

Research Paper

Identification of Claisen cyclase domain in fungal polyketide synthase WA, a naphthopyrone synthase of *Aspergillus nidulans*Isao Fujii, Akira Watanabe, Ushio Sankawa¹, Yutaka Ebizuka*

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Abstract

Background: Based on the homology with fatty acid synthases and bacterial polyketide synthases (PKSs), thioesterase domains have been assigned at the C-terminus regions of fungal iterative type I PKSs. We previously overexpressed *Aspergillus nidulans* *wA* PKS gene in a heterologous fungal host and identified it to encode a heptaketide naphthopyrone synthase. In addition, expression of C-terminus-modified WA PKS gave heptaketide isocoumarins suggesting that the C-terminus region of WA PKS is involved in the cyclization of the second aromatic ring of naphthopyrone. To unravel the actual function of the C-terminus region, we carried out functional analysis of WA PKS mutants by C-terminus deletion and site-directed mutagenesis.

Results: Only the 32 amino acid deletion from the C-terminus of WA PKS caused product change to heptaketide isocoumarins from heptaketide naphthopyrone, YWA1 1, a product of intact WA PKS. Further C-terminus deletion mutant of WA PKS up to Ser¹⁹⁶⁷, an active site residue of so far called thioesterase, still produced isocoumarins. Site-directed mutagenesis of amino acid residues in this C-terminus region showed that even a single

mutation of S1967A or H2129Q caused production of isocoumarin instead of naphthopyrone. Furthermore, the role of tandem acyl carrier proteins (ACPs), a typical feature of fungal aromatic PKSs, was examined by site-directed mutagenesis and the results indicated that both ACPs can function as ACP independently.

Conclusions: Claisen-type cyclization is assumed to be involved in formation of aromatic compounds by some fungal type I PKSs. These PKSs have a quite identical architecture of active site domain organization, β -ketoacyl synthase, acyltransferase, tandem ACPs and thioesterase (TE) domains. Since the C-terminus region of WA PKS of this type was determined to be involved in Claisen-type cyclization of the second ring of naphthopyrone, we propose that the so far called TE of these PKSs work not just as TE but as Claisen cyclase. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Polyketide synthase; Claisen cyclase; Naphthopyrone; Deletion mutant; Site-directed mutagenesis; Thioesterase

1. Introduction

Simple carboxylic acids, such as acetate, malonate, are basic building blocks in the biosynthesis of natural products. Especially, polyketides are assembled by direct condensation of these short chain acids as their activated form

of CoA esters in a similar manner to that of fatty acid biosynthesis. In contrast to fatty acids, polyketides are diversified in their structures and biological activities, providing an important source of pharmaceutical and agrochemical agents [1,2]. By recent advances in our understanding of polyketide biosynthesis, genetic manipulation of polyketide biosynthesis genes is becoming a promising tool to produce novel compounds [3–7].

Polyketide chain assembly is catalyzed by polyketide synthases (PKSs) that are structurally and functionally analogous to fatty acid synthases (FASs). PKS-mediated condensation of acyl starter and malonyl unit results in the formation of a β -ketoacyl thioester. The subsequent sequential reactions of ketoreduction, dehydration and enoyl reduction, which normally occur in fatty acid biosynthesis, are partly or fully omitted in polyketide assembly cycles in highly programmed manners, which are key

Abbreviations: ACP, acyl carrier protein; FAS, fatty acid synthase; PCR, polymerase chain reaction; PKS, polyketide synthase; TE, thioesterase

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to the structural diversity in polyketide natural products [4].

The modular type I PKSs, which catalyze the biosynthesis of reduced macrolide polyketides such as erythromycin, are large multifunctional proteins consisting of so-called modules. Each reaction necessary for one cycle of chain elongation and reduction is catalyzed by unique active site domains assembled linearly on a module [8]. In contrast, most of aromatic polyketides in actinomycetes are assembled by a complex of small, discrete, monofunctional proteins (type II PKS) by the iterative use of a single set of active sites [3], as exemplified by actinorhodin PKS [9,10]. Fungal PKSs are formally classified into type I consisting of a single large polypeptide with a set of active site domains similar to a module of macrolide type I PKSs, but they work iteratively to produce their specific product compounds, including both aromatic and reduced complex-type compounds such as 6-methylsalicylic acid [11,12], tetrahydroxynaphthalene [13], T-toxin [14], lovastatin [15,16], etc. (Fig. 1) Thus, they might be classified as a separate group of PKSs [17].

The architectures of fungal PKSs are very similar to each other as shown in Fig. 2. Their linear active site organization is much more similar to that of mammalian FASs than to fungal FASs [18]. In most cases, functions of the fungal PKS genes have been identified by complemen-

tation experiments of blocked mutants. Although direct product identification of fungal PKSs had been rarely demonstrated, we have reported successful expression of *atX* gene from *Aspergillus terreus* [12], *wA* gene from *Aspergillus nidulans* [19,20], *PKS1* gene from *Colletotrichum lagenarium* [13,21], and *alb1* gene from *Aspergillus fumigatus* [22]. Heterologous expression of lovastatin PKS was also reported by the Hutchinson and Vederas group [16].

A. nidulans wA gene was first cloned and characterized as the PKS gene involved in pigment biosynthesis of mature green spores [23,24]. Our recent analysis of *wA* function by expression in a heterologous host *Aspergillus oryzae* unambiguously identified that the *wA* gene encodes a PKS for naphthopyrone compound YWA1 **1** [20]. In the course of the *wA* expression study, an interesting result was also obtained with the C-terminus-truncated WA PKS which was accidentally constructed due to an error in the previously reported nucleotide sequence [19]. This truncated WA PKS produced heptaketide isocoumarins, citreoisocoumarin **2**, as a major product instead of a heptaketide naphthopyrone, indicating that the truncated PKS still retained the ability to control chain-length but lost that for second aromatic ring cyclization. In C-termini of fungal iterative type I PKSs, acyl carrier protein (ACP) and thioesterase (TE) domains are assigned based on their homology with other types of PKS and FAS. However,

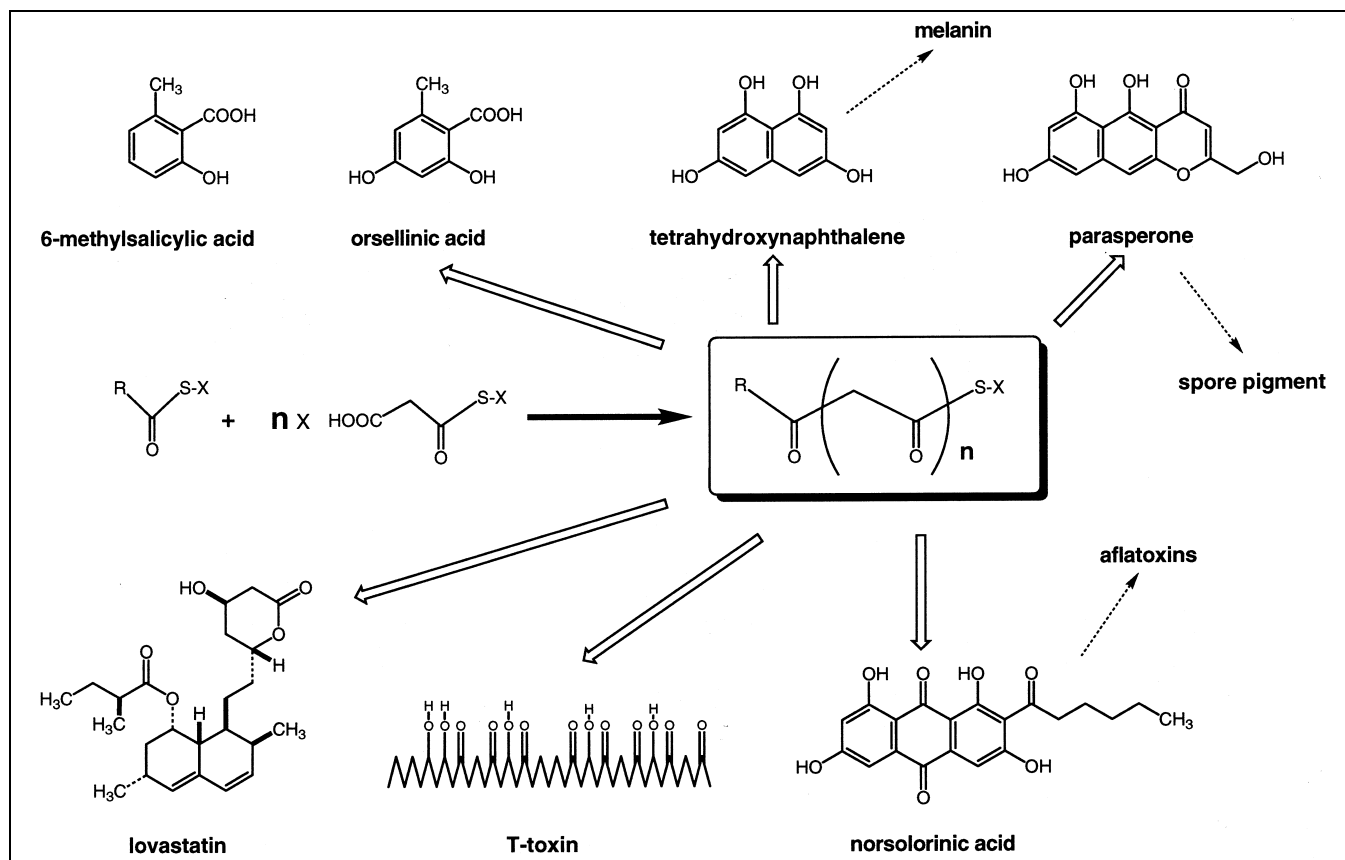


Fig. 1. Typical compounds produced by iterative type I fungal PKSs.

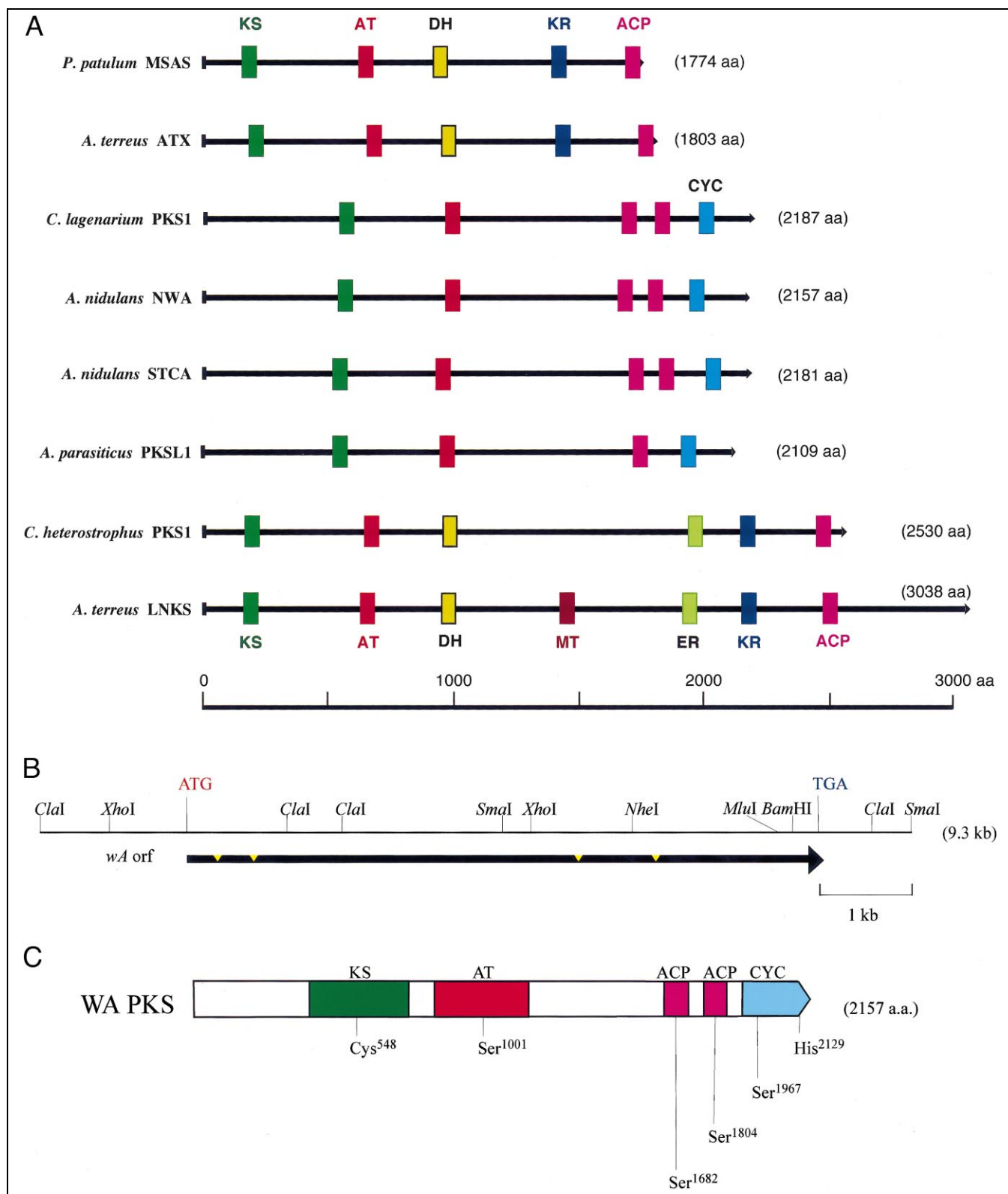


Fig. 2. Architecture of fungal PKSs. (A) Active site organizations on fungal PKSs deduced from the reported gene sequences are schematically shown. KS, β -ketoacyl synthase; AT acyltransferase; DH, dehydratase; KR, β -keto reductase; ER, enoyl reductase; MT, methyltransferase; CYC, Claisen cyclase. *P. patulum* MSAS, 6-methylsalicylic acid synthase gene (X5576) [11]; *A. terreus* ATX, 6-methylsalicylic acid synthase gene (D85860) [12]; *C. lagenarium* PKS1, 1,3,6,8-tetrahydroxynaphthalene synthase gene (D83643) [32]; *A. nidulans* NWA, naphthopyrone synthase (X65866) [20,24]; *A. nidulans* STCA, *stcA* gene for sterigmatocystin biosynthesis (L39121) [33]; *Aspergillus parasiticus* PKSL1, *pksL1* gene for aflatoxin biosynthesis (L42765) [34]; *Cochliobolus heterotrophus* PKS1, *PKS1* gene for T-toxin biosynthesis (U68040) [14]; *A. terreus* LNKS, lovastatin nonaketide synthase gene (AF151722) [15]. (B) A map of the *wA* gene with its open reading frame shown as an arrow. Introns are shown in yellow triangles. (C) Architecture of WA PKS with amino acid residues involved in its catalytic activity.

actual function of TE in fungal PKS has not been confirmed. In general, TE is believed to be necessary for releasing product compounds from PKS [25], but is not always present in fungal PKSs, e.g., 6-methylsalicylic acid synthase [26]. The role of tandem ACP motifs which is not unique to *A. nidulans* WA PKS but is found in some other fungal PKSs [17,27] has not been understood yet (Fig. 2). These facts prompted us to conduct more detailed functional analysis of the C-terminus region of fungal iterative type I PKSs using WA PKS as a model. Here, we report experimental evidence that the C-terminus region of WA PKS of *A. nidulans* works not as TE but as Claisen cyclase.

2. Results

2.1. Polyketide folding pattern of naphthopyrone YWA1

In our previous work, YWA1 **1** and citreoisocoumarin **2** were identified to be the products of WA PKS [20] and WAF (C-terminus-truncated WA PKS [19]), respectively. Apparently, both compounds are heptaketides. As shown in Fig. 3, the polyketide folding pattern for citreoisocoumarin **2** is obvious, but two different folding and cyclization patterns **b** and **c** for naphthopyrone are possible. In fact, both patterns have been reported for fungal naphthopyrones by feeding experiments [28,29]. In order to obtain clues for how WA PKS controls its reaction, it was necessary for the first place to identify the actual polyketide folding pattern for YWA1 **1**. [1,2-¹³C₂]Sodium acetate was fed to the induction culture of *A. oryzae* transformant

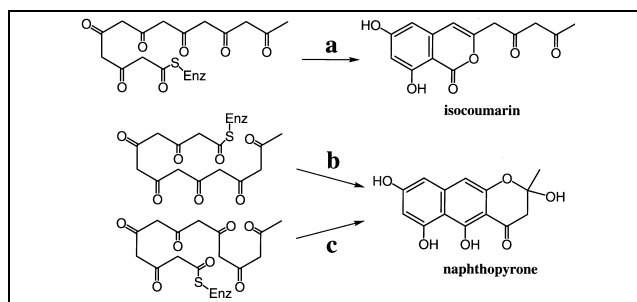


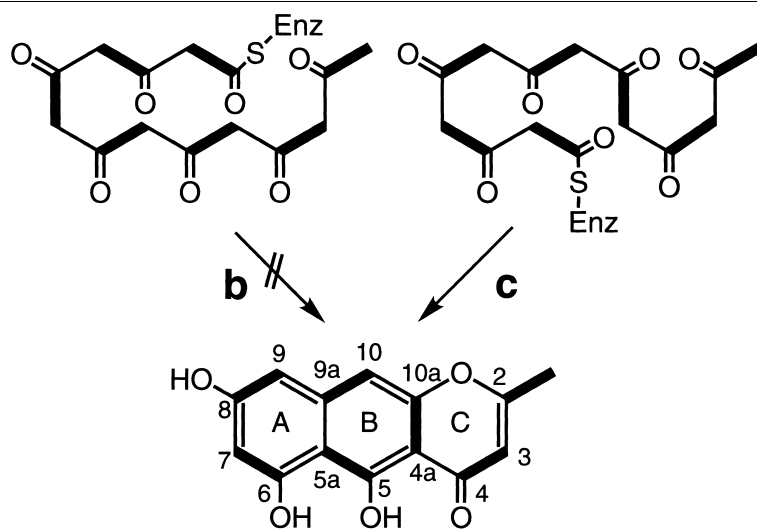
Fig. 3. Polyketetomethylene folding patterns for heptaketide isocoumarin and naphthopyrone.

with pTA-nwA [20]. After further 2 days culture, YWA1 **1** was extracted and converted to more stable YWA2 **3** by acid-catalyzed dehydration. Observed ¹³C NMR data of YWA2 **3** are shown in Table 1. From ¹J (¹³C–¹³C) coupling constant data, the folding pattern for naphthopyrone YWA1 **1** was established as pattern **c**.

After the formation of heptaketomethylene-ACP intermediate, aldol type condensation could give a monocyclic intermediate, and if it is released from WA PKS by hydrolysis, citreoisocoumarin **2** is produced. To form a naphthopyrone carbon skeleton, ring B closure by Claisen-type cyclization must take place following ring A formation by aldol type cyclization.

Since WAF produced no naphthopyrone but citreoisocoumarin **2** and its derivatives [19], it is deduced that WAF is unable to catalyze the second ring B cyclization and just releases heptaketide monocyclic intermediate to give isocoumarin derivatives. On the other hand, the intact WA PKS can catalyze the closure of ring B that leads to

Table 1
¹³C NMR data of naphthopyrone YWA2 **3** fed with [1,2-¹³C₂]sodium acetate



YWA2 (**3**)

| position | ¹³ C δ ppm | ¹ J (¹³ C– ¹³ C) in Hz |
|-----------------|-----------------------|--|
| 2 | 169.5 | 50 |
| 3 | 105.6 | 58 |
| 4 | 183.1 | 58 |
| 4a | 101.5 | 63 |
| 5 | 162.3 | 67 |
| 5a | 105.3 | 67 |
| 6 | 158.3 | 73 |
| 7 | 100.6 | 73 |
| 8 | 160.7 | 69 |
| 9 | 100.7 | 69 |
| 9a | 140.2 | 58 |
| 10 | 99.6 | 58 |
| 10a | 151.8 | 63 |
| CH ₃ | 20.1 | 50 |

naphthopyrone formation. Thus, the C-terminus region of WA, which is deleted in WAF, is assumed to be involved in Claisen-type cyclization to form ring B of naphthopyrone.

2.2. Construction of C-terminus deletion mutants of WA PKS and their product analysis

The WAF protein has a deletion of 67 amino acids from the C-terminus of WA PKS and additional modification with 24 amino acids derived from the ligated vector sequence in this position [20]. To identify the essential region of the C-terminus of WA PKS for ring B Claisen-type cyclization, C-terminus deletion mutants were expressed and their product compounds were analyzed. As shown in Fig. 4, the mutant WAC1 with 32 amino acids deletion from C-terminus produced citreoisocoumarin **2** instead of normal naphthopyrone YWA1 **1**, indicating the presence of amino acid residue(s) essential for Claisen-type cyclization in the deleted region. WAC2 is the mutant with further deletion of 274 amino acids including TE motif (–G W S¹⁹⁶⁷ A G–). This mutant also produced citreoisocoumarin **2** and its productivity was not affected significantly. In mammalian fatty acid biosynthesis, TE releases palmitate from FAS [30]. In polyketide biosynthesis, it is known that DEBS1 without TE showed severe reduction of productivity [31]. However, the comparable production level of citreoisocoumarin **2** by WAC2 suggested that TE domain of fungal aromatic PKSs does work not just as a simple TE.

In fungal PKSs, the TE motif is not always present and 6-methylsalicylic acid synthase that lacks this motif is a typical example [17,27]. (Fig. 2) To the best of our knowledge, all fungal aromatic PKSs with TE motifs involve Claisen-type cyclization in their PKS reactions as shown in Fig. 5. Furthermore, their protein architecture, such as *C. lagenarium* PKS1 [32], and *Aspergillus* STCA [33], are quite similar as shown in Fig. 2. Thus, TE motifs in these

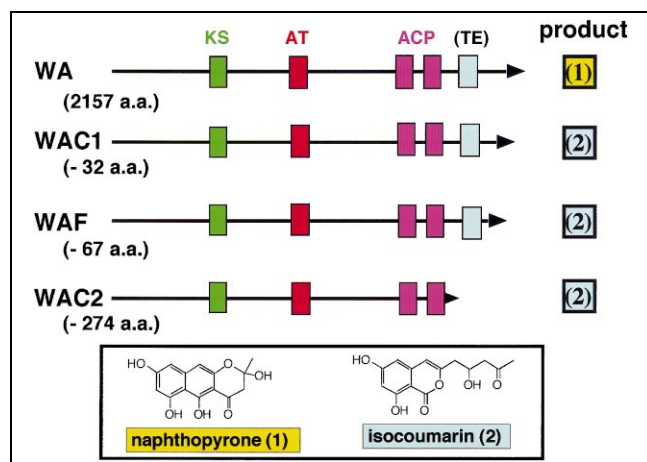


Fig. 4. Protein architecture of WA and its C-terminus truncated mutants with their product compounds. TE indicates a catalytic domain.

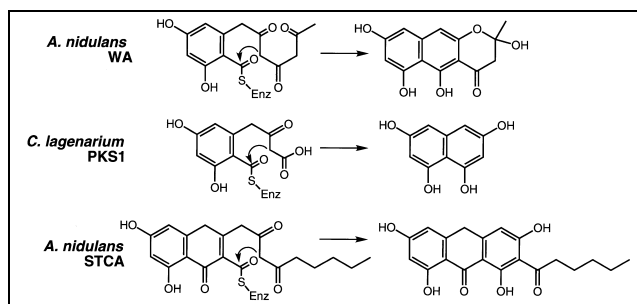


Fig. 5. Claisen-type cyclization reactions involved in fungal PKS reactions.

fungal PKSs may not be simple TE to release their products from PKS enzymes, but possibly be involved in Claisen-type cyclization as was found in WA PKS.

2.3. Site-directed mutagenesis of the WA PKS C-terminus

To date, four TEs have been structurally characterized by X-ray crystallography [35–38]. Of these, the *Vibrio harveyi* myristoyl ACP TE [35] and the mammalian palmitoyl protein TE [36] contain a classic Ser–His–Asp triad [39] in the active site. Similar catalytic triad might be operative in fungal PKS Claisen-type cyclization. When amino acid sequences of these Claisen cyclization type fungal PKSs, WA, PKS1, and STCA, are aligned, presence of several highly conserved amino acid residues becomes apparent in their C-terminus regions (Fig. 6). Thus, a series of site-directed mutagenesis was carried out in this region to identify the amino acid residue(s) essential for Claisen cyclization.

The mutant S1967A in which putative TE active site residue Ser¹⁹⁶⁷ was mutated to Ala did not produce naphthopyrone at all but produced citreoisocoumarin **2** instead. This is in accord with the result of WAC2 which has a deletion up to the TE motif. Also, the single mutation of His²¹²⁹ into Gln resulted in the production of citreoisocoumarin **2**, which is also in accord with the result of deletion mutant WAC1 lacking the amino acid residues after Asp²¹²⁶ just three residues upstream of His²¹²⁹ (Table 2). These site-directed mutagenesis results indicated that at least both Ser¹⁹⁶⁷ and His²¹²⁹ are essential for Claisen-type cyclization to form ring B of naphthopyrone YWA1 **1**.

2.4. Deletion of the N-terminus of WA PKS

Compared with 6-methylsalicylic acid synthase and reduced complex-type fungal PKSs, also conserved in these Claisen cyclization fungal PKSs are relatively longer N-terminus regions upstream of β -ketoacyl synthase domains, suggesting their importance in Claisen cyclase reactions (Fig. 2). If these fungal PKSs have homodimeric subunit structures with head-to-tail interaction as mammalian FASs [18], C-terminus Claisen cyclase domains might

| | | | |
|-------------|------|---|------|
| WA | 1785 | GVSADE IKSDENLNEL GMDSL SLTLVLGKIRESLDMDLPGEFFIENQTLDDQIETALDLKPKAVPTAVPQSQPITLPQSQ----- | 1863 |
| PKS1 | 1807 | GV EVDEIIAAPDLAAL GMDSL MSLSILGTL REK SGQDIPNDLFVTNPSSLLEVEKALGIGPKPKPAAAPKPAKSAPAASRREKVEPTKEIN | 1896 |
| STCA | 1843 | GVALDE LSAETVFADIG IDL SSMVITSRF RED LGMSLDSSFNLFEEVPTVARLQEFFGTTSGSTTGSSGSGSSEDETDSIPSTPEAYTT | 1932 |
| | 1864 | -----STKQLSTRPTSSSDNHPPAT S ILLQGNPRT A SKTLFLF PDGSGS AT S YAT I PGVSPNV AVYGLNCPYMKAP EKL T GS L DSL | 1944 |
| | 1897 | THPGNTTASITKPPPTTEIIDNYPHRKAT S ILLQGSTRT A T K NLWMV PDGSGC AT S Y T E I SQVSSNW AVWGLFSP FMKT P E E Y K GGVYGM | 1986 |
| | 1933 | ADTRVPECRPT----- T SVVLQGLPQ MA KQILF MLPDGGS AS S Y L T I PR L HADVA I V GLNCPYARD PEN M NG T HQSM | 2005 |
| | 1945 | TTPYLAEIR R QPT GPYNLGGWS AGGICAYDAARKLV L Q Q GEIVET L LL L DT P F I GLE K LP P RLYSFFNS I GL F GEGK--AAP----- P | 2027 |
| | 1987 | AAKFIEAMK A RQ S K GPY SLAG WS AGG V IAYEIVNQLTKA-GETVEN L II D AP CP VT I E L PRSLHAWFAS I GL L GEGDDEAKKI--- P | 2075 |
| | 2006 | IQSFCNEIK R Q PE GYHL GGWS SGGAFAYVTAEALINA-GNEV H SLII D AP VP Q V ME K L P TSFYECNN L GL F SNQPGGTTDGT A Q P | 2095 |
| | 2028 | AW L PHFLAFIDSLDAYKAV P LPFNEQEWKGLPKTYL V WAKDGVC PK PGDPWPEPAEDGSKDPREMVLLSN R T D L G PNG W DTLVGKEN | 2117 |
| | 2076 | SW L PHFAASVTALSN Y TAE P IPKEKCPNVMAIWCEGD V CHLPT-----DPRDPYPTGHAF-----LLDN R T D F G PN R W E YLDVNK | 2151 |
| | 2096 | PY L I P H F QATVDVMDL Y RV A PLKTNRM P KVGIIWASE T VMD E DNAP K M K GM H FMVQK----- R W D F G P D G W D V CPGAV | 2168 |
| | 2118 | IGGITVIHD A N H FTMTKG E KAKELATFMKNALGVCERRLV | 2157 |
| | 2152 | FRTRHMPG-- N H F SMMHG D YVSQTTLSPYND D NL T RSF | 2187 |
| | 2169 | FDILRAEGA- N HLR | 2181 |

Fig. 6. Alignment of amino acid sequences of C-terminus regions of Claisen cyclization fungal PKSs. Conserved amino acid residues are shown in red. Ser and His residues involved in Claisen cyclization are shown in green and blue, respectively.

interact with N-termini of complementary subunits. To assess this possibility, N-terminus deletion mutants of WA PKS were expressed and analyzed.

To our surprise, the mutant WAN1 with just five amino acid deletions from its N-terminus apparently lost PKS activity and no product was detected at all. None of mutants with further deletion from the N-terminus produced any detectable polyketide compounds. These results indicated the important role of N-termini of fungal Claisen-type PKSs, that has not been recognized so far.

2.5. Role of tandem ACP motifs

Another characteristic feature conserved in Claisen-type fungal PKSs is that most of them possess tandem ACP motifs where the Ser residue is known as the site to which the 4'-phosphopantethein group is attached as an anchor for polyketomethylene chain elongation. (Fig. 2) Significance of this feature was probed by site-directed mutagenesis. Both Ser¹⁶⁸² and/or Ser¹⁸⁰⁴, in two ACP motifs present in WA PKS, were mutated to Cys or Ala. As shown in Table 3, presence of at least a single intact Ser residue, either Ser¹⁶⁸² or Ser¹⁸⁰⁴, is good enough for WA PKS to function as naphthopyrone synthase. Although S1804A mutant produced heptaketide isocoumarins, it could be attributed to secondary structural change of the C-terminus. Thus, the characteristic feature of tandem ACPs in these PKSs is not related with Claisen cyclase function. It is interesting to know whether both Ser residues are modified with 4'-phosphopantethein in intact WA PKS, or just one specific residue is attached.

2.6. Mechanism of Claisen-type cyclization in fungal PKS reaction

Deletion and site-directed mutagenesis of WA PKS identified that at least two amino acid residues, Ser¹⁹⁶⁷ and His²¹²⁹, are essential for Claisen-type cyclization to form naphthopyrone YWA1 1. Ser¹⁹⁶⁷ and His²¹²⁹ may form a so-called catalytic triad type proton transfer system [39] with an acidic residue of yet unidentified Asp or Glu residue. A two-step mechanism could well explain Claisen-

Table 2

Products of WA PKS and its mutants with site-directed mutagenesis in possible Claisen cyclase domain

| | (1) | (2) |
|------------------|-----|-----|
| native WA | + | - |
| H2129Q | - | + |
| S1967A | - | + |

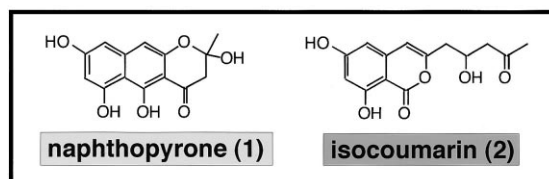
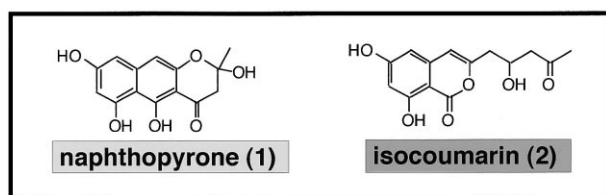


Table 3
Products of WA PKS and its mutants with site-directed mutagenesis in ACPs

| | (1) | (2) |
|-----------------------|-----|-----|
| native WA | + | - |
| S1682C | + | - |
| S1804C | + | - |
| S1682C, S1804C | - | - |
| S1682A | + | - |
| S1804A | - | + |
| S1682A, S1804A | - | - |



type cyclization and product release mechanism as shown in Fig. 7. In the first step, polyketomethylene intermediate anchored on phosphopantetheinyl residue is transferred on to Ser¹⁹⁶⁷ with aid of proton transfer from Asp (or Glu) via His²¹²⁹. This step immobilizes the monocyclic intermediate from highly mobile ACP to facilitate the following ring B cyclization reaction. Then, His²¹²⁹ imidazole base abstracts the methylene proton at the position corresponding to C4a of YWA1 **1** and aids its attack to ester carbonyl to cyclize. Following C–O bond cleavage releases the product compound. Formation of hemiketal proceeds non-enzymatically since its stereochemistry is not controlled (data not shown).

It is needless to say that further biochemical analysis is necessary not only to confirm this cyclization mechanism but also for mechanistic issues such as how these fungal PKSs control condensation cycles, stabilization of chemically active polyketomethylene intermediates, aromatization and cyclization.

3. Significance

Fungal PKSs are high molecular weight proteins containing multiple catalytic domains and thus classified as type I. However, different from bacterial modular PKSs, they apparently use their catalytic domains iteratively to elaborate product polyketide compounds. Catalytic domains such as β -ketoacyl synthase, acyltransferase, ACP in fungal PKSs are assigned based on homology with FASs and bacterial PKSs. In this work, actual function of so far assumed TE domain of fungal PKSs was identified to be Claisen cyclase using *A. nidulans* WA PKS. Based on the results of site-directed mutagenesis, a mechanism of the Claisen cyclization and following product release was proposed that could be general in Claisen cyclization type fungal PKSs.

4. Materials and methods

4.1. Feeding experiment with [1,2-¹³C₂]sodium acetate

A. oryzae transformant harboring pTA-nwA which produces naphthopyrone YWA1 **1** was first shake-cultured in 10 ml Czapek–Dox medium containing glucose as carbon source for 4 days at 30°C and then transferred into 200 ml of the same medium and shake-cultured for 4 days at 28°C. After filtration, mycelia were transferred into 2×200 ml Czapek–Dox medium containing

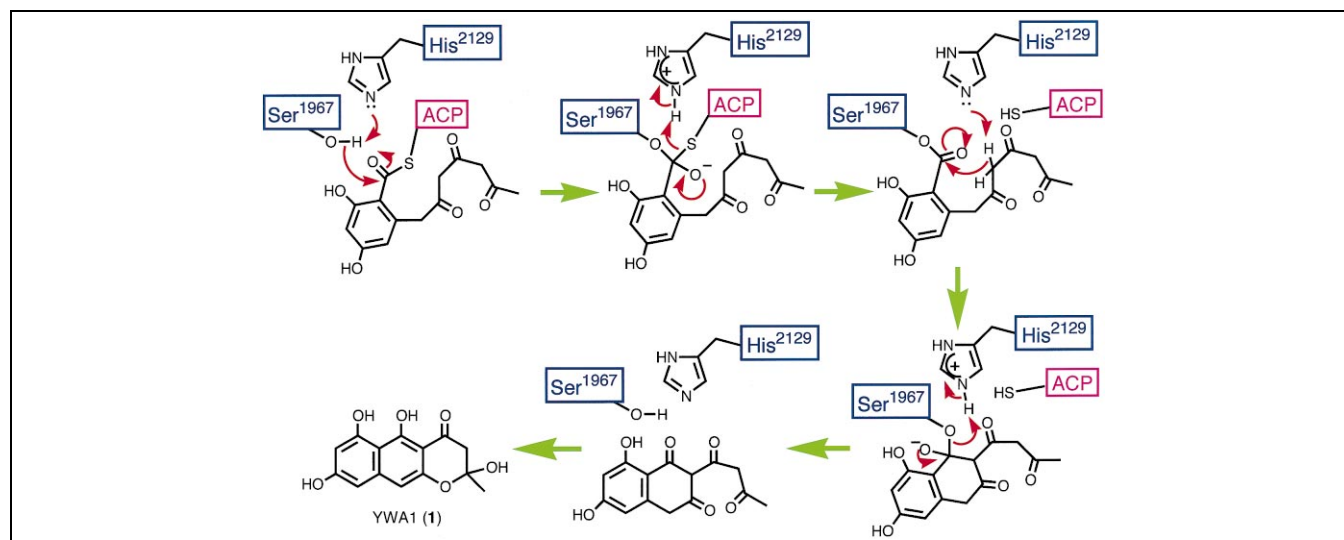


Fig. 7. Claisen cyclase mechanism involved in the second ring B formation of naphthopyrone.

starch as carbon source (induction medium) and shake-cultured for 1 day at 28°C. Then mycelia were collected on a Buchner funnel, washed with sterile water, transferred into 3×200 ml fresh induction medium, and then fed 3×100 mg of [1,2-¹³C₂]sodium acetate. After another 1 day shake-culture at 28°C, culture medium was collected and extracted with ethyl acetate. Five hundred mg of crude extract was suspended in 50 ml of concentrated HCl and kept at room temperature for one night, then poured into excess water and extracted with ethyl acetate. Dehydration product YWA2 **3** was then isolated by oxalic-acid-impregnated silica gel column chromatography (benzene–acetone solvent system) and recrystallized from ethanol. NMR spectra were obtained as DMSO-*d*₆ solution with a JEOL Alpha-500 instrument.

4.2. Fungal expression system

Fungal expression plasmid pTAex3 possessing the α -amylase promoter (*amyB*) of *A. oryzae* and auxotrophic marker *argB* of *A. nidulans* was used. Protoplast preparation and transformation procedures were described previously [40].

4.3. Construction of C-terminus deletion mutants

The C-terminal fragment of *wA* gene between unique *MluI* site and His²¹²⁵ was amplified by polymerase chain reaction (PCR) using *MluI*-site sense primer and antisense primer with a designed stop codon at the Asp²¹²⁶ and *MluI* sites. After subcloning into pT7-Blue T-vector, this fragment was cut out with *MluI* and inserted at *MluI* site of pTA-nwA [20] to construct pTA-wAC1 which could express WAC1 truncated PKS.

The C-terminal fragment of *wA* gene between unique *NheI* site and Thr¹⁸⁸³ was amplified by PCR using *NheI*-site sense primer and antisense primer with designed stop codon at Ser¹⁸⁸⁴. After subcloning into pT7-Blue T-vector, this fragment was cut out with *NheI* and *XbaI* (site in the vector), and replaced with *NheI* fragment of pTA-nwA to construct pTA-wAC2.

4.4. Construction of N-terminus deletion mutants

The region after N-terminus deletion of the *AflII* region of the *wA* gene was amplified with a designed sense primer with an *EcoRI* site and start codon and specific antisense primer. The amplified fragment was cut with *EcoRI* and *AflII* was replaced with the *EcoRI*–*AflII* region of pTA-nwA to construct pTA-wAN1 and other expression plasmids of N-terminus deletion mutants.

4.5. Site-directed mutagenesis

PCR-based site-directed mutagenesis was carried out. pTA-nwAH2129Q to express the H2129Q mutant was constructed as follows. The *MluI*-site to the His²¹²⁹ region of the *wA* gene was amplified with *MluI*-site sense primer and H2129Q antisense primer. This fragment was then used as a primer to amplify the *MluI* site to the stop codon region with antisense stop codon

primer with a *MluI* site. The amplified fragment was then subcloned into pT7-Blue T-vector and its sequence was confirmed. After cutting with *MluI*, the fragment was introduced into the *MluI* site of pTA-wA to construct pTA-nwAH2129Q as was carried out to construct pTA-nwA [20].

pTA-nwAS1967A to express S1967A mutant was constructed as follows. Ser¹⁹⁶⁷–*MluI* region of *wA* gene was amplified with S1967A sense primer and *MluI*-site antisense primer. This fragment was then used as a primer to amplify the *NheI*-site to *MluI*-site region with *NheI*-site sense primer. Amplified fragment was then subcloned into pT7-Blue T-vector and its sequence was confirmed. This *NheI*-site to *MluI*-site region was introduced into pTA-nwA to construct pTA-nwAS1967A.

Site-directed mutagenesis to construct ACP mutant expression plasmids was also carried out by PCR-based mutagenesis. PCR fragments with mutations were replaced with the corresponding region of pTA-nwA.

4.6. Detection of WA and mutant PKS products

A. oryzae transformants with WA PKS or its mutant expression plasmid were pre-cultured in Czapek–Dox medium containing glucose as the carbon source and then transferred into an induction medium (Czapek–Dox containing starch). After 1 day induction culture, the culture medium was acidified and extracted with ethyl acetate. Then, ethyl acetate extract was analyzed by HPLC with a reverse-phase column (Tosoh ODS-80Ts, 4.6×150 mm) maintained at 40°C. The solvent mixtures were (A) water/acetic acid (98/2) and (B) acetonitrile/acetic acid (98/2). The chromatography was run with a linear gradient of solvent from 5% B to 40% B in 30 min with detection at 254 nm. The retention time of YWA1 **1** was 27 min and that of citreoisocoumarin **2** was 22 min. Product analysis was carried out on each representative *A. oryzae* transformant, but production was not analyzed quantitatively because copy numbers of integrated expression cassette may vary in each *A. oryzae* transformant.

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References

- [1] D. O'Hagen, The polyketide metabolites, Ellis Horwood, New York, 1991.
- [2] R.L. Monaghan, J.S. Tkacz, Bioactive microbial products: Focus

- upon mechanism of action, *Annu. Rev. Microbiol.* 44 (1990) 271–301.
- [3] C.R. Hutchinson, I. Fujii, Polyketide synthase gene manipulation: a structure–function approach in engineering novel antibiotics, *Annu. Rev. Microbiol.* 49 (1995) 201–238.
 - [4] D.A. Hopwood, Genetic contributions to understanding polyketide synthase, *Chem. Rev.* 97 (1997) 2465–2497.
 - [5] C.R. Hutchinson, Combinatorial biosynthesis for new drug discovery, *Curr. Opin. Microbiol.* 1 (1998) 319–329.
 - [6] C.W. Carreras, D.V. Santi, Engineering of modular polyketide synthases to produce novel polyketides, *Curr. Opin. Biotechnol.* 9 (1998) 403–411.
 - [7] R. McDaniel, A. Thamchaipenet, C. Gustafsson, H. Fu, M. Betlach, M. Betlach, G. Ashley, Multiple genetic modifications of the erythromycin gene cluster to produce a library of novel ‘unnatural’ natural products, *Proc. Natl. Acad. Sci. USA* 96 (1999) 1846–1851.
 - [8] S. Donadio, M.J. Staver, J.B. McAlpine, S.J. Swanson, L. Katz, Modular organization of genes required for complex polyketide biosynthesis, *Science* 252 (1991) 675–679.
 - [9] M.A. Fernández-Moreno, E. Martínez, L. Boto, D.A. Hopwood, F. Malpartida, Nucleotide sequence and deduced functions of a set of co-transcribed genes of *Streptomyces coelicolor* A3 including the polyketide synthase for the antibiotic actinorhodin, *J. Biol. Chem.* 267 (1992) 19278–19290.
 - [10] S.E. Hallam, F. Malpartida, D.A. Hopwood, Nucleotide sequence, transcription and deduced function of a gene involved in polyketide antibiotic synthesis in *Streptomyces coelicolor*, *Gene* 74 (1988) 305–320.
 - [11] J. Beck, S. Ripka, A. Signer, E. Schiltz, E. Schweizer, The multifunctional 6-methylsalicylic acid synthase gene of *Penicillium patulum*. Its gene structure relative to that of other polyketide synthases, *Eur. J. Biochem.* 192 (1990) 487–498.
 - [12] I. Fujii, Y. Ono, H. Tada, K. Gomi, Y. Ebizuka, U. Sankawa, Cloning of the polyketide synthase gene *atX* from *Aspergillus terreus* and its identification as the 6-methylsalicylic acid synthase gene by heterologous expression, *Mol. Gen. Genet.* 253 (1996) 1–10.
 - [13] I. Fujii, Y. Mori, A. Watanabe, Y. Kubo, G. Tsuji, Y. Ebizuka, Heterologous expression and product identification of *Colletotrichum lagenarium* polyketide synthase encoded by the *PKS1* gene involved in melanin biosynthesis, *Biosci. Biotechnol. Biochem.* 63 (1999) 1445–1452.
 - [14] G. Yang, M.S. Rose, B.G. Turgen, O.C. Yoder, A polyketide synthase is required for fungal virulence and production of the polyketide T-toxin, *Plant Cell* 8 (1996) 2139–2150.
 - [15] L. Hendrickson, C.R. Davis, C. Roache, D.K. Nguyen, T. Aldrich, P.C. McAda, C.D. Reeves, Lovastatin biosynthesis in *Aspergillus terreus*: characterization of blocked mutants, enzyme activities and multifunctional polyketide synthase gene, *Chem. Biol.* 6 (1999) 429–439.
 - [16] J. Kennedy, K. Auclair, S.G. Kendrew, C. Park, J.C. Vederas, C.R. Hutchinson, Modulation of polyketide synthase activity by accessory proteins during lovastatin biosynthesis, *Science* 284 (1999) 1368–1372.
 - [17] I. Fujii, Polyketide biosynthesis in filamentous fungi, in: U. Sankawa, (Ed.), *Comprehensive Natural Products Chemistry*, Vol. 1, Elsevier, Oxford, 1999, pp. 409–441.
 - [18] S. Smith, The animal fatty acid synthase: one gene, one polypeptide, seven enzymes, *FASEB J.* 8 (1994) 1248–1259.
 - [19] A. Watanabe, Y. Ono, I. Fujii, U. Sankawa, M.E. Mayorga, W.E. Timberlake, Y. Ebizuka, Product identification of polyketide synthase encoded by *Aspergillus nidulans* *wA* gene, *Tetrahedron Lett.* 39 (1998) 7733–7736.
 - [20] A. Watanabe, I. Fujii, U. Sankawa, M.E. Mayorga, W.E. Timberlake, Y. Ebizuka, Re-identification of *Aspergillus nidulans* *wA* gene to code for a polyketide synthase of naphthopyrone, *Tetrahedron Lett.* 40 (1999) 91–94.
 - [21] I. Fujii, Y. Mori, A. Watanabe, Y. Kubo, G. Tsuji, Y. Ebizuka, Enzymatic synthesis of 1,3,6,8-tetrahydroxynaphthalene solely from malonyl coenzyme A by a fungal iterative Type I polyketide synthase *PKS1*, *Biochemistry* 39 (2000) 8853–8858.
 - [22] . Watanabe, I. Fujii, H.-F. Tsai, Y.C. Chang, K.J. Kwon-Chung, Y. Ebizuka, *Aspergillus fumigatus* *alb1* encodes naphthopyrone synthase when expressed in *Aspergillus oryzae*, *FEMS Microbiol. Lett.* 192 (2000) 39–44.
 - [23] M.E. Mayorga, W.E. Timberlake, Isolation and molecular characterization of the *Aspergillus nidulans* *wA* gene, *Genetics* 126 (1990) 73–79.
 - [24] M.E. Mayorga, W.E. Timberlake, The developmentally regulated *Aspergillus nidulans* *wA* gene encodes a polypeptide homologous to polyketide and fatty acid synthases, *Mol. Gen. Genet.* 235 (1992) 205–212.
 - [25] R. Aggarwal, P. Caffrey, P.F. Leadlay, C.J. Smith, J. Staunton, The thioesterase of the erythromycin-producing polyketide synthase: mechanistic studies in vitro to investigate its mode of action and substrate specificity, *J. Chem. Soc. Chem. Commun.* (1995) 1519–1520.
 - [26] P.M. Shoolingin-Jordan, I.D.G. Campuzano, Biosynthesis of 6-methylsalicylic acid, in: U. Sankawa (Ed.), *Comprehensive Natural Products Chemistry*, Vol. 1, Elsevier, Oxford, 1999, pp. 345–365.
 - [27] I. Fujii, A. Watanabe, Y. Mori, Y. Ebizuka, Structures and functional analyses of fungal polyketide synthase genes, *Actinomycetology* 12 (1998) 1–14.
 - [28] F. Leeper, J. Staunton, The biosynthesis of rubrofusarin, a polyketide naphthopyrone from *Fusarium culmorum*: ¹³C NMR Assignments and incorporation of ¹³C- and ²H-labelled acetates, *J. Chem. Soc. Perkin Trans. I* (1984) 2919–2925.
 - [29] J.L. Bloomer, T.J. Caggiano, C.A. Smith, Demonstration of an alternative chain folding in the pyranonaphthalene fonsecin, *Tetrahedron Lett.* 23 (1982) 5103–5106.
 - [30] N. Singh, S.J. Wakil, J.K. Stoops, On the question of half- or full-site reactivity of animal fatty acid synthetase, *J. Biol. Chem.* 259 (1984) 3605–3611.
 - [31] C.M. Kao, G. Luo, D.E. Cane, C. Khosla, Manipulation of macro-lide ring size by directed mutagenesis of a modular polyketide synthase, *J. Am. Chem. Soc.* 117 (1995) 9105–9106.
 - [32] Y. Takano, Y. Kubo, K. Shimizu, K. Mise, T. Okuno, I. Furusawa, Structural analysis of *PKS1*, a polyketide synthase gene involved in melanin biosynthesis of *Colletotrichum lagenarium*, *Mol. Gen. Genet.* 249 (1995) 162–167.
 - [33] J.-h. Yu, T.J. Leonard, Sterigmatocystin biosynthesis in *Aspergillus nidulans* requires a novel type I polyketide synthase, *J. Bacteriol.* 177 (1995) 4792–4800.
 - [34] G.H. Feng, T.J. Leonard, Characterization of the polyketide synthase gene (*pksL1*) required for aflatoxin biosynthesis in *Aspergillus parasiticus*, *J. Bacteriol.* 177 (1995) 6246–6254.
 - [35] D.M. Lawson, U. Derewenda, L. Serre, S. Ferri, R. Szttnner, Y. Wei, E.A. Meighen, Z.S. Derewenda, Structure of a myristoyl-ACP-specific thioesterase from *Vibrio harveyi*, *Biochemistry* 33 (1994) 9382–9388.
 - [36] J.J. Bellizzi, J. Widom, C. Kemp, J.-Y. Lu, A.K. Das, S.L. Hofmann, J. Clardy, The crystal structure of palmitoyl protein thioesterase 1 and the molecular basis of infantile neuronal ceroid lipofuscinosis, *Proc. Natl. Acad. Sci. USA* 97 (2000) 4573–4578.
 - [37] M.M. Benning, G. Wesenberg, R. Liu, K.L. Taylor, D. Dunaway-Mariano, H.M. Holden, The three-dimensional structure of 4-hydroxybenzoyl-CoA thioesterase from *Pseudomonas* sp. strain CBS-3, *J. Biol. Chem.* 273 (1998) 33572–33579.
 - [38] J. Li, U. Derewenda, Z. Dauter, S. Smith, Z.S. Derewenda, Crystal structure of the *Escherichia coli* thioesterase II, a homolog of the human Nef binding enzyme, *Nature Struct. Biol.* 7 (2000) 555–559.
 - [39] D.M. Blow, J.J. Birktoft, B.S. Hartley, Role of a buried acid group in the mechanism of action of chymotrypsin, *Nature* 221 (1969) 337–340.
 - [40] K. Gomi, Y. Iimura, S. Hara, Integrative transformation of *Aspergillus oryzae* with a plasmid containing the *Aspergillus nidulans* *argB* gene, *Agric. Biol. Chem.* 51 (1987) 2549–2555.