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Discovery and elucidation of the biosynthesis of aspernidgulenes, novel polyenes from *Aspergillus nidulans*, using serial promoter replacement

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Abstract

Through serial promoter exchanges, we isolated several novel polyenes, the aspernidgulenes, from *Aspergillus nidulans* and uncovered their succinct biosynthetic pathway involving only four enzymes. An enoyl reductase (ER)-less highly reducing polyketide synthase (HR-PKS) putatively produces a 5,6-dihydro- α -pyrone polyene, which undergoes bisepoxidation, epoxide ring opening, cyclization, and hydrolytic cleavage by three tailoring enzymes to generate aspernidgulene A1 and A2. Our findings demonstrate the prowess of fungal-tailoring enzymes to transform a polyketide scaffold concisely and efficiently into complex structures. Moreover, comparison with citreoviridin and aurovertin biosynthesis suggests that methylation of the α -pyrone hydroxy group by methyltransferase (CtvB or AurB) is the branching point at which the biosynthesis of these two classes of compounds diverge. Therefore, scanning for the presence or absence of the gatekeeping α -pyrone methyltransferase gene in homologous clusters might be a potential way to classify the product bioinformatically as belonging to methylated α -pyrone polyenes or polyenes containing rings derived from the cyclization of the unmethylated 5,6-dihydro- α -pyrone, such as 2,3-dimethyl- γ -lactone and oxabicyclo[2.2.1]heptane.

Keywords

aspernidgulenes; biosynthesis; natural products; polyenes; polyketides

The rapid accumulation of publicly available genome data and recent advancements in molecular biology have opened up unprecedented opportunities to discover new chemical entities from natural sources and to investigate their biosynthesis.^[1] In filamentous fungi, the vast majority of secondary metabolite gene clusters remain unelucidated owing to their cryptic nature.^[2] For even a well-studied model fungus such as *Aspergillus nidulans*, less

than half of its secondary metabolite gene clusters have been linked to downstream products despite intensive research,^[3] and this suggests that the potential for secondary metabolites to be a source of antibiotics and therapeutics is vast and still largely unexploited.^[4]

The genome of *A. nidulans* encodes 27 polyketide synthases (PKSs), 15 of which are nonreducing polyketide synthases (NR-PKSs) and 12 of which are highly reducing polyketide synthase (HR-PKSs).^[3] The microarray transcription data of *Aspergillus nidulans* indicated that one enoyl reductase (ER)-less HR-PKS AN1784 is co-regulated with three other putative biosynthesis genes.^[5] This four-gene cluster attracted our attention for two reasons. First, three of its four genes share sequence homology with the biosynthetic genes of citreoviridin (*ctv*)^[6] and aurovertin (*aur*)^[7] (Table 1). Citreoviridin and aurovertin belong to a class of potent α -pyrone polyene F1-ATPase inhibitors and have been tested as antitumor agents.^[8–11] We thus speculated that the cluster could produce a novel variant given that *A. nidulans* was not known to produce any α -pyrone polyenes at the time of this study.^[3] Second, the genes in the cluster are divergently transcribed. AN1784 and AN1785 are adjacent genes that have opposite orientations, and so are AN1786 and AN1787 (Figure 1). Divergent transcription would allow us to activate the entire cluster quickly in just two rounds of transformation with a previously described strategy.^[12,13] Here, we report the discovery and isolation of the aspernidgulenes, which unexpectedly fall into an entirely different class of compounds than citreoviridin and aurovertin, from this cluster (*sdg*).

Similar to the *ctv* cluster, the *sdg* cluster encodes an ER-less HR-PKS and two enzymes putatively responsible for cyclization of the polyketide product (Table 1). The HR-PKS AN1784 is homologous to *ctvA*, and the encoded enzymes share the same domain architecture of KS-AT-DH-CMet-KR-ACP (KS, ketosynthase; AT, acyltransferase; DH, dehydratase; CMet, C-methyltransferase; KR, ketoreductase; ACP, acyl carrier protein).^[6,14] We thus named AN1784 *sdgA*. AN1785 encodes a putative membrane-associated hydrolase and exhibits homology with *ctvD*, so it was named *sdgD*. AN1786 encodes a flavin-dependent monooxygenase and is homologous to *ctvC*; thus, it was named *sdgC*. Previous work demonstrated that CtvC and CtvD and their homologs AurC and AurD are responsible for the assembly of the tetrahydrofuran ring in citreoviridin and the 2,6-dioxabicyclo[3.2.1]-octane ring in aurovertin, respectively (Table 1).^[6,7] Unlike the *ctv* cluster, however, the *sdg* cluster does not contain methyltransferase (*ctvB*) or resistance (*ctvE*) genes. Instead, AN1787, which we named *sdgF*, encodes a putative FAD-linked oxidase and shows homology with *sol5* of the solanapyrone biosynthesis cluster (Table 1).^[15]

We first replaced the promoters of both *sdgA* and *sdgD* with the inducible *alcA* promoter [*alcA*(p)] in a single transformation^[12,13] to create an *alcA*(p)-*sdgAD* strain (see Table S2 in the Supporting Information for the designation and genotype of strains created in this study). Under inducing conditions, three new peaks representing compounds **1** – **3** not present in the host strain were detected in its media (Figure 1, i and ii). Next, we replaced the promoters of the other divergently transcribed pair, *sdgC* and *sdgF*, to generate an *alcA*(p)-*sdgADCF* strain. In this strain, three new peaks representing compounds **4** – **6** emerged (Figure 1, iii). In addition, we also replaced the promoter of AN1783, which encodes a putative dehydrogenase and shows homology with *sol3* from the solanapyrone pathway (Table 1).^[15]

However, we did not detect any new peaks in the *alcA(p)-sdgADCF*+ AN1783 strain under the inducing conditions (Figure 1, iv); this suggests that AN1783 is not part of the *sdg* cluster as the transcription data predicted.^[5] Therefore, **4** – **6** are likely the final products.

We purified compounds **4** – **6** from large-scale fermentation and elucidated their structures on the basis of their spectroscopic data. To our surprise, **4** – **6** bear little structural resemblance to either citreoviridin or aurovertin. Instead, all three compounds contain a 2,3-dimethyl- γ -lactone substituted with a 1-carboxyethyl group (Scheme 1A), a moiety found in several other fungal natural products (Scheme 1B).^[16–19] Compound **4** had a molecular formula of C₂₄H₃₄O₆. Its ¹H, ¹³C, DEPT, and HSQC NMR spectra exhibit signals for seven methyl groups, three olefins, and three carbonyl groups. The conjugated triene moiety could be assigned unambiguously from the ¹H-¹H COSY and ¹H-¹³C HMBC correlations, whereas the trimethylcyclopentanone and 2,3-dimethyl- γ -lactone moieties were constructed mainly from the ¹H-¹³C HMBC correlations. From the NOESY correlations, we assigned relative configurations of (2*R**,5*S**,6*R**) and (14*S**,16*R**,17*R**), as shown in Scheme 1A (see the Supporting Information for detailed structural elucidation of all compounds). Compound **5** has the same molecular formula as **4** and the NMR spectral data of the former are very similar to that of the latter. From these data and the NOESY correlations, we established **5** to be a 16-epimer of **4**. We named compounds **4** and **5** aspernidgulene A1 and A2, respectively. Compound **6** had a molecular formula of C₂₄H₃₄O₅. Its ¹H, ¹³C, and 2D NMR spectral data indicate that **6** also contains the same 2,3-dimethyl- γ -lactone as that found in **4** and **5** but lacks the trimethylcyclopentanone moiety and is a tetraene instead of a triene. We named it aspernidgulene B1.

Having identified the products of the *sdg* cluster, we proceeded to investigate the function of each individual gene. First, we created two promoter replacement strains: *alcA(p)-sdgA* and *alcA(p)-sdgADF*. We found that the metabolite profiles of both strains were identical to that of the *alcA(p)-sdgAD* strain (Figure 1, ii and v, vi). These data indicate that SdgD and SdgF do not act immediately downstream of SdgA and that the HR-PKS SdgA alone is sufficient for the production of the peaks representing compounds **1** – **3**. Purification of compounds **1** – **3** was difficult, possibly because of the instability of polyenes.^[20] However, we were able to deduce the chemical structures of **1** and **2** from the spectra we obtained (see the Supporting Information for details). They were 5,6-dihydro- α -pyrone-containing hexaenoic acids, which we named preaspernidgulene A1 (**1**) and A2 (**2**) (Scheme 1A). As SdgA does not contain an oxygenase domain, it is likely that the terminal carboxylic acid functional groups on **1** and **2** were installed by unknown endogenous enzymes.^[21] Despite repeated attempts, we were unable to obtain compound **3** for NMR spectral analysis owing to its instability.

After concluding that SdgD and SdgF do not act immediately downstream of SdgA, we turned our attention to SdgC. We created an *alcA(p)-sdgAC* strain, which produced compounds **7** – **9** (Figure 1, vii). Compound **7** has the same molecular formula as **6**. Both molecules have similar ¹H and ¹³C NMR spectral data, except for signals from the polyene portion. We elucidated the structure of **7** on the basis of ¹H-¹³C HMBC correlations, as shown in Scheme 1A, and we named **7** aspernidgulene B2 as a result of its structural similarity to aspernidgulene B1 (**6**). The structures of **8** and **9** were revealed by NMR

characterization (Scheme 1A, see the Supporting Information for details). As these compounds resemble **1** and **2**, we named **8** and **9** preaspermidgulene A3 and A4, respectively. That all three compounds contain a secondary alcohol on their side chain suggests that SdgC acts on the polyene tail.

Lastly, to establish the roles of the predicted hydrolase SdgD and the oxidase SdgF, we generated two strains: *alcA(p)-sdgADC* and *alcA(p)-sdgACF*. The *alcA(p)-sdgADC* strain produced **7** without detectable amounts of **8** and **9** (Figure 1, viii), unlike the *alcA(p)-sdgAC* strain (Figure 1, vii). These data suggest that SdgD works in concert with SdgC to facilitate the complete conversion of the polyketide precursor into **7** and that **8** and **9** are presumably shunt products. In the *alcA(p)-sdgACF* strain, we observed the reemergence of **4** – **6** (Figure 1, ix); this indicates that SdgF is involved in trimethylcyclopentanone synthesis.

Interestingly, the *alcA(p)-sdgACF* strain also produced minor products such as **8** and **9** as well as **10**, **13**, and **16** – **19** that were not found in the *alcA(p)-sdgADC* strain (Figure S1). Compounds **10** and **13** are likely epimers of **4** and **5** on the basis of their UV-Vis and mass spectra (Figure S2 and S3), thus suggesting that SdgD could contribute to the stereoselectivity.

On the basis of our data, we propose a biosynthetic pathway of aspermidgulene A1 (**4**) (Scheme 2). The carbon backbone is synthesized by the HR-PKS SdgA, which accepts acetyl-CoA as the starter unit and performs malonyl-CoA extensions as well as regioselective methylation and reduction. The resulting nonaketide offloads the HR-PKS by intramolecular lactonization to yield a 5,6-dihydro- α -pyrone polyene. SdgC then installs the first epoxide on the penultimate double bond. Subsequently, a ketone intermediate is generated through Meinwald rearrangement involving a hydride shift. As **4** and **5** are only detected in *sdgF* promoter replacement strains, SdgF is presumably involved in this rearrangement. Next, SdgC introduces another epoxide on the last olefin of the ketone intermediate after *E/Z* isomerization, reminiscent of the actions of its homologs CtvC and AurC in iterative polyene epoxidation.^[6,7] SdgD then catalyzes stereospecific cyclization of the 5,6-dihydro- α -pyrone and opening of the epoxide ring to form an oxygenated trimethylcyclopentanone and an oxabicyclo[2.2.1]heptane unit. Finally, the bicyclic unit undergoes hydrolytic cleavage, either spontaneously or catalyzed by SdgD, to assemble the dimethyl- γ -lactone moiety in compound **4**. The presence of **4** and its epimers in strains not expressing *sdgD* (Figure 1, ix and Figure S1) likely results from non-stereoselective, spontaneous cyclization of the epoxidated ketone intermediate and subsequent spontaneous hydrolytic cleavage of the oxabicyclo[2.2.1]heptane. This hypothesis is supported by the results of Sofiyev *et al.*, who obtained oxabicyclo[2.2.1]heptanes by treating the β -keto lactone substrate with camphorsulfonic acid.^[22] Moreover, treatment of prugosene A1 with diluted NaOH led to the production of prugosene B1 (Scheme 1B).^[16] Thus, bicyclic unit formation and hydrolysis can happen non-enzymatically under acidic and basic conditions, respectively.

Interestingly, SdgD is implicated in several stereocontrol steps. The relative configurations of the trimethylcyclopentanone moieties in compounds **4** (14*S**,16*R**,17*R**) and **5** (14*S**,16*S**,17*R**) are determined by the configurations of the last two double bonds and the two epoxides installed on them. Given that each double bond could be either *E* or *Z* and could be

epoxidized in two different orientations, 16 combinations are possible (Figure S4 – S7). Of them, four would result in the configurations of **4** and another four would result in the configurations of **5**. The conformation depicted in Scheme 2 represents only one of the possible ways by which **4** could be formed. How SdgD works in concert with SdgC in isomerization and epoxidation to achieve the stereoselectivity remains to be investigated biochemically. Additionally, the NOESY data indicated that the methyl groups attached to C-6 in compounds **4** and **5** (CH₃-22) (Table S8 and Figure S9) and wortmannilactone G are all on the β face, whereas the methyl group on C-6 in prugosene B1 is on the α face. The stereochemistry at C-6 is determined by how the β -keto lactone intercepts the positive charge on C-6 after opening of the epoxide ring. Because no C-6 epimers were isolated in any of the three studies,^[16,17] this reaction is also presumably enzymatically controlled by SdgD and its homologs in wortmannilactone and prugosene biosynthesis.

Perhaps the most interesting step in our proposed biosynthesis is the Meinwald rearrangement following the first SdgC epoxidation. Until the recent discovery of PenF by Zhou *et al.*, no enzyme had been known to perform Meinwald rearrangement.^[23] Although our data indicate that SdgF could play a role in this rearrangement, further biochemical verification is needed because SdgF is predicted to be a FAD-containing dehydrogenase whereas PenF belongs to the CrtC (carotenoid 1,2-hydratase) superfamily. Furthermore, compounds **6** – **9** were off-pathway products that were likely generated before Meinwald rearrangement. Reduction of the epoxide intermediate, presumably by endogenous enzymes, would lead to the formation of **8** and **9**. Spontaneous cyclization and hydrolytic cleavage of the epoxide intermediate would produce **7**, after which hydride shift, possibly catalyzed by SdgF, would yield **6**.

In conclusion, using serial promoter replacement, we discovered eight polyketides, the aspernidgulenes, that are new to science and elucidated their succinct biosynthesis. Conversion of the 5,6-dihydro- α -pyrone polyene into **4** and **5** required only three enzymes; this demonstrated the conciseness of the Sdg biosynthetic pathway. As similar enzymes are likely employed in the biosynthesis of related natural products such as the prugosenes,^[16] the wortmannilactones,^[17,24] shimalactone A,^[18] and coccidiostatin A,^[19] this study paves the way for future genome mining endeavors. Moreover, the characterization of SdgA contributes to the growing database of fungal ER-less HR-PKS gene-product connections (e.g., the first characterization of a mushroom ER-less HR-PKS was recently described^[25]) which will lay the foundation for predicting polyene structure from phylogenetic analysis.

Notably, although the *sdg* cluster exhibits significant homology with the *ctv* and *aur* clusters, it lacks an *O*-methyltransferase gene. In compounds related to citreoviridin and aurovertin, *O*-methylation of the α -pyrone is universally observed.^[26] In fact, downstream tailoring enzymes have been shown to be selective for methylated as opposed to unmethylated α -pyrones.^[6,7] On the other hand, our results here demonstrated that unmethylated 5,6-dihydro- α -pyrone could undergo cyclization to form another class of compounds. Thus, α -pyrone methylation is a biosynthetic branching point at which remarkable chemical diversity is generated by homologues of *ctvC/sdgC* and *ctvD/sdgD* as well as of *sdgF*. We propose that for clusters homologous to *ctv* and *sdg* (also containing homologs of *ctvC/sdgC* and *ctvD/sdgD*), the *O*-methyltransferase gene is a genetic indicator of whether the product

belongs to methylated α -pyrone polyenes or polyenes that undergo further 5,6-dihydro- α -pyrone cyclization to generate moieties including dimethyl- γ -lactone and oxabicyclo[2.2.1]heptane. If proven to be applicable, such accurate and rapid structural prediction from genetic information would bypass laborious genetic and chemical interrogation, and would be essential for the fully and efficient untapping of the potential of secondary metabolite as a source of novel chemicals.^[27]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Organization of the aspernidgulene biosynthesis gene cluster and total scan UV-Vis HPLC profiles of culture media of *A. nidulans* strains expressing aspernidgulene biosynthesis genes under the control of *alcA*(p).

Scheme 1.

(A) New compounds isolated from the study. (B) Representative compounds containing the oxabicyclo[2.2.1]heptane unit or their derivatives.

Scheme 2.
Proposed biosynthetic pathway for asperniduglene A1 (**4**).

Table 1.

Aspernidgulene biosynthesis genes in *A. nidulans*, their homologs in other species, and gene function predictions.

gene	putative function	<i>Aspergillus terreus</i> var. <i>aureus</i>	<i>Calcarisporium arbuscula</i>	<i>Alternaria solani</i>
AN1783	dehydrogenase	---	---	Sol3 (25, 45) ^a
<i>sdgA</i> (AN1784)	highly reducing polyketide synthase	CtvA (44, 59) ^a	AurA (45, 63) ^a	Sol1 (28, 44) ^a
<i>sdgD</i> (AN1785)	hydrolase	CtvD (24, 40) ^a	AurD (23, 41) ^a	---
<i>sdgC</i> (AN1786)	flavin-dependent monooxygenase (FMO)	CtvC (39, 56) ^a	AurC (37, 54) ^a	---
<i>sdgF</i> (AN1787)	FAD linked oxidase	---	---	Sol5 (29, 44) ^a

^aNumbers in parentheses represent the percentage of identity and similarity to Sdg proteins.