Pergamon

Tetrahedron Letters 40 (1999) 91–94

TETRAHEDRON LETTERS

Re-identification of *Aspergillus nidulans wA* Gene to Code for a Polyketide Synthase of Naphthopyrone

Akira Watanabe, Isao Fujii, Ushio Sankawa,[1] María E. Mayorga,[2] William E. Timberlake,[3] Yutaka Ebizuka*

Graduate School of Pharmaceutical Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan
[1]Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, Sugitani 2630, Toyama 930-0194, Japan
[2]Millennium Pharmaceuticals Inc., Building 300, One Kendall Square, Cambridge, MA 02139, USA
[3]Cereon Genomics, LLC, Building 300, One Kendall Square, Cambridge, MA 02139, USA

Received 10 September 1998; revised 16 October 1998; accepted 23 October 1998

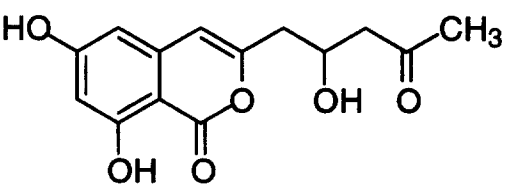
Abstract

By reconstruction and expression of the full-length WA (NWA) polyketide synthase of *A. nidulans* based on the revised *wA* gene sequence, its product was re-identified to be a naphthopyrone compound YWA1 instead of citreoisocoumarin and its derivatives, which were found to be products of C-terminus truncated WA polyketide synthase. YWA1 shows yellow color and is considered to be a true intermediate of *A. nidulans* asexual spore pigments. Thus, the *wA* gene is identified to code for a polyketide synthase of heptaketide naphthopyrone YWA1.

Keywords: biosynthesis, fungi, pigments, polyketides, pyrones.

Structural diversity and biological activities of polyketide compounds promoted their biosynthetic studies and led to isolation of genes for polyketide synthases (PKSs) from a number of organisms [1]. Several fungal PKS genes have been cloned and found to code for so-called type I PKS polypeptides [2]. Of these, only a few were identified their direct PKS reaction products. We previously reported expression of the *atX* gene from *Aspergillus terreus* [3] and the *wA* gene from *Aspergillus nidulans* [4] and identified their products to be 6-methylsalicylic acid and citreoisocoumarin (**1**), respectively.

The *wA* gene was assumed to code for a PKS involved in conidial spore pigment biosynthesis and its expected PKS product was a yellow pigment which could be polymerized by laccase to mature green spore pigments [5,6]. Production of citreoisocoumarin (**1**) and its derivatives was observed in our previous expression experiment [4]. However, these compounds showed light yellow-brownish color or colorless. Thus, it was skeptical whether citreoisocoumarin (**1**) and/or its derivatives are the true spore pigment intermediates.



citreoisocoumarin (**1**)

0040-4039/99/$ - see front matter
PII: S0040-4039(98)02285-0

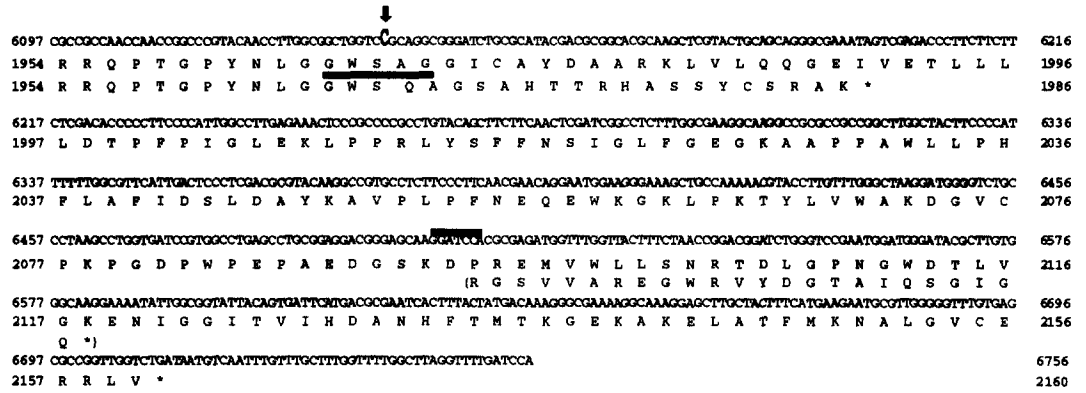


Fig. 1 Nucleotide and deduced amino acid sequences of *A. nidulans wA* gene C-terminus.
The arrow indicates the nucleotide missing in the original report [6]. Underlined amino acid sequence is the thioesterase motif found in the revised amino acid sequence. Overlined is the *Bam*HI site which was used for the expression plasmid pTA-wA construction in the previous report [4]. The additional C-terminal amino acid sequence of previously expressed WA PKS derived from the ligated expression vector pTAex3 [7] is shown in parentheses.

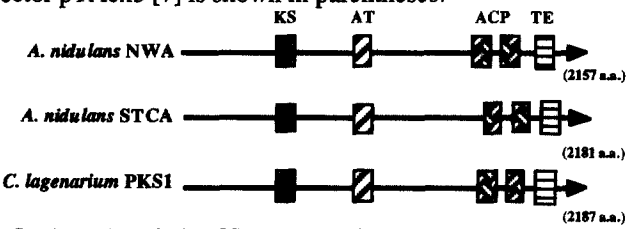


Fig. 2 Architecture of fungal type I polyketide synthases. KS, β-ketoacyl synthase; AT, acyltransferase; ACP, acyl carrier protein; TE, thioesterase.

In the *wA* coded PKS polypeptide (WA), presence of tandem acyl carrier protein (ACP) motifs were reported [6]. Similar feature is found in the reported fungal PKSs like PKS1 of *Colletotrichum lagenarium* for melanin biosynthesis [8] and STCA of *A. nidulans* for sterigmatocystin biosynthesis [9]. Comparison of active site organizations of these tandem ACP type fungal PKSs revealed that WA PKS alone lacks thioesterase (TE) motif at its C-terminus and have shorter polypeptide length (1986 amino acids). To confirm this discrepancy, we carried out resequencing of the *wA* gene around the C-terminal region and found an error, missing one base, in the original report by Timberlake's group [6]. That is the 6137th C in the original sequence (accession no. X65866) and this should be two consecutive Cs. This error caused an apparent frame shift in the deduced amino acid sequence as shown in Fig. 1. The corrected WA polypeptide (NWA) has a similar polypeptide length with TE motif at its C-terminus and thus the quite similar active site organization to those of PKS1 and STCA as shown in Fig. 2.

For the construction of expression plasmid in the previous report, the gene was cut at the *Bam*HI site just downstream of a stop codon at nucleotide position 6196 of the original sequence. However, actual open reading frame continues further down over the *Bam*HI site. Thus, the previous expression plasmid pTA-wA lacked a part of C-terminus and had additional extra amino acids derived from the ligated vector sequence. To express the correct full-length NWA polypeptide, a new expression plasmid pTA-nwA was constructed by replacing the C-terminus region in pTA-wA as shown in Fig. 3. The *A. oryzae* transformant with pTA-nwA obtained as in the same way as previously reported [4] showed yellow pigmentation on agar plates. This observation was quite a contrast to the pTA-wA transformant that showed white mycelia. From the one liter induction culture medium of the pTA-nwA transformant, 100 mg of yellow compound was isolated by silica gel column chromatography and recrystallized from ethylacetate / n-hexane (m.p.: 175 °C). By HR-EIMS, the compound showed a peak at 258.0484 for $C_{14}H_{10}O_5$ corresponding to an M^+-H_2O ion (calcd: 258.0528). Its ^1H and ^{13}C NMR spectral data are

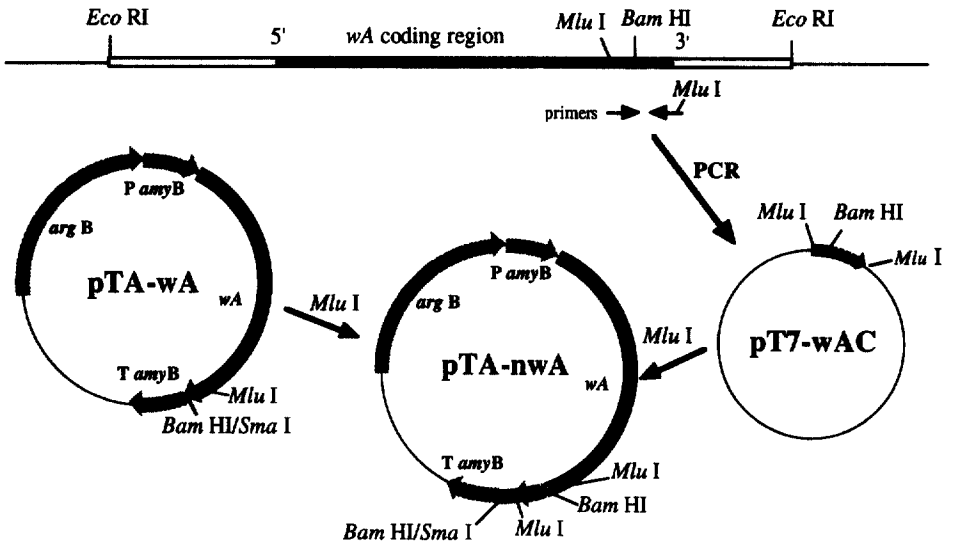
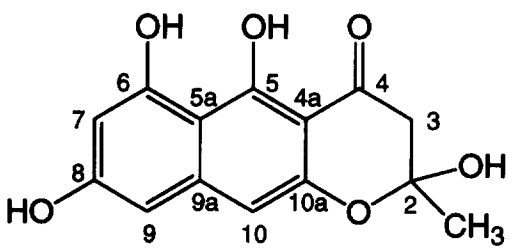
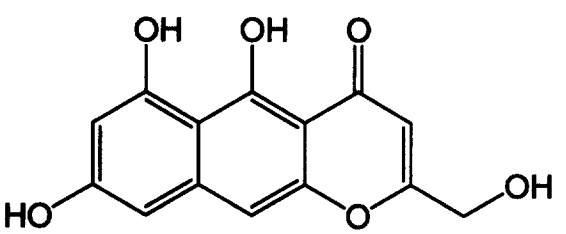


Fig. 3 Construction of expression plasmid pTA-nwA



YWA1 (2)



parasperone A (3)

Table 1
^{13}C and ^{1}H NMR data of YWA1 (2)

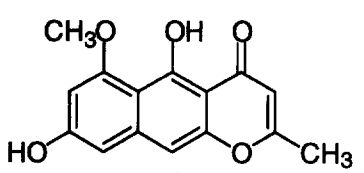
position	^{13}C δppm	^{1}H δppm (J in Hz)
2	101.4	
3	47.9	2.87 (1H, d, J=17.1)
		3.17 (1H, d, J=17.1)
4	198.9	
4a	103.0	
5	165.0	
5a	105.0	
6	160.8	
7	100.9	6.28 (1H, d, J = 1.8)
8	162.8	
9	102.5	6.51 (1H, d, J = 1.8)
9a	143.6	
10	102.8	6.45 (1H, s)
10a	154.2	
CH_3	28.4	1.72 (3H, s)
2-OH		6.05 (1H, s)
5-OH		15.34 (1H, brs)
6-OH		9.42 (1H, brs)
8-OH		9.14 (1H, brs)

Proton (500.00 MHz) and carbon (125.65 MHz) NMR spectra were obtained in acetone-d_6.

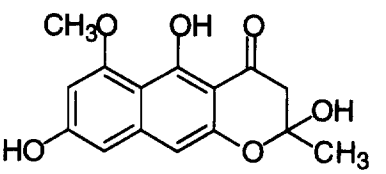
shown in Table 1. From these data and HMBC correlation, the product compound was identified to be a novel naphthopyrone compound named YWA1 (**2**). The characteristic UV/VIS absorption (λmax at 411, 335, 323, 281 and 229 nm in CH_3CN) and the unusual low field resonance at 15.3 ppm of 5-OH proton indicated the linear-type structure of naphthopyrone similar to parasperone A (**3**). Naphthopyrone compounds such as rubrofusarin (**4**), fonsecin (**5**), *etc*. were isolated from several fungal origins. Among them, isolation of parasperone A (**3**) [10] from a laccase deficient strain of *Aspergillus parasiticus* as an intermediate of asexual spore pigments should be noted. Thus, naphthopyrone compound YWA1 (**2**) is considered to be an actual intermediate of *A. nidulans* conidial spore pigments, and the *wA* gene is identified to code for a polyketide synthase of heptaketide naphthopyrone YWA1 (**2**).

Production of citreoisocoumarin (**1**) derivatives was not observed in *A. oryzae*/pTA-nwA transformant culture. It is quite interesting that the C-terminal modified WA PKS produces citreoisocoumarin (**1**) and its derivatives which are heptaketides as is YWA1 (**2**), the product of a full-length NWA PKS. These results strongly indicate that condensation of acetate units to form the specific chain-length, heptaketides in NWA and WA PKSs, is controlled by β-ketoacyl synthase (KS), acyltransferase (AT), and ACP regions of the PKS polypeptides and the TE might be involved in cyclization and product release from the PKS polypeptides. The fact that 6-methylsalicylic acid synthase (MSAS) of *Penicillium patulum* does not possess TE motif [11] might suggest that TE has a specific role other than simple product release.

In naphthopyrone biosynthesis, two contradictory folding patterns for heptaketide intermediates were reported for rubrofusarin (**4**) [12] and fonsecin (**5**) [13]. For further mechanistic analysis of NWA PKS reaction to form YWA1 (**2**), it is necessary to identify which folding pattern is for YWA1 (**2**) biosynthesis. This experiment is now under way in this laboratory.



(**4**)



(**5**)

Acknowledgment: We are grateful to Prof. Katsuya Gomi of Tohoku University, Graduate School of Agriculture, Japan, for his kind supply of plasmid and fungal strains. This work was in part supported by Research for the Future Program (No. JSPS-RFTF 96I00302), The Japan Society for the Promotion of Science.

REFERENCES:

[1] Hopwood DA. Chem. Rev. 1997;97:2465-2497.

[2] Fujii I, Watanabe A, Mori Y, Ebizuka Y. Actinomycetol. 1998;12:1-14.

[3] Fujii I, Ono Y, Tada H, Gomi K, Ebizuka Y, Sankawa U. Mol. Gen. Genet. 1996;253:1-10.

[4] Watanabe A, Ono Y, Fujii I, Sankawa U, Mayorga ME, Timberlake WE, Ebizuka Y. Tetrahedron Lett. 1998;39:7733-7736.

[5] Mayorga ME, Timberlake WE. Genetics 1990;126:73-79.

[6] Mayorga ME, Timberlake WE. Mol. Gen. Genet. 1992;235:205-212.

[7] Fujii T, Yamaoka H, Gomi K, Kitamoto K, Kumagai C. Biosci. Biotech. Biochem. 1995;59:1869-1874.

[8] Takano Y, Kubo Y, Shimizu K, Mise K, Okuno T, Furusawa I. Mol. Gen. Genet. 1995;249:162-167.

[9] Yu Jh, Leonard TJ. J. Bacteriol. 1995;248:270-277.

[10] Brown DW, Hauser FM, Tommasi R, Corlett S, Salvo JJ. Tetrahedron Lett. 1993;34:419-422.

[11] Beck J, Ripka S, Signer E, Schiltz E, Schweizer E. Eur. J. Biochem. 1990;192:487-498.

[12] Leeper FJ, Staunton JJ. Chem. Soc. Perkin Trans. I. 1984:2919-2925.

[13] Bloomer JL, Caggiano TJ, Smith CA. Tetrahedron Lett. 1982;23:5103-5106.