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Molecular Genetic Characterization of Terreic Acid Pathway in *Aspergillus terreus*

Chun-Jun Guo,[†,∥] Wei-Wen Sun,[†,∥] Kenneth S. Bruno,[‡] and Clay C. C. Wang*[,†,§]

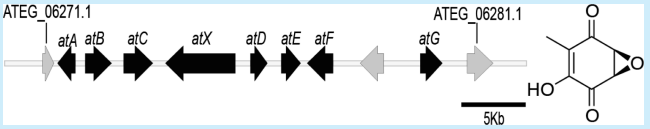
[†]Department of Pharmacology and Pharmaceutical Sciences, School of Pharmacy, University of Southern California, Los Angeles, California 90089, United States

[‡]Chemical and Biological Process Development Group, Energy and Environment Directorate, Pacific Northwest National Laboratory, Richland, Washington 99352, United States

[§]Department of Chemistry, Dornsife College of Letters, Arts, and Sciences, University of Southern California, Los Angeles, California 90089, United States

Supporting Information

ABSTRACT: Terreic acid is a natural product derived from 6-methylsalicylic acid (6-MSA). A compact gene cluster for its biosynthesis was characterized. Isolation of the intermediates and shunt products from the mutant strains, combined with bioinformatic analyses, allowed for the proposition of a biosynthetic pathway for terreic acid.



Filamentous fungi are well-known producers of a wide variety of secondary metabolites with interesting biological activity and important pharmaceutical potential. Terreic acid (TA, **1**) (Figure 1), a quinone epoxide, is one such compound isolated from *Aspergillus terreus*. Prior studies have recognized the inhibitory effect of TA (**1**) against bacteria.[1] TA (**1**) has attracted attention because it selectively inhibits the catalytic activity of Bruton's tyrosine kinase (Btk), the kinase that plays vital roles in mast cell activation and B cell development.[2] Because of its inhibitory effect, TA (**1**) has been used as a chemical probe to examine the function of Btk.[2] Ibrutinib, also known as PCI-32765, is a selective inhibitor of Btk and has been approved by the FDA recently in the treatment of mantle cell lymphoma and chronic lymphocytic leukemia.[3] One of the research interests in our laboratory is in the discovery of natural product inhibitors of Btk such as TA (**1**) as potential cancer therapeutics.

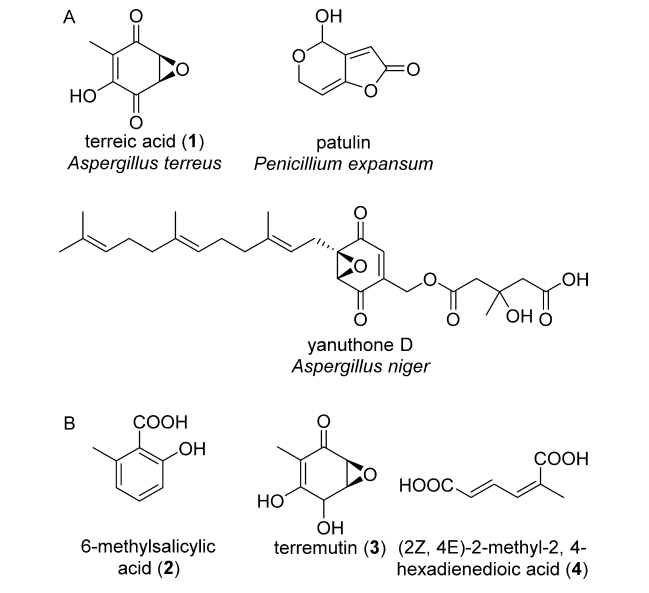


Figure 1. (A) Compounds derived from 6-MSA. (B) Intermediates or shunt products isolated in this study.

Compared to its biological activity, knowledge of the biosynthesis pathway for TA (**1**) in *A. terreus* is quite limited. Pioneering work using a radiolabeled precursor approach demonstrated that the carbon skeleton of TA (**1**) originated from 6-methylsalicylic acid (6-MSA, **2**) (Figure 1).[4] The 6-MSA (**2**) precursor undergoes decarboxylation and a series of oxidation steps to give TA (**1**).[4,5] Recent genome sequencing of *A. terreus* enabled us to use a genome-mining approach to decipher the biosynthesis of TA (**1**). We can use the genes for TA (**1**) biosynthesis as probes to uncover structurally similar compounds with potential Btk inhibitory activity from other genome-sequenced fungi. A previous study reported the cloning of the gene *atX* in *A. terreus* and its identification as a 6-MSA synthase (6-MSAS).[6] Although *atX* is the only *6-msas* gene identified in *A. terreus*, the genetic linkage between *atX* and TA (**1**) has not been confirmed experimentally until this study.

In this study, we analyzed the *A. terreus* NIH 2624 genome and identified the *at* cluster responsible for TA (**1**) biosynthesis. *A. terreus* was cultivated on yeast extract agar (YAG), a medium on which TA (**1**) is consistently produced. The compound TA (**1**) was identified in the ethyl acetate (EA) layer after extraction from the acidified water layer (for details, see the Supporting Information). The structure of TA (**1**) was

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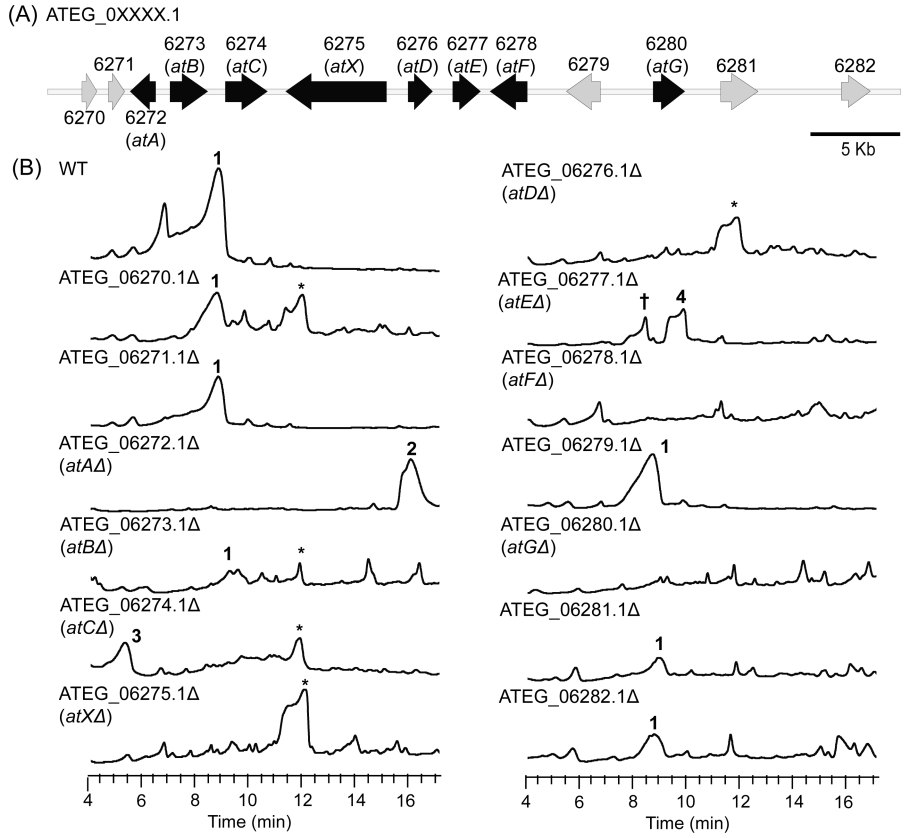

Figure 2. (A) Organization of the *at* gene cluster involved in TA (**1**) biosynthesis in *A. terreus*. Black open reading frames (ORFs) are involved in TA (**1**) biosynthesis while gray ones are not. (B) DAD traces of extracts from the wildtype and mutants as detected by UV (200−600 nm). Numbers on peaks correspond to compounds in Figure 1. *This metabolite is not related to TA (**1**) (for more details, see Figure S1, Supporting Information). †This metabolite is related to TA (**1**), and its structure is similar to **4** according to UV absorption spectra but not isolated due to low production yield.

confirmed by comparing its ^{1}H and ^{13}C NMR spectra with published data.[7] Next, we deleted the gene ATEG_06275.1 (*atX*) using an efficient gene targeting system we developed for *A. terreus*.[8] As expected, examination of the secondary metabolite profile of the *atX*Δ strain showed that the production of TA (**1**) was eliminated (Figure 2), providing direct evidence that AtX synthesizes 6-MSA (**2**) that incorporates into the TA (**1**) pathway.

Secondary metabolite genes are often clustered in filamentous fungi.[9] Previous bioinformatic analyses suggested that the *at* cluster contains seven genes from ATEG_06272.1 to ATEG_06278.1, but there was no experimental proof.[10] To explicitly characterize the entire gene cluster, a total of 12 genes flanking *atX*, from ATEG_06270.1 to ATEG_06282.1 were individually deleted. The LC/MS profiles of the verified gene deletion mutants indicated that the production of TA (**1**) was impaired in the strains ATEG_06272.1Δ (*atA*Δ), ATEG_06273.1Δ (*atB*Δ), ATEG_06274.1Δ (*atC*Δ), ATEG_06276.1Δ (*atD*Δ), ATEG_06277.1Δ (*atE*Δ), ATEG_06278.1Δ (*atF*Δ) and ATEG_06280.1Δ (*atG*Δ), suggesting that these genes are involved in the biosynthesis of TA (**1**) (Figure 2). TA (**1**) was produced in the ATEG_06270.1Δ, ATEG_06271.1Δ, ATEG_06281.1Δ and ATEG_06282.1Δ mutants indicating we have defined the left and right borders of the entire gene cluster.

Among the genes involved in TA (**1**) production, the *atA*Δ, *atC*Δ, and *atE*Δ strains produced intermediates or shunt products that were UV active (Figure 2). Compound **2** (Figure 1B) was purified from large-scale cultures of the *atA*Δ strain (Figure 2). The structure of compound **2** was confirmed to be 6-MSA (**2**) by comparing the ^{1}H and ^{13}C NMR spectra with those of the published data.[6] The *atC*Δ strain accumulated chemically stable compound **3** (Figures 1B and 2). The structure of compound **3** was identifed as terremutin (**3**), a dihydroquinone epoxide intermediate proposed to be involved in the TA (**1**) pathway (see the Supporting Information for detailed structural information).[5] The structure of compound **4**, purified from the *atE*Δ strain, was found to be (2*Z*,4*E*)-2-methyl-2,4-hexadienedioic acid. Since no published NMR data are available for compound **4**, thorough 1D and 2D NMR analyses enabled us to determine its structure (see the Supporting Information for detailed information). The NOE correlation between H-3 and the 2-methyl protons, as well as the coupling consant (J = 15.2 Hz) between H-4 and H-5, led us to determine the conformation of **4** to be 2*Z*,4*E*. Interestingly, compound **4** no longer maintains the six-carbon ring moiety as identified in other characterized intermediates (**2** and **3**), suggesting that it might be a shunt product in the TA (**1**) pathway.

Elucidation of the above intermediates' structures, combined with the sequence analyses of the involved genes, allowed us to propose a biosynthetic pathway for TA (**1**) (Figure 3, Table 1). The first step of the pathway is the synthesis of 6-MSA (**2**) by the 6-MSAS AtX. The domain architecture of AtX includes β-ketoacyl synthase (KS), acyltransferase (AT), thioester hydrolase (TH), ketoreductase (KR), and acyl carrier protein (ACP)

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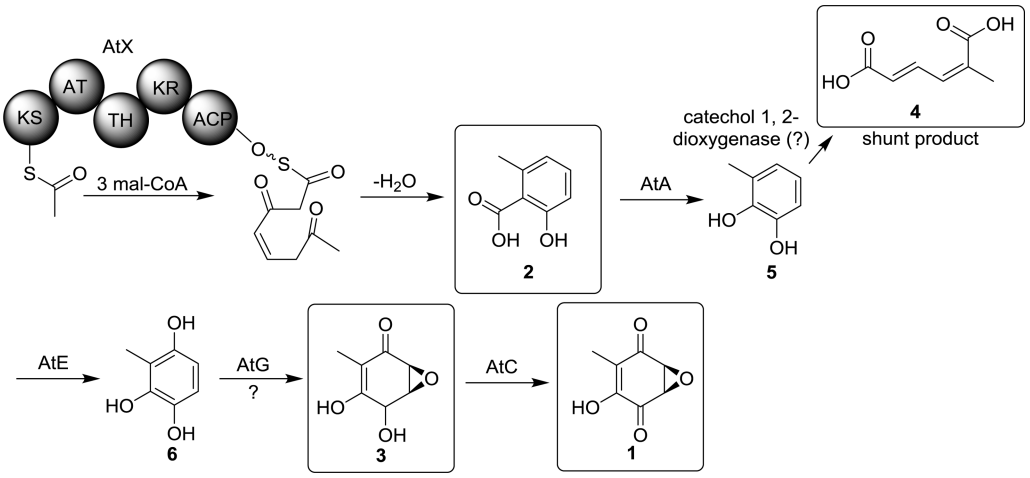

Figure 3. Proposed biosynthetic pathway for TA (**1**). Isolated and structurally characterized compounds are boxed.

Table 1. *at* Gene Cluster (ATEG_062XX.1) and Gene Function Prediction[a]

gene	cofactors (putative)	blast homologues (% identity)	putative function
72 (*atA*)	FAD/NAD(P)	*salA* (27%)[13]	6-MSA decarboxylase
73 (*atB*)	unknown	unknown	MFS transporter
74 (*atC*)	FAD	VBS (40%)[24]	GMC oxidoreductase
75 (*atX*)			6-MSAS
76 (*atD*)	unknown	*patJ* (67%)[22]	unknown
77 (*atE*)	NADH/NADPH	*patI* (64%)[18] *patH* (55%)[18]	cytochrome P450 monooxygenase
78 (*atF*)	unknown	*patL* (38%)[22]	transcription factor
80 (*atG*)	NADH/NADPH	unknown	cytochrome P450 monooxygenase

[a]The proteins of *patJ* and *patL* are not characterized.

(Figure 3). The previous study reveals that the TH domain in AtX acts as a product-releasing domain which catalyzes a thioester hydrolysis to give 6-MSA (**2**), representing a first example of a product-releasing domain embedded in the middle of a PR−PKS.[11] In the biosynthesis of 6-MSA (**2**), AtX utilizes one acetyl-CoA and three malonyl-CoAs as its substrates and catalyzes a series of programmed reactions including Claisen condensation, dehydration, reduction, and cyclization to yield compound **2**.[11]

A recent bioinformatic study by Boruta et al. predicted that AtA catalyzes the conversion of **2** to **5** and AtE catalyzes hydroxylation of **5** to **6** (Figure 3). They predicted that AtC is involved in the dehydrogenation of **6** to a quinone precursor of terreic acid (**1**). They did not propose the enzyme responsible for the final epoxidation. The study also suggested that the cluster contains a putative transporter gene (*atB*) and a regulatory gene (*atF*).[10]

In our study, only compound **2** (but no other intermediates) was accumulated in the *atA*Δ strain (Figure 2). Conserved domain search of AtA indicated that the putative protein contains a salicylate 1-monooxygenase domain. Salicylate 1-monooxygenase is an enzyme that catalyzes the converion of salicylate to catechol and is FAD dependent (Table 1).[12] AtA shares around 30% amino acid identity with a characterized salicylate 1-monooxygenase gene, *salA*, in *A. nidulans*.[13] This suggests that AtA might catalyze the decarboxylative hydroxylation of **2** to **5**. Previous literature reported the characterization of two FAD-dependent monooxygenases, TropB and SorbC, that catalyze the hydroxylative dearomatization of their substrates.[14,15] In comparison, the aromaticity of compound **2** still remains after the decarboxylative hydroxylation by AtA.

The conversion of 3-methylcatechol (**5**) to terremutin (**3**) requires a series of oxidation modifications such as hydroxylation and epoxidation (Figure 3). We were able to isolate a shunt product (**4**) in the 2 L scaled-up cultures (original scale: 25 mL) of the *atE*Δ (Figure 2). We could identify compound **4** after feeding the *atX*Δ strain with compound **5** (Figure S4, Supporting Information), suggesting that the accumulation of **4** is likely due to the decomposition of **5**. This degradation is probably catalyzed by an unidentified catechol 1, 2-dioxygenase,[16] the coding gene of which is not located in the terreic acid (**1**) cluster. A similar cleavage and degradation of aromatic acids has also been identified in *A. terreus*.[17] Homology analysis of AtE showed that the putative protein has 80% similarity in amino acid sequence with CYP619C2 characterized in *A. clavatus*.[18] CYP619C2 is involved in the biosynthesis of patulin (Figure 1A), another natural product derived from 6-MSA (**2**). The study showed that CYP619C2 is capable of catalyzing the p-hydroxylation of both *m*-cresol to toluquinol and *m*-hydroxybenzyl alcohol to gentisyl alcohol.[18] On the basis of the hydroxylation function of its homologue, we propose that AtE catalyzes the hydroxylation of **5** to **6**. Lastly, since the deletion of *atC* accumulated terremutin (**3**), we propose that AtC is required for the oxidation of terremutin (**3**) to TA (**1**) (Figure 3).

The *at* cluster contains one gene, *atF*, that encodes a putative zinc family transcription factor (Table 1). Deletion of *atF* eliminated the production of only TA (**1**) but not other types of secondary metabolites in *A. terreus*, suggesting that AtF specifically regulates the expression of genes in the *at* cluster. The cluster also contains one gene, *atB*, coding for a putative major facilitator superfamily (MFS) transporter (Table 1).

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These MFS transporter genes, identified in several secondary metabolite gene clusters, have been shown to be responsible for transporting the metabolites out of its producing organisms. Tri12, for example, is involved in the transport of trichothecene in *Fusarium* species.[19] However, there are cases in which deletion of the MFS transporter gene in the pathway does not lead to significant loss of the metabolites.[20,21] Production of TA (**1**) is greatly diminished in the *atB*Δ strain (Figure 2), indicating that AtB might play a role in the transport of TA (**1**).

There are two genes (*atD* and *atG*) in the cluster to which we could not assign their functions. Although they are involved in the biosynthesis of TA (**1**), we could not detect any intermediates or shunt products accumulated in the mutant strains. A homologous protein of AtD could be identified in the patulin cluster (PatJ)[22] but the functions of both putative enzymes are unknown. Conserved domain analysis of AtG showed that it contains a cytochrome P450 monooxygenase domain, but no characterized homologue can be identified in a BLASTp analysis. It is possible that AtG catalyzes one of the oxidation steps occurred in the conversion of **6** to terremutin (**3**). Thus, for AtD and AtG, further effort is necessary to elucidate their specific functions in the biosynthesis of TA (**1**).

In conclusion, we report the identification of a cluster of eight genes that are responsible for the biosythesis of TA (**1**). The biosynthetic pathway is proposed on the basis of the bioinformatic analyses of the involved genes and the chemical analyses of the gene deletion strains. The cluster contains a key gene *atX* that codes for a 6-MSAS. BLASTp analysis of AtX showed that its homologues are widespread in Ascomycetes (Table S7, Supporting Information), but in most cases, only one *6-msas* gene resides in the genome of each single fungus. All 6-MSASs have high similarity in the amino acid sequence and domain architecture, strongly suggesting that they share a common origin. The diversity of 6-MSA (**2**) derivatives is enriched due to the tailoring proteins, with specific catalytic activities in each pathway.[18,23] Thus, understanding the function of these tailoring proteins will expand our knowledge of this important class of natural products.

■ ASSOCIATED CONTENT

Supporting Information

General methods, compound characterization with spectral data, and diagnostic PCR results. This material is available free of charge via the Internet at http://pubs.acs.org

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: clayw@usc.edu.

Author Contributions

[‖]These authors contributed equally to this project.

Notes

The authors declare no competing financial interest.

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