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Article

Ketoreductase Domain-Catalyzed Polyketide Chain Release in Fungal Alkyl Salicylaldehyde Biosynthesis

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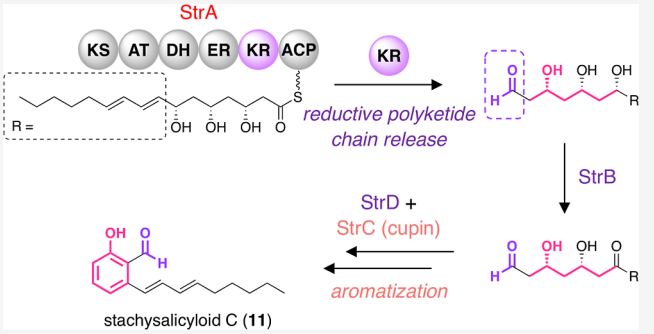
ABSTRACT: Alkyl salicylaldehyde derivatives are polyketide natural products, which are widely distributed in fungi and exhibit great structural diversity. Their biosynthetic mechanisms have recently been intensively studied; however, how the polyketide synthases (PKSs) involved in the fungal alkyl salicylaldehyde biosyntheses release their products remained elusive. In this study, we discovered an orphan biosynthetic gene cluster of salicylaldehyde derivatives in the fungus *Stachybotrys* sp. g12. Intriguingly, the highly reducing PKS StrA, encoded by the gene cluster, performs a reductive polyketide chain release, although it lacks a C-terminal reductase domain, which is typically required for such a reductive release. Our study revealed that the chain release is achieved by the ketoreductase (KR) domain of StrA, which also conducts cannonical β-keto reductions during polyketide chain elongation. Furthermore, we found that the cupin domain-containing protein StrC plays a critical role in the aromatization reaction. Collectively, we have provided an unprecedented example of a KR domain-catalyzed polyketide chain release and a clearer image of how the salicylaldehyde scaffold is generated in fungi.



INTRODUCTION

Polyketides are one of the representative classes of natural products with great structural diversity and a wide range of biological activities, as exemplified by the antiparasitic agents avermectins and the cholesterol-lowering medication lovastatin. Polyketide biosynthesis occurs via repeated condensation of acetate (or other carboxylate) units and exemplifies how nature synthesizes a diverse array of molecules from a limited number of starting materials.[1] The majority of fungal polyketides are synthesized by iterative type-I polyketide synthases (PKSs).[2] The structural diversity of fungal PKS products is attributable to many factors, such as the number of elongation cycles, the degree of carbonyl reduction, and the cyclization mode of a polyketide chain. In addition, polyketide chain release mechanisms are among the major contributors to diversity generation (Figure 1A).[3] A typical chain release mechanism involves the hydrolysis of a polyketide chain, which is catalyzed by either a thioesterase (TE) domain found at the C-terminus of a PKS or by a transacting TE.[4] TE domains can also achieve chain release via Claisen (Dieckmann) condensation or macrolactonization[5] or undergo additional reactions prior to hydrolysis.[6] In addition, many fungal PKSs possess a reductase (R) domain at the C-terminus to yield an aldehyde or an alcohol product.[7] Other commonly reported chain release mechanisms include nonenzymatic pyrone formation[8] and polyketide chain transfer to another PKS or a small molecule.[9] Pyridoxal 5′-phosphate-dependent enzymes are also known to catalyze chain release using an amino acid.[10] Although the chain release mechanisms of PKSs have been well investigated, given the structural diversity of polyketides, additional mechanisms may exist.

Alkyl salicylaldehyde derivatives are one type of fungal metabolites with diverse molecular architectures (Figure 1B), including the phytotoxin pyriculol and varitriol that exhibits potent anticancer activity.[11] Their biosynthesis has been intensively investigated in recent years.[12] In particular, the biosynthetic study of trichoxide has provided important insights into how the salicylaldehyde moiety is generated (Figure 1C).[12c] First, the highly reducing (HR)-PKS VirA, possibly with the assistance of the cupin domain-containing protein VirC, synthesizes the linear polyketide aldehyde, which undergoes two sequential alcohol dehydrogenations catalyzed by the two short-chain dehydrogenases/reductases (SDRs) VirB and VirD. The subsequent aldol reaction followed by dehydration and enolization results in the formation of an

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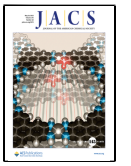

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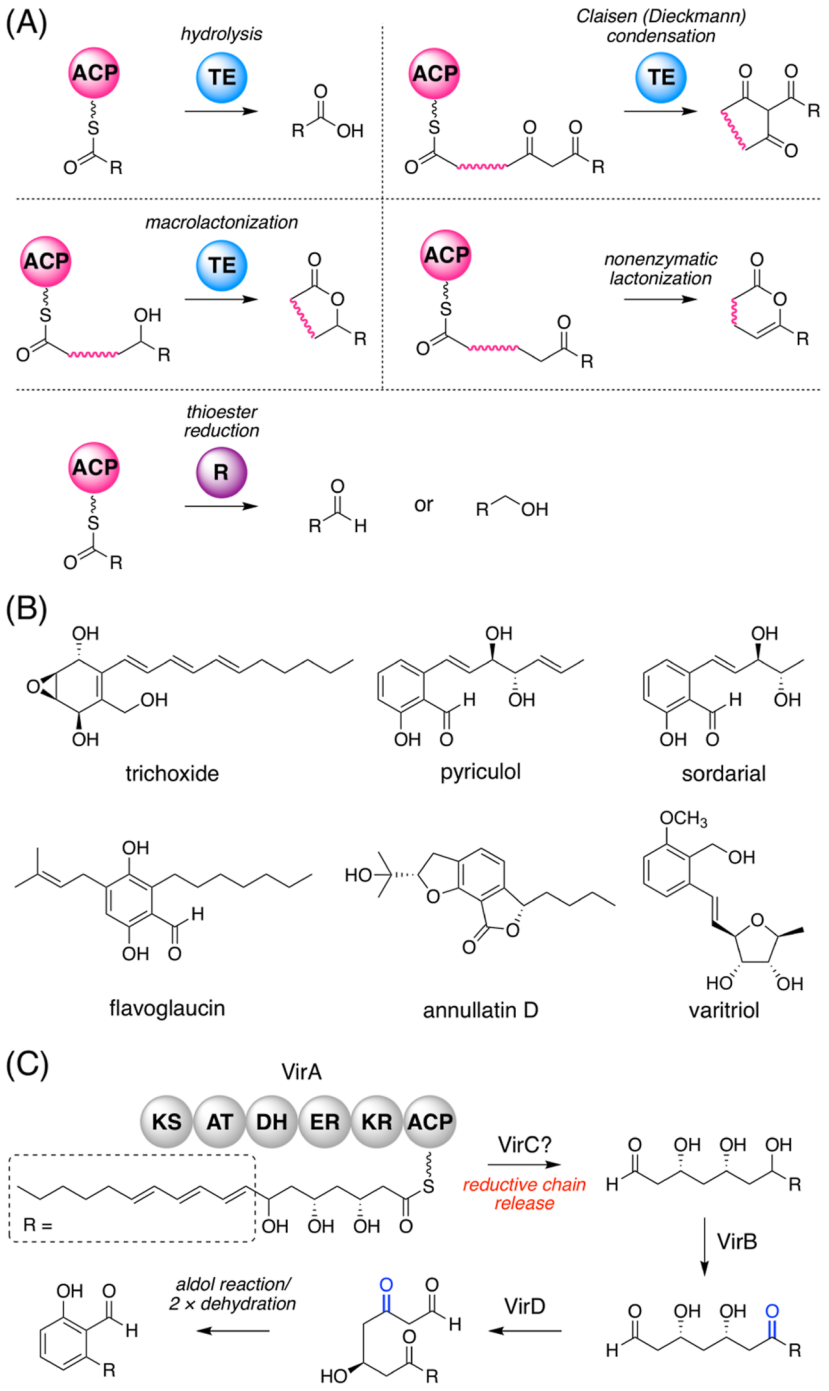

Figure 1. (A) Representative chain release mechanisms in fungal type I PKSs. (B) Selected alkyl salicylaldehyde derivatives from fungi. (C) Formation of a salicylaldehyde moiety in trichoxide biosynthesis.[12c]

aromatic ring. Despite the apparent requirement of reductive release, VirA lacks an R domain and so do the other identified VirA homologues.[12] Thus, the chain release by VirA possibly occurs via an unidentified mechanism, which remains a major question in alkyl salicylaldehyde biosynthesis.

Herein, we report the identification of the biosynthetic gene cluster for an uncharacterized alkyl salicylaldehyde derivative in the fungus *Stachybotrys* sp. g12 and the characterization of StrA, the PKS encoded by the gene cluster. It was revealed that StrA, which lacks a C-terminal R domain, performs reductive polyketide chain release using the ketoreductase (KR) domain. Furthermore, we found that the cupin domain-containing protein StrC is the key player in the aromatization process to afford the salicylaldehyde scaffold.

RESULTS AND DISCUSSION

Search for Uncharacterized Biosynthetic Gene Clusters for Fungal Alkyl Salicylaldehyde Derivatives. To further elucidate the underlying chemistry of salicylaldehyde formation, we searched for gene clusters potentially responsible for the biosynthesis of alkyl salicylaldehyde derivatives in our in-house fungal genome database. One gene cluster from the fungus *Stachybotrys* sp. g12, which was designated as the *str* cluster, was somewhat different from the characterized clusters

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with more putative tailoring enzyme genes and might be involved in the production of a new salicylaldehyde derivative (Figure 2A, Table S1, and Figure S1; DDBJ/EMBL/GenBank accession number: LC754785). The *str* cluster encodes the HR-PKS StrA and the two SDRs, StrB and StrD, which are homologous to VirB and VirD,[12c] respectively. These three enzymes should be essential for the formation of the salicylaldehyde skeleton. In addition, the two cupin domain-containing proteins, StrC and StrE, are also encoded by the *str* cluster, which might also be involved in the early stage of biosynthesis. Furthermore, several putative tailoring enzyme genes also occur within the cluster.

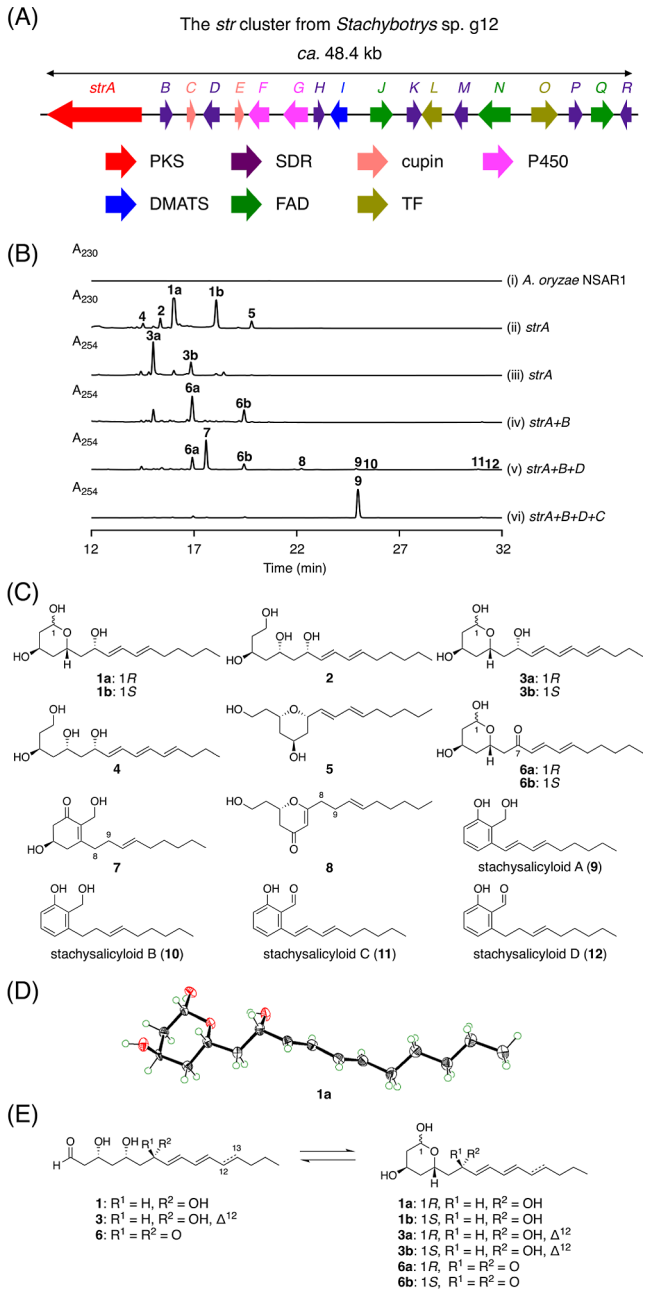

Figure 2. (A) Schematic representation of the *str* cluster. Cupin: cupin domain-containing protein; P450: cytochrome P450 monooxygenase; DMATS: dimethylallyltryptophan synthase-type prenyltransferase; FAD: FAD-dependent monooxygenase/oxidoreductase; and TF: transcription factor. (B) High-performance liquid chromatography profiles of the metabolites of the *A. oryzae* transformants. The chromatograms were monitored at 230 or 254 nm. (C) Structures of **1**−**12**. The stereochemistry in **2**−**8** was deduced according to that of **1**. (D) X-ray crystal structure of **1a**. (E) Structures of **1**, **3**, and **6**, the linearized forms of **1a**/**1b**, **3a**/**3b**, and **6a**/**6b**, respectively.

Heterologous Reconstitution of the Early-Stage Biosynthesis. To investigate whether the *str* cluster is responsible for the biosynthesis of an alkyl salicylaldehyde derivative, we performed heterologous expression experiments using *Aspergillus oryzae* NSAR1,[13] which has been utilized for the characterization of orphan biosynthetic gene clusters.[14] First, the PKS gene *strA* was solely expressed in *A. oryzae*, producing several specific metabolites (Figure 2B, traces i to iii, and Figure 2C). The major products **1a**/**1b** were isolated as an epimeric mixture, and the structural analysis of **1a**/**1b** revealed the presence of a six-membered ring hemiacetal with a side chain containing 11 carbon atoms. The structure of **1a** was further confirmed via X-ray crystallographic analysis, which established its absolute structure (Figure 2D; CCDC 2242213). Compounds **1a**/**1b** appear to be derived from linear aldehyde **1** (Figure 2E). Compared with the VirA product, **1** had two fewer carbon atoms and a conjugated diene, instead of a triene, but the overall structures of the two products were similar, indicating that StrA was also responsible for the biosynthesis of a salicylaldehyde derivative. Compound **2** was determined to be a reduced form of **1**, with a hydroxy group at the end of its carbon chain, and **3a**/**3b** [hemiacetal forms of **3** (Figure 2E)] and **4** were characterized as analogues of **1** and **4** with a triene instead of a diene. Moreover, **5** possesses a tetrahydropyran ring.

We then coexpressed *strA* and the SDR gene *strB* in *A. oryzae*. The transformant yielded **6a**/**6b** as major products (Figure 2B, trace iv), which was also obtained as an epimeric mixture and found to be the C-7 keto form of **1a**/**1b** (Figure 2C) and should be derived from linear form **6** (Figure 2E). Further introduction of the other SDR gene *strD* resulted in a complicated metabolic profile with six new isolated products **7**−**12** (Figure 2B, trace v, and Figure 2C). The structural characterization of **7** elucidated that it had a cyclohexenone ring and that the double bond at C-8/C-9 was reduced in **7**. Compound **8** resembled **5** but featured the dihydro-γ-pyrone moiety and a single bond at C-8/C-9. The other products **9**−**12**, which we named stachysalicyloids A−D, harbored either a salicylaldehyde or salicyl alcohol moiety with different unsaturation degrees in their alkyl chain. The PKS StrA and the two SDRs, StrB and StrD, were thus found to produce salicylaldehyde derivatives, although at a low yield.

In-Depth Characterization of the PKS StrA. We next characterized the PKS StrA to clarify the mechanism of the reductive chain release. The enzyme was purified using the *Escherichia coli* expression system by coexpressing the promiscuous phosphopantetheinyl transferase Sfp.[15] The purified StrA was incubated with malonyl-coenzyme A (CoA) and nicotinamide adenine dinucleotide phosphate (NADPH), which yielded **1a** and **3a** as major products, along with a small amount of **1b**, **2**, **3b**, and **4** (Figure 3A), indicating that StrA was solely responsible for both polyketide chain synthesis and reductive chain release. Thus, one of the reductive PKS domains, namely, the KR and enoylreductase (ER) domains, was possibly responsible for the chain release, and the KR and ER domains were expressed and obtained from the *E. coli* expression system (Table S2). We then

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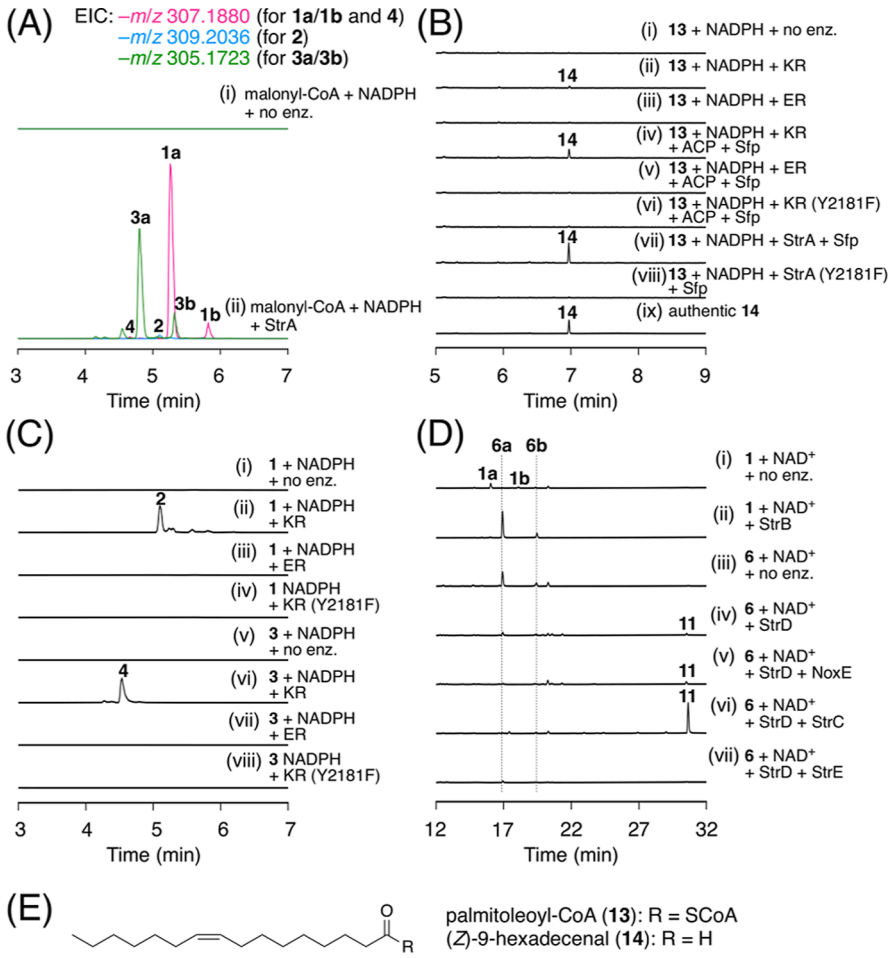

Figure 3. (A) Liquid chromatography–mass spectrometry (LC–MS) analysis of the products from the enzymatic reactions of StrA. (B) GC–MS chromatograms of the products from the enzymatic reactions of the KR and ER domains. (C) LC–MS analysis of the enzymatic reactions of KR and ER domains. Traces i to iv and v to viii were extracted at *m*/*z* 309.2036 and 307.1880, respectively. Compounds **1** and **3** were given as a racemic mixture of **1a**/**1b** and **3a**/**3b**, respectively. (D) HPLC profile of the products from the enzymatic reactions of StrB, StrC, StrD, and StrE. The chromatograms were monitored at 254 nm. (E) Structures of **13** and **14**.

confirmed that KR and ER domains were obtained in active forms by performing reactions utilizing acetoacetyl-*N*-acetylcysteamine thioester (acetoacetyl-SNAC) and crotonyl-SNAC, respectively (Figure S2). Because we could not successfully synthesize the SNAC or CoA derivative of the polyketide chain generated by StrA, we utilized the commercially available palmitoleoyl-CoA (**13**) (Figure 3E) as an acyl carrier protein (ACP)-bound substrate mimic to investigate the functions of the two domains. The domains were individually reacted with **13** in the presence of NADPH, and the reaction products were analyzed via gas chromatography–mass spectrometry (GC–MS). (*Z*)-9-Hexadecenal (**14**) was formed when the KR domain was used for the reaction, whereas no significant reduction occurred in the reaction with the ER domain (Figure 3B, traces i to iii; Figure S3). We also initiated enzymatic reactions with the ACP domain (Table S2) and Sfp to load the fatty acyl chain onto the ACP. The yield of **14** increased in the reaction with the KR domain, whereas **14** was not formed in the reaction with the ER domain (Figure 3B, traces iv and v). The replacement of the catalytic tyrosine[16] (Tyr2181) of the KR domain with phenylalanine prevented the reductase activity to afford **14** (Figure 3B, trace vi). This indicates that the KR domain was responsible for the reductive chain release using the same catalytic site as the regular ketoreduction. Additionally, a similar enzymatic reaction was conducted using the full-length StrA. Consequently, StrA successfully yielded reduced product **14**, whereas no reduction was observed from the Y2181F variant of StrA (Figure 3B, traces vii and viii), further confirming that the KR domain is critical for the reductive chain release. Moreover, the KR domain, but not the ER domain, could accept **1** and **3** to provide **2** and **4**, respectively (Figure 3C), indicating that the KR domain allowed for the occurrence of four-electron reduction to convert the thioester into the hydroxy group.

Investigation of the Mechanism for the Generation of the Salicylaldehyde Scaffold. Subsequently, we examined the mechanism of the aromatization to form the salicylaldehyde scaffold, using the two SDRs, StrB and StrD, obtained from the *E. coli* expression system. The incubation of StrB with **1** in the presence of NAD^+ efficiently produced **6** (Figure 3D, traces i and ii), confirming that StrB was the C-7 alcohol dehydrogenase. We then initiated another in vitro enzymatic reaction of StrD with **6** and NAD^+. The reaction yielded the expected aromatized product **11**, although with low productivity (Figure 3D, traces iii and iv). Considering that a previous study on VirD, an StrD homologue, revealed that VirD also performs a reductive reaction and that the addition of the NADH oxidase NoxE[17] increased the product yield,[12c]

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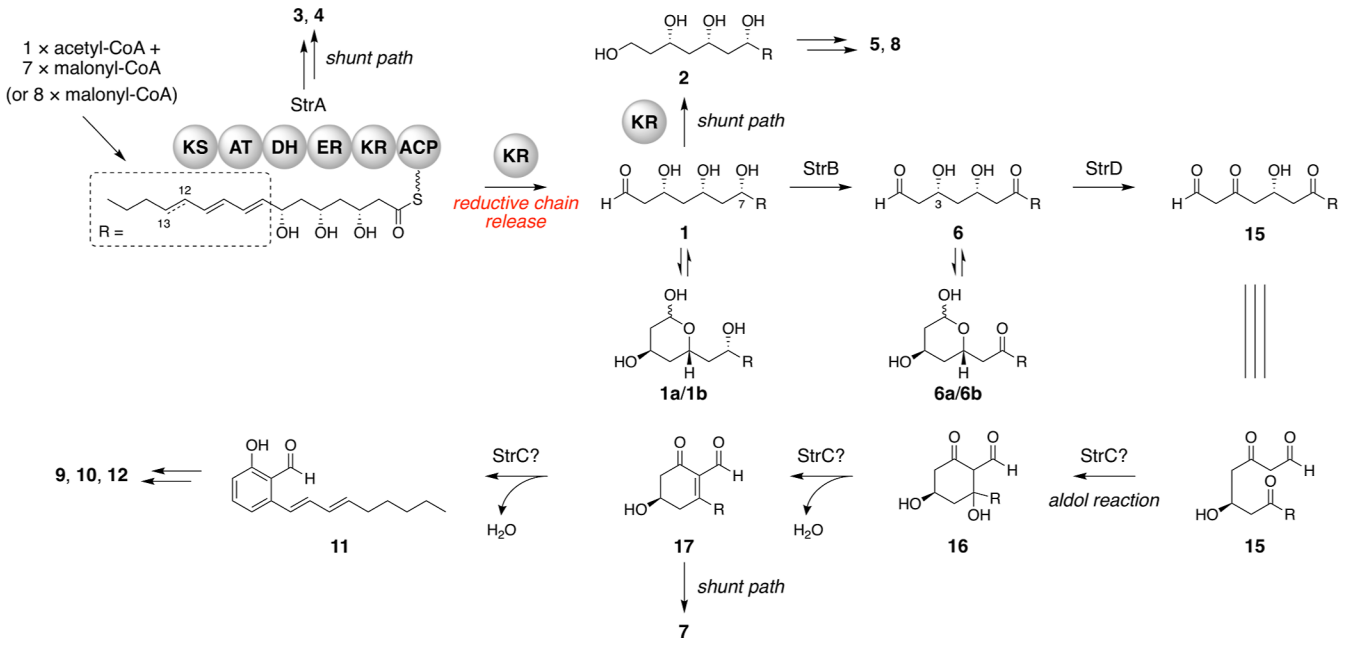

Figure 4. Proposed biosynthetic pathways for the formation of **11** and other metabolites obtained in this study.

we conducted another enzymatic reaction with NoxE. In this case, **6** was more efficiently consumed; however, the yield of **11** was still low (Figure 3D, trace v). Given this observation and the poor productivity of the aromatic compounds in the *A. oryzae* transformant with *strA*, *-B*, and *-D*, we speculate that an additional enzyme(s) is required for the efficient synthesis of **11**. Thus, two cupin domain-containing proteins StrC and StrE, which are homologues of VirC, may be involved in this process, given that *virC* homologues are universally conserved in known biosynthetic gene clusters for fungal alkyl salicylaldehydes and that their functions remain enigmatic.[12] The addition of StrC markedly increased the yield of **11**, whereas StrE addition showed no significant effect (Figure 3D, traces vi and vii), demonstrating that StrC is the critical enzyme for the aromatization reaction. The function of StrE, which shows a 52% amino acid sequence identity with StrC, remains unclear but is expected to be involved in a later stage of biosynthesis. We also constructed the *A. oryzae* transformant expressing *strA*−*D* and found that the transformant efficiently affords the aromatized product **9** (Figure 2B, trace vi), further illuminating the importance of StrC for the aromatization process. We reasoned that **11** was reduced by an endogenous enzyme of *A. oryzae* to give **9** because **2** cannot be converted to **9** by StrB, -C, and -D (Figure S4).

The proposed pathways of the probable intermediates and shunt products in the stachysalicyloid biosynthesis are summarized in Figure 4. Initially, the PKS StrA produces linear aldehyde **1**, in which the KR domain is responsible for the reductive chain release. Considering no shorter chain length polyketide was obtained from StrA, the KR domain should perform the reductive release only when the chain elongation completes, whereas it conducts canonical *β*-keto reduction in every elongation cycle. Compound **1** is then oxidized by the SDR StrB to afford **6**. If the ER domain of StrA does not work in the second round of the chain elongation, this leads to the formation of **3** and **4**, which are shunt pathway products with the C-12/C-13 double bond. For the synthesis of **11** from **6**, **6** is the first oxidized at C-3 by the SDR StrD to yield **15**, which then undergoes an aldol reaction to yield cyclohexanone **16**. Subsequent dehydration results in the formation of cyclohexenone **17**, which undergoes additional dehydration and enolization to yield salicylaldehyde **11**. The LC−MS analysis of the enzymatic reaction products revealed that compounds with molecular formulas corresponding to that of **15**, **16**, or **17** were detected in the StrC-free reaction, whereas these molecules were not detected in the presence of StrC (Figure S5). Thus, StrC possibly played a role in the aldol reaction and subsequent aromatization, which slowly occurs under the nonenzymatic condition.

In this study, we elucidated the biosynthesis of fungal alkyl salicylaldehyde derivatives (Figure 4) and solved some unanswered questions raised in previous studies. The most uncharacteristic feature of stachysalicyloid biosynthesis is the KR domain-catalyzed reductive polyketide chain release. Such reductive releases have only been known to be enabled by C-terminal R domains, and this work provides the first example in which a KR domain allowed for polyketide chain release. Phylogenetic analysis revealed that the KR domain of StrA is distantly related to R domains and is most closely related to its homologues found in other known fungal alkyl salicylaldehyde derivative biosyntheses[12] (Figure S6). Thus, these enzymes are likely to perform the KR-catalyzed reductive release as well.

We revealed that the cupin domain-containing protein StrC is the key enzyme for the aromatization step of the biosynthesis rather than for the chain release or modification of the enzyme-bound polyketide chain, as speculated in previous studies.[12c,d] StrC homologues are encoded by all the known gene clusters for the biosynthesis of fungal alkyl salicylaldehyde derivatives.[12] Moreover, a cblaster[18] analysis revealed that homologues of *strA*, *-B*, *-C*, and *-D* are colocalized in numerous fungi (Figure S7), and therefore, StrC homologues should universally serve as a critical aromatase in fungal natural product biosynthesis. A biosynthetic study of flavoglaucin revealed that the deletion of *fogC*, an *strC* homologue, prevented flavoglaucin biosynthesis,[12d] further demonstrating the importance of StrC homologues.

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Cupin domain-containing proteins are widely distributed in natural product biosynthetic pathways and include both metal-dependent and -independent enzymes.[19] They are known to catalyze diverse reactions, such as epoxidation,[20] oxidative rearrangement,[21] dehydration,[22] nitrogen−nitrogen bond formation,[23] and sulfonamide formation.[24] It should be noted that some type-II PKS cyclases (aromatases) are cupin domain-containing enzymes,[25] although they are not closely related to StrC (Figure S8). Comparison of the crystal structure of RemF, the polyketide cyclase involved in resistomycin biosynthesis,[26] and the modeled structure of StrC generated by ColabFold[27] identified possible metal-binding residues in StrC (His117, Asp123, and His157) (Figure S9), which are completely conserved in the predicted aromatases for fungal alkyl salicylaldehyde derivative biosynthesis (Figure S10). Thus, the cyclization/aromatization by StrC might proceed in a similar manner to that by type-II PKS cyclases. Because the pre-aromatized biosynthetic intermediates can undergo over-reductions to form byproducts that are not suitable for aromatization,[12c] StrC and its homologues might protect such intermediates from being over-reduced and facilitate the aldol reaction and/or the following aromatization reaction.

CONCLUSIONS

In this study, we discovered an unreported chemical characteristic of a fungal PKS domain and revealed the function of the previously uncharacterized cupin domain-containing proteins. Our study has defined the minimal components required for the efficient biosynthesis of alkyl salicylaldehyde in fungi and solved the mystery regarding the formation of the salicylaldehyde scaffold. We are currently conducting a structural biological study on the KR domain of StrA and the probable aromatase StrC, which we hope will provide a detailed mechanism of these unusual chemical transformations.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.3c02011.

Experimental details, analytical data, tables of gene cluster information, DNA and protein sequences, primers used and plasmids constructed, and NMR data, gene cluster comparison, mass spectra, LC−MS profiles, cblaster analysis, SDS-PAGE analysis, and NMR spectra (PDF)

Accession Codes

CCDC 2242213 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.

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Notes

The authors declare no competing financial interest.

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