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Mpp7 controls regioselective Knoevenagel condensation during the biosynthesis of *Monascus* azaphilone pigments

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ABSTRACT

Targeted inactivation of the *mpp7* gene in the *Monascus* azaphilone pigment (MAzP) biosynthetic gene cluster resulted in the accumulation of monasfluol A (**7**) and B (**8**), of which the latter was a novel compound, and the abolition of the main MAzPs. It is thus proposed that **7** and **8** are the products of non-enzymatic Knoevenagel condensation followed by a reduction and that Mpp7 assists in regioselective Knoevenagel aldol condensation during MAzP biosynthesis.

Monascus extract has served as a traditional food colorant in eastern Asia, and the main colored substances of this extract are azaphilone compounds. These *Monascus* azaphilone pigments (MAzPs), produced from some *Monascus* species, such as *Monascus ruber*, *Monascus pilosus*, and *Monascus purpureus*, comprise ankaflavin (**1**), monascin (**2**), rubropunctatin (**3**), monascorubrin (**4**), rubropuctamine (**5**), and monascorubramine (**6**), along with various minor compounds (Scheme 1).[1] MAzPs can be divided into three classes depending on their colors: yellow, orange, and red. MAzPs **1** and **2** are yellow-colored, and the orange pigments **3** and **4** turn into the red pigments **5** and **6**, respectively, when reacted with ammonia. The yellow and orange MAzPs display diverse biological properties including anti-diabetic, anti-inflammatory, anti-atherosclerosis, and anti-cancer activities.[2]

T-DNA random mutagenesis studies have previously localized the MAzP biosynthesis loci in *M. ruber* and *M. purpureus*,[3] the latter of which was used to identify the MAzP biosynthetic gene cluster from the *M. pilosus* genome sequence (GenBank accession no. KC148521).[3b] The genome sequences of *M. ruber* and *M. purpureus* recently became available in the genome portal of the Department of Energy-Joint Genome Institute (DOE-JGI). These *M. ruber* and *M. purpureus* genome sequences were used to compare the genetic organizations of the MAzP biosynthesis clusters from these three *Monascus* species (Fig. 1). The clusters encode the blueprints for four oxidoreductases, two transcription factors, an acyltransferase, and an esterase between *MpPKS5* and *MpfasB2* (Fig. 1).[4] *MpPKS5* encodes a non-reducing fungal type I polyketide synthase (NR-fPKS) with a reductive releasing domain and its inactivation resulted in the loss of MAzP biosynthesis.[3b] *MpfasA2*/*MpfasB2* is predicted to encode a canonical fungal fatty acid synthase and is proposed to be involved in the formation of the short chain fatty acyl thioester for MAzP biosynthesis.[3b] Among the genes located outside this central region, *mppF* is suggested to mediate the hydroxylation step for the pyran-ring closure (Scheme 1 and Fig. 1). This type of hydroxylation is a prerequisite for the formation of the azaphilone structure.[5] Between *MpfasB2* and *mppF*, seven ORFs (*mpp1–7*) are found in *M. pilosus* and *M. ruber*, but their roles in MAzP biosynthesis are elusive. The genetic organization of the *M. purpureus* cluster bears a notable difference to that of *M. pilosus* and *M. ruber* in this region, as the *M. purpureus* cluster shares *mpp7* with the two other clusters, but not *mpp1* through *6*. A putative MAzP biosynthetic gene cluster was also previously identified in the genome sequence of *Talaromyces stipitatus*,[3b] and this putative cluster harbors an *mpp7* homologue at the right border of the cluster (Fig. 1). A conserved domain search in the National Center for Biotechnology Information (NCBI) interface[6] predicts that Mpp7 belongs to an uncharacterized protein family with a non-ribosomal peptide synthase (NRPS) condensation domain (COG4908).

In the present study, we generated the Δ*mpp7*::*hyg* mutant to assess the role of *mpp7* in MAzP biosynthesis (see Supplementary

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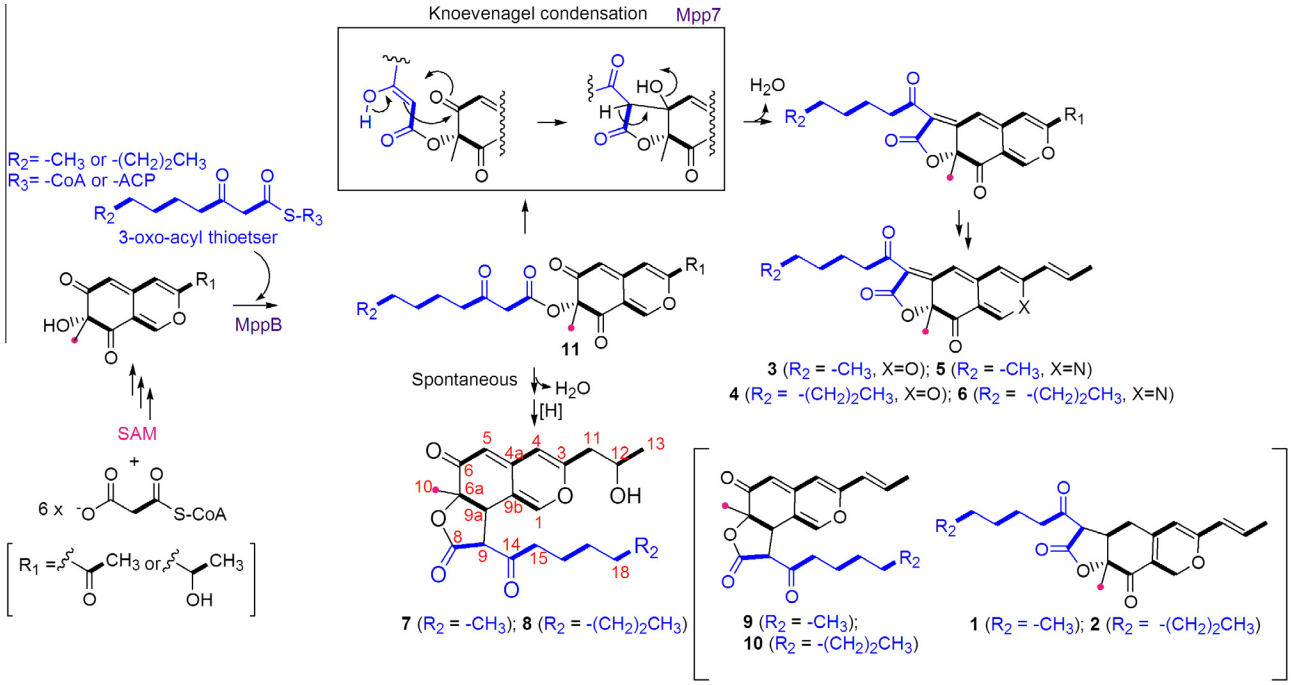

Scheme 1. Proposed biosynthetic pathway of azaphilone pigment in *Monascus* spp. depicting the role of Mpp7 in Knoevenagel aldol condensation.

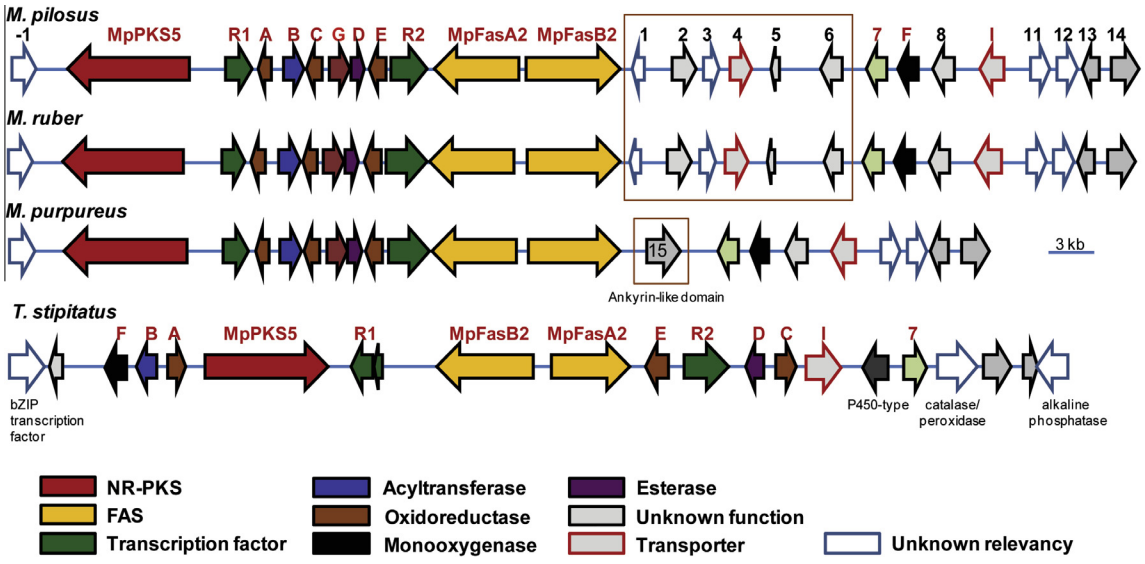

Figure 1. Organization of the azaphilone pigment biosynthetic gene clusters from *M. pilosus*, *M. ruber*, and *M. purpureus*, with the putative cluster gene from *T. stipitatus*. The *M. purpureus* MAzP biosynthetic gene cluster is included in the genome draft scaffold 1 at the nucleotide position from 368,313 to 301,716 (http://genome.jgi.doe.gov/Monpu1/Monpu1.home.html).

data for experimental procedures). HPLC analysis of the *mpp7* mutant showed that the main MAzPs **1**–**4** were not detectable and that new compounds **7** and **8** accumulated (Fig. 2). It appeared that **7** and **8** were minor components in the wile-type culture and highly accumulated upon the loss of **1**–**4**. Notably, **8** appeared abundant inside the cell, and **7** is readily secreted into the medium, whereas **1** to **4** generally accumulated in the cell. Citrinin was contained in the organic extracts of the supernatants, eluting at the same retention time as **8** under the given HPLC conditions, but silica gel chromatography clearly separated **8** from citrinin. Compound **7** and **8** were isolated through silica gel chromatography of the supernatant extracts; approximately 120 mg and 30 mg of **7** and **8**, respectively, were obtained from a 1.5 L culture of the *mpp7* mutant. In ^{1}H NMR analysis (Table 1), **7** and **8** appear closely related, showing three olefinic singlet hydrogen signals at 7.38, 6.10/6.09 (**7**/**8**), and 5.38 ppm. These downfield signals, together with a singlet methyl hydrogen signal at 1.58 ppm, are characteristic to the azaphilone structure (Scheme 1).[5a] Several ^{1}H NMR upfield signals indicate saturated acyl chains in **7** and **8**. The ^{13}C NMR spectra are also comparable between **7** and **8**, except for two additional methylene carbons (29.0 and 28.8 ppm) in **8**, as determined by HSQC and ^{13}C DEPT experiments. In the HMBC spectrum of **8**, the singlet methyl hydrogen signal of C10 (1.58 ppm) correlates to a carbonyl carbon at 191.5 ppm (C6), a quaternary carbon at