatic activity was determined using Ac-NAC as a substrate, as described above.

A standard reaction contained 50 mM potassium phosphate buffer (pH 8.5), Ac-NAC (13–150 μM), and 300 nM ACTE in a total volume of 100 μl for the determination of kinetic values for Ac-NAC. After the reaction mixture had been preincubated at 37°C for 2 min, reactions were initiated by adding the substrate and continued for 5 min. We observed that the product formation was linear throughout this period. The reactions were stopped with 10 μl 6 N HCl, and the material in the mixture was extracted with ethyl acetate. The organic layer was collected and evaporated. The residual materials were dissolved in 100 μl DMSO, and 20 μl of the material was used for HPLC analysis. The velocity of the reaction was measured by monitoring the amount of anthraquinone-2-carboxylic acid. HPLC analysis was carried out using a LaChrom ELITE system with a DOCOSIL-B reverse-phase HPLC column (4.6 × 250 mm; Shenshu). The products were eluted with a linear gradient of 45%–55% CH_3CN in water (each containing 0.1% trifluoroacetic acid) at a flow rate of 1 ml/min. Anthraquinone-2-carboxylic acid was used to generate the standard curve for the quantification of the products. Steady-state parameters were determined by fitting the curve to $v = V_{max}[S]/(K_m + [S])$.

SUPPLEMENTAL DATA

Supplemental Data include seven figures and Supplemental Experimental Procedures and can be found with this article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(09)00137-9.

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