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A novel hexaketide naphthalene synthesized by a chimeric polyketide synthase composed of fungal pentaketide and heptaketide synthases

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Abstract—*Aspergillus nidulans wA* and *Colletotrichum lagenarium pks1* code for iterative type I polyketide synthases (PKSs) and synthesize heptaketide naphthopyrone and pentaketide naphthalene, respectively. A chimeric PKS, called SW-B, composed of WA and PKS1 synthesized mainly hexaketide isocoumarin and an unknown yellow compound. The latter was identified as the novel hexaketide 2-acetyl-1,3,6,8-tetrahydroxynaphthalene. These findings indicate broad substrate specificity of the WA C-terminal domain for Claisen-type cyclization and an unprecedented chain length determination mechanism of fungal aromatic PKSs.

Fungal aromatic polyketides constitute a structurally diverse class of natural products which exhibits various types of biological activity. For example, aflatoxin B_1 is known to be a hepatotoxic mycotoxin, griseofulvin is an antifungal drug, and DHN-melanin is an essential compound for pathogenicity of *Aspergillus fumigatus* causing pulmonary aspergillosis in humans[1] and of *Colletotrichum lagenarium* causing anthracnose in Cucurbitaceous plants.[2] Biosynthesis of these compounds is achieved by an iterative condensation of acetate units to form a polyketomethylene chain and its subsequent cyclization. Both of those processes are catalyzed by polyketide synthase (PKS), followed by modifications by various tailoring enzymes, such as P-450s and oxidoreductases. Compared with the number of diverse fungal aromatics, only a few aromatic PKS genes have been cloned from fungi. They code for multifunctional enzymes, the so-called iterative type I PKSs, similar to a vertebrate fatty acid synthase. In contrast to bacterial type II PKSs and modular PKSs, it is not clear how fungal aromatic PKSs control the chain length of polyketomethylene intermediate and its cyclization.

A. nidulans wA[3] and *C. lagenarium pks1*[4] have been cloned as genes for spore pigment synthesis, which code for iterative type I PKS producing a heptaketide naphthopyrone (YWA1, **1**)[5] and a pentaketide 1,3,6,8-tetrahydroxynaphthalene (T4HN, **2**),[6] respectively. They show identical domain architecture (Fig. 1) with an extended N-terminal domain (domain A), a central domain (domain B) and C-terminal thioesterase-like

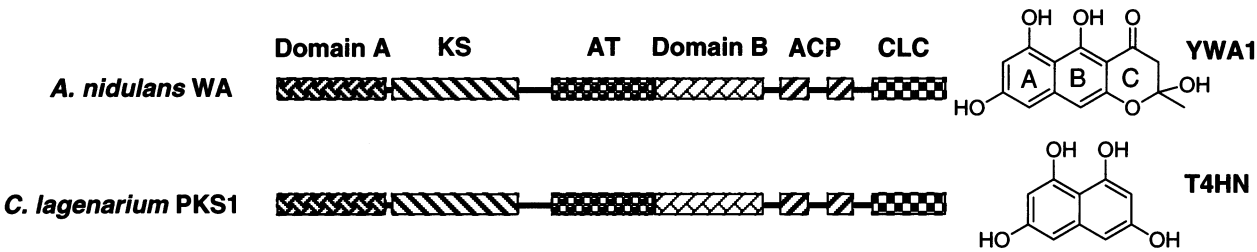

Figure 1. Domain architecture of WA and PKS1. KS, β-ketoacylsynthase; AT, acyltransferase; ACP, acyl carrier protein; CLC, Claisen-type cyclase.

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domain in addition to β-ketoacylsynthase (KS), acyltransferase (AT) and two tandem acyl carrier proteins (ACPs). Recently, we have determined that the C-terminal domain of WA (CLC), which has high homology with the known thioesterase domains, functions as a Claisen-type cyclase for B-ring formation of YWA1 **1**.[7] To obtain further insights into the mechanism of PKS-catalyzed reactions, we have constructed several deletion and chimeric mutant PKSs using *A. nidulans* WA and *C. lagenarium* PKS1. Among them, the chimeric PKS SW-B (Fig. 2), a PKS1 derivative of which the C-terminal region including two ACPs and a thioesterase-like domain was replaced with that of WA, synthesized an unknown yellow pigment when expressed in the heterologous fungus *Aspergillus oryzae*. Here we report the identification of the structure of this yellow pigment and some features of the SW-B-catalyzed reaction.

The chimeric PKS gene *sw*-B was amplified through the polymerase chain reaction (PCR) with a primer set including a chimeric oligonucleotide positioned on the C-terminal region of domain B (Fig. 2). The PCR amplified *sw*-B was flanked by the *att* recombination site on both ends, and cloned into the fungal expression vector pTAex3 using GATEWAY cloning technology (GIBCO BRL) and then introduced into *A. oryzae* M-2-3, an arginine auxotrophic mutant, by a method reported previously.[8] The *A. oryzae* M-2-3/pTA-sw-B transformant exhibited yellow pigmentation, which was not observed in a control transformant with pTAex3. After preculture for 1 day in Czapeck-Dox medium supplemented with glucose, expression was induced by shaking the culture in minimal medium supplemented with starch for an additional day.[9] The culture medium was then extracted with ethyl acetate. Thin-layer chromatographic analysis of the crude extract indicated the production of an unknown yellow pigment in addition to other colorless compounds. Silica gel (impregnated with 0.5N oxalate) column chromatography with a benzene–acetone solvent system afforded 7 mg of yellow compound from 600 ml of culture medium.

The molecular formula of the yellow compound (λ_{max} at 408, 330, 318, 278, 230 and 220 nm in CH_3CN) was determined to be $C_{12}H_{10}O_5$ by high-resolution electron-impact mass spectrometry (HR-EIMS) (*m*/*z* 234.0512 [M^+], Δ −1.7 mmu). Its ^{13}C and ^1H NMR data are summarized in Table 1. These spectral data and heteronuclear multiple-bond correlations (HMBC) easily led to the identification of its structure as the novel hexaketide 2-acetyl-1,3,6,8-tetrahydroxynaphthalene (ATHN, compound **3**) (Fig. 3). The unusually low proton resonance at 17.20 ppm indicates that 1′-carbonyl oxygen forms a hydrogen bond with 1-OH and not with 3-OH. Although derivatives of the heptaketide naphthopyrone **1** and pentaketide naphthalene **2** occur widely among bacteria, fungi and plants, derivatives of **3** are rare in nature. In echinoderms, a few naphthazarins, such as spinochrome A (**4**), have been identified,[10] while in fungi, the presence of compound **5** has been reported after a mass spectra library search for gas chromatography–mass spectrometry (GC–MS) peaks of pyrolysis products of truffle melanins (Fig. 4).[11]

Table 1. ^{13}C and ^1H NMR data of compound **3** (ATHN)

Position	^{13}C δppm	^1H δppm (*J* in Hz)
1	169.9	
2	105.6	
3	156.4	
4	100.3	6.35 (1H, s)
4a	142.7	
5	101.1	6.35 (1H, d, *J*=2.2)
6	162.6	
7	99.8	6.19 (1H, d, *J*=2.2)
8	161.1	
8a	104.9	
1′	204.5	
2′	31.7	2.72 (3H, s)
1-OH		17.20 (1H, brs)
3,6,8-OH		9.90, 9.33, 9.00 (1H, brs)

^1H (500.00 MHz) and ^{13}C (125.65 MHz) NMR spectra were obtained in acetone-d_6.

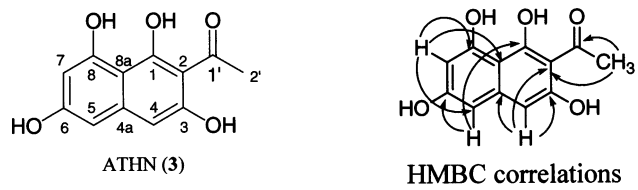

Figure 3. Structure and HMBC correlations of compound **3** (ATHN).

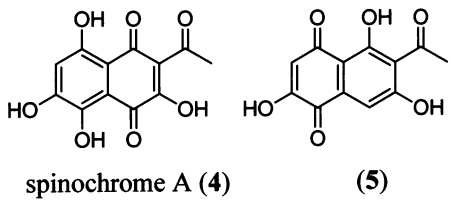

Figure 4. Derivatives of compound **3** (ATHN).

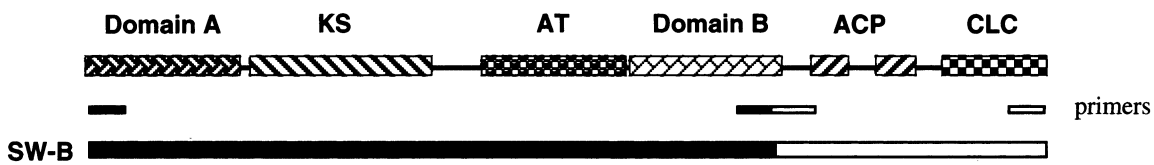

Figure 2. Structure of SW-B. The three short bars above SW-B indicate primers used for the construction of the SW-B gene by PCR. Black and white bars indicate parts of PKS1 and WA, respectively.

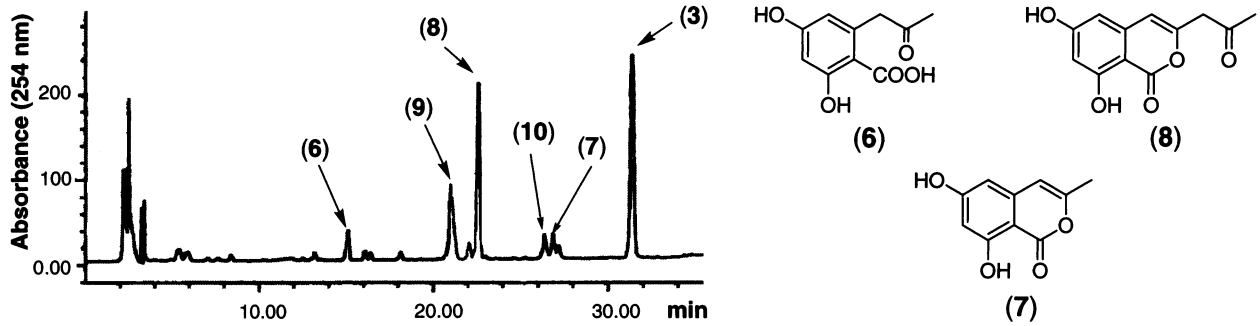

Figure 5. HPLC analysis of the culture medium of *A. oryzae* M-2-3/pTA-sw-B. A reverse-phase column (TOSOH ODS-80Ts, 4.6×150 mm) was eluted with a linear gradient of 5–40% of CH_3CN in 2% AcOH (aq.) over 30 min at a flow rate of 0.8 ml/min, with detection at 254 nm.

In addition to pentaketide carboxylic acid (**6**), pentaketide isocoumarin (**7**) and hexaketide isocoumarin (**8**), the presence of two unknown compounds (**9** and **10**) in the culture medium was detected by reverse-phase HPLC analysis (Fig. 5). However, no trace of YWA1 **1** and T4HN **2** were detected. Compounds **6**–**8** were identified by LC–ESIMS through comparison with authentic samples.

Compound **9** was converted to **7** by heating at 100°C for 10 min in HCl aq. (pH 1). Negative LC–ESIMS of **9** exhibited two prominent peaks at m/z 271 and at m/z 191, and the latter corresponded to **7**. The $[M-H]^-$ peak at m/z 271 was accompanied by a minor peak at m/z 273 (intensity approximately 5%), indicating the presence of one sulfur atom in **9**. From these observations, compound **9** was assumed to be *O*-sulfated pentaketide isocoumarin, although the position of the sulfate attachment remains to be determined. The similar chemical and LC–MS behaviour of compound **10** suggests that it is *O*-sulfated ATHN. Sulfate conjugation of SW-B products could be attributed to the host strain *A. oryzae* M-2-3. This is the first example of sulfate conjugation of polyketide compounds in *A. oryzae*.

The formation of compound **3**, a Claisen-type cyclization product, indicates that the WA CLC domain can cyclize a hexaketide intermediate as efficiently as an intrinsic heptaketide intermediate. On the other hand, the absence of T4HN **2** among pentaketide products implies that a pentaketide intermediate is not a good substrate for WA CLC domain. It will be interesting to elucidate more detailed functions of the WA CLC domain, such as its tolerance for substrates and how it catalyzes Claisen-type cyclization.

Another interesting feature of SW-B-catalyzed reactions is the chain length of the products. SW-B, a derivative of pentaketide synthase PKS1, produces hexaketides, such as **3** and **8**, as major products. The dramatic shift in the chain length of SW-B products was unexpected, because the WA CLC domain had previously been demonstrated to have no relevance to chain length determination,[7] and there has been no evidence that ACP controls the chain length of products in fungal PKSs or in bacterial type II and modular type I PKSs. The results reported in this paper suggest the presence of an unprecedented mechanism for chain length determination in fungal aromatic PKSs that is apparently very different from that of bacterial PKSs. To resolve this interesting and important issue, further analysis of other mutants is now in progress.

Acknowledgements

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