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Signaling the Induction of Sporulation Involves the Interaction of Two Secondary Metabolites in *Aspergillus nidulans*

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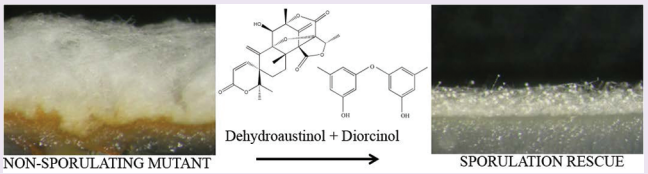
Supporting Information

ABSTRACT: When growing *Aspergillus nidulans* hyphae encounter the atmosphere, they initiate a morphogenetic program leading to the production of spores. Mutants that are defective in the *fluG* gene fail to undergo sporulation because they lack an endogenous diffusible factor that purportedly accumulates on aerial hyphae, thus signaling the initiation of development. In this study, the defect could be reversed by adding culture extracts from a wild-type strain onto a mutant colony. Moreover, a bioassay-guided purification of the active culture extract resulted in the identification of the active agent as dehydroaustinol. However, this meroterpenoid was active only when administered in conjunction with the orsellinic acid derivative diorcinol. These two compounds formed an adduct that was detected by HRMS in an LC−MS experiment. The diorcinol-dehydroaustinol adduct prevented crystal formation of the signal on the surface of aerial hyphae and on an artificially prepared aqueous film and also increased the signal lipophilicity.



Filamentous fungi are a diverse group microorganisms that can be found in most environments, including living organisms. Their widespread distribution is largely due to a fundamental part of the life cycle, i.e., their ability to undergo prolific production of aerial spores for dispersion. The process of spore production in filamentous fungi has many practical implications in agriculture, food preservation, livestock management, and health.[1]

The process by which growing fungal cells (hyphae) sense their emergence into the aerial environment and initiate the process of spore production has been the subject of many studies in *Aspergillus nidulans*, an accepted model organism with a publically available genome sequence.[2] In addition, *A. nidulans* is closely related to species that are involved in food spoilage, industrial processes, and human disease.[3,4]

The mechanism underlying the perception of the aerial stimulus and the first responses leading to spore production are being intensively studied.[5] Early screenings in the search for genetic defects at early stages of sporulation yielded non-sporulating mutants that produced masses of undifferentiated aerial hyphae (*flu*, for *fluffy mutants*). The mutants that are affected in the *fluG* gene were shown to be defective in the synthesis of an extracellular diffusible factor (known as the FluG factor), which is necessary to initiate the process. Interestingly, this factor could be transferred from a neighboring wild-type colony to a mutant *fluG*$^-$ colony, thus restoring sporulation at the contact site.[6] The fluffy phenotype of *fluG*$^-$ mutants could also be overcome under carbon and nitrogen limitations and osmotic stress.[7] Later studies showed that other fluffy mutants (*flbA-E*$^-$) were capable of producing the FluG factor, but they lacked a second diffusible signal. Hence, these mutants could complement *fluG*$^-$ mutants upon contact, but not *vice versa*. This finding was interpreted as evidence that the *flbA-E*-dependent signal was produced downstream of the *fluG*-dependent factor.[8,9] The chemical identities of the two factors were not elucidated in these studies.

A more recent evaluation of the role of a 4-phosphopantetheinyl transferase (PPTase) in steroid and polyketide synthesis revealed that a functional form of the PPTase CfwA/NpgA was required for the production of several polyketides and meroterpenoids [hybrid molecules of both polyketide and terpenoid origin[10]] including the meroterpenoid dehydroaustinol. Mutants that are defective in the *cfwA* gene function also presented severe sporulation defects. This characteristic opened the possibility that one of these compounds was the FluG factor. However, supplementing a null mutant of the *fluG* gene (Δ*fluG*) with the aforementioned compounds, including dehydroaustinol, did not reverse the fluffy mutant phenotype.[11]

In the work described here, a bioassay-guided purification of wild-type extracellular fractions was undertaken in the search for the diffusible factor that rescued the sporulation phenotype of a Δ*fluG* mutant. The study revealed that all fractions

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containing dehydroaustinol were capable of rescuing sporulation except for the stage at which the meroterpenoid was purified to homogeneity. Further analysis of active fractions revealed that the other component required for activity was diorcinol, a compound with which dehydroaustinol formed an adduct. The adduct prevented the crystallization of dehydroaustinol in an aqueous film comparable to the hypha surface and conferred higher lipophilicity to the signal, both of which have possible functional implications.

RESULTS AND DISCUSSION

Wild-Type Culture Extracts Restore Aerial Sporulation in Δ*fluG* Colonies. In order to assess the sporulation-inducing activity of mature medium [a medium that has previously sustained growth[12]], the medium from a wild-type sporulating strain (FGSC26; Table 1) was prepared and tested as follows.

Table 1. *Aspergillus nidulans* Strains Used in This Study

strain	genotype	source or reference
FGSC26	*biA1 veA1*	Fungal Genetic Stock Center
TTA127.4	*pabaA1, yA2;* Δ*fluG::trpC*$^+$; *trpC*801	6
BD85	*biA1* Δ*fluG::trpC veA1*	cross between FGSC26 and TTA127.4
CRO1	*cfwA2 pabaA1 yA2 veA1*	11
BD143	*pyrG89* Δ*nkuA::argB argB* Δ*flbB::pyrG pyroA4 veA1*	13

Shaken flask cultures containing different proportions of fresh and mature medium were prepared and adjusted for glucose and nitrate concentration. The appearance of conidiophores [specialized structures bearing asexual spores[14]] was followed throughout a culture period of 30 h. This experiment showed that, as the proportion of mature medium increased, the time required for conidiophores to appear became shorter. The earliest appearance (17 h) took place 6 h sooner in 100% mature medium than in fresh medium (23 h; Supplemental Table 1). When the same experiment was performed with mature medium obtained from a Δ*fluG* strain (Table 1), conidiophores were detected after 23 h of culture, as in the case of the fresh medium. Thus, it was concluded that the wild-type mature medium contained a chemical factor that reduced the period required for the induction of sporulation and that the Δ*fluG* mutation prevented the production of that factor.

Mature media from wild-type and a Δ*fluG* mutant were fractionated with ethyl acetate and applied as a methanol extract to 23 h-old Δ*fluG* surface colonies. After an additional incubation period of 17 h, colonies that were administered extracts from the Δ*fluG* mutant had a fluffy phenotype and low spore counts ($(2.3 \pm 1.2) \times 10^3$ spore mm^{-2}). In contrast, the colonies that had been administered extracts from the wild-type strain presented visible sporulation (lack of aerial hyphae and conidiophores, Figure 1A) and a 10-fold increase in spore production ($(2.9 \pm 0.9) \times 10^4$ spore mm^{-2}), although this level of production is moderate in comparison to wild-type colonies ($(9.7 \pm 1) \times 10^4$ spore mm^{-2}).

The bioassay was also performed on a *cfwA2* strain (Table 1), which is defective in PPTase activity and has been reported to be affected in the biosynthesis of several polyketides, sterols, and meroterpenoids. This strain also displayed a defect in asexual development.[11] The results show that the wild-type extracts partially complemented the defects of the *cfwA2* strain ($(1.1 \pm 0.8) \times 10^4$ spore mm^{-2}, Figure 1B). When the extract was applied on a Δ*flbB* strain (Table 1), which had been reported to produce the FluG factor but to lack the second diffusible signal,[9,13] a sporulating response was not observed ($(1.1 \pm 0.5) \times 10^3$ spore mm^{-2}). The addition of salts restored a sporulation response in Δ*flbB* colonies through an already characterized stress response ($(6.8 \pm 0.9) \times 10^4$ spore mm^{-2}).[13,15]

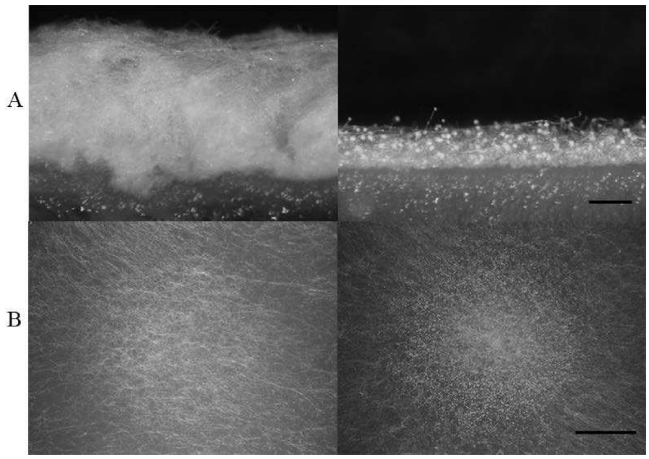

Figure 1. (A) Cross section of the mycelia of the Δ*flu*G mutant after the addition of an ethyl acetate extract of the culture medium from a Δ*flu*G strain (left) and the wild-type strain FGSC 26 (right). The Δ*flu*G extract-treated colonies show masses of undifferentiated hyphae (left, 793 ± 93 μm in height). FGSC 26 extract-treated colonies rescue the sporulating phenotype and do not show masses of undifferentiated hyphae (right, 354.5 ± 44 μm in height). (B) Extracellular complementation of the sporulation defect of mutant *cfwA2*. Left, a Δ*flu*G extract-treated colony; right, a sporulating FGSC extract-treated colony. Scale bar = 1.5 mm.

Taken together, these experiments provide evidence that extracts from the mature medium of the wild-type strain contained one or more factors that are capable of inducing both premature sporulation in the wild-type strain in submerged culture and of reverting the fluffy phenotype of the Δ*fluG* and *cfwA2* strains in surface cultures through specific complementation of their respective defects. The specificity of the response was confirmed by the observation that the extracts were ineffective on a Δ*flbB* mutant, which requires a different signaling factor.

Dehydroaustinol and Diorcinol Are Jointly Required for Activity. The ethyl acetate extract from a mature medium of wild-type strain cultures was used for further fractionation by reverse phase C18 solid phase extraction (SPE, see Methods). Each fraction was subsequently screened for sporulation-inducing activity by applying it onto a Δ*fluG* colony (Supplemental Table 2). A single active fraction eluting between 50% and 60% methanol/water (v/v) showed activity, as can be seen in Figure 2a. The HPLC profile of this fraction contained a prominent single peak with a retention time (Rt) of 36.5 min, which was absent or very attenuated in the equivalent fraction from a Δ*fluG* mutant culture (Figure 2b,c). This particular peak was examined in more detail as it appeared to be the most likely sporulation signal.

Further purification by semipreparative reverse phase HPLC and subsequent analysis by NMR of the fraction indicated the

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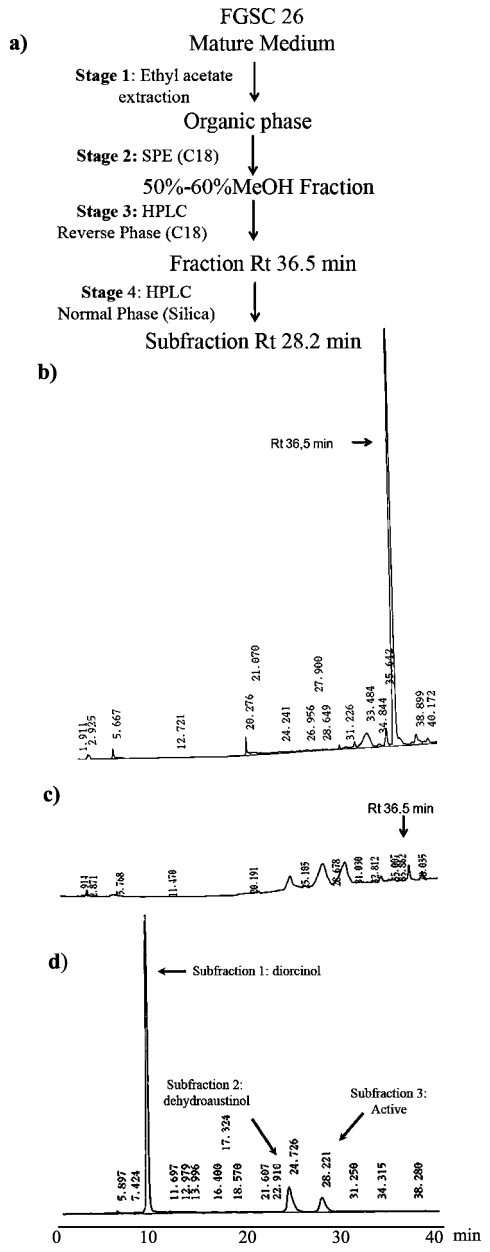

Figure 2. Stages of purification and corresponding HPLC chromatograms. (a) Simplified flowchart for the experimental design used for the purification of the sporulation-inducing factor from the mature medium of an FGSC26 culture. The stages bearing no sporogenic activity have been omitted. SPE = solid phase extraction. Rt = retention time. (b) HPLC chromatogram of the FGSC 26 extract at stage 2, showing a prominent peak (arrow). (c) HPLC chromatogram of a Δ*fluG* strain extract at stage 2, showing a minimal peak at the same retention time (arrow). (d) HPLC chromatogram of the compounds separated by normal phase chromatography at stage 4. The active fraction was eluted with a Rt value of 28.2 min.

presence of more than one compound. Further attempts to isolate the components of the mixture by additional reverse phase HPLC steps proved unsuccessful. Therefore, the fraction components were separated by normal phase chromatography on a silica gel column, which yielded three clearly discernible subfractions (Figure 2d). Analysis of the NMR and MS data along with LC−MS experiments on these subfractions yielded positive identifications as diorcinol, dehydroaustinol, and a mixture of the former with austinol (Figure 3; Supplemental Figure 1 and Supplemental Table 3). When all three subfractions were subjected to bioassay, only the third one showed substantial levels of morphogenetic activity ($(2.5 \pm 0.6) \times 10^4$ spore mm^{-2}).

The result suggests that either austinol was the active signaling compound or a mixture of the aforementioned compounds was needed for activity. An assay for activity of all three compounds alone and combined in the proportions found in subfraction 3 (dehydroaustinol/diorcinol/austinol, 2:1:1) clearly showed that dehydroaustinol and diorcinol were jointly required for activity and that austinol was inactive (Figure 4).

Closer examination by light microscopy of mycelial preparations that had been administered dehydroaustinol revealed the presence of crystals on and among hyphae. In contrast, the mycelia that had been administered both dehydroaustinol and diorcinol did not show the presence of crystals (not shown). The formation of dehydroaustinol crystals in an aqueous film manually prepared on a microscope slide was observed to take place within 5 min at RT, as shown in Figure 5A. When the meroterpenoid was added to the aqueous film with diorcinol, however, the sample remained as an emulsion (Figure 5B). The interaction between the two molecules appeared to be specific, as the simpler but structurally related orcinol had no significant effect on dehydroaustinol crystal formation when added at the same concentration as diorcinol (Figure 5C). This result supports the hypothesis that diorcinol interacts with dehydroaustinol to form an adduct that prevented crystal formation at the hypha surface, thus facilitating the spreading of the signal over aerial hyphae and improving access of the meroterpenoid to its putative receptor. Indeed, the formation of an adduct between these two compounds is consistent with their coelution in reverse phase HPLC and the fact that only partial separation was achieved by normal phase HPLC. The presence of this adduct was also confirmed by the negative mode HRESIMS of the pseudomolecular ion $[M_{diorcinol} + M_{dehydroaustinol} - H]^-$ at m/z 685.2656 (Δ 0.7 ppm) observed in the LC−MS chromatogram (Figure 3) of the active subfraction (third fraction eluted in normal phase HPLC). Studies to determine the nature of this interaction are currently under way.

Activity of Other Austin Derivatives. In order to determine the specificity of dehydroaustinol as a signaling molecule, a number of austin derivatives, which were not found in *A. nidulans* extracts, were tested in the bioassay both in the presence and absence of diorcinol. The results are illustrated in Figure 6 and they clearly show that diorcinol was required as an accompanying molecule to induce spore production in all instances. Dehydroaustinol and dehydroaustin (compounds **1** and **3** in Figure 7), which differ only in the presence of an acyl group that forms an ester with the hydroxyl group at C11, showed comparable levels of activity. However, austinol and austin (compounds **2** and **4** in Figure 7), which are similar to dehydroaustinol and dehydroaustin, respectively, but lack the ether bond between C9 and C6′, were inactive. In the case of acetoxydehydroaustin (compound **5** in Figure 7), which showed insignificant activity, the ether bond is present but this compound differs from the active compounds in the presence of an acetoxy group at C7. These results indicate the requirement of two important structural features for activity: a similar substitution to austinol and the ether bridge bond between C9 and C6′.

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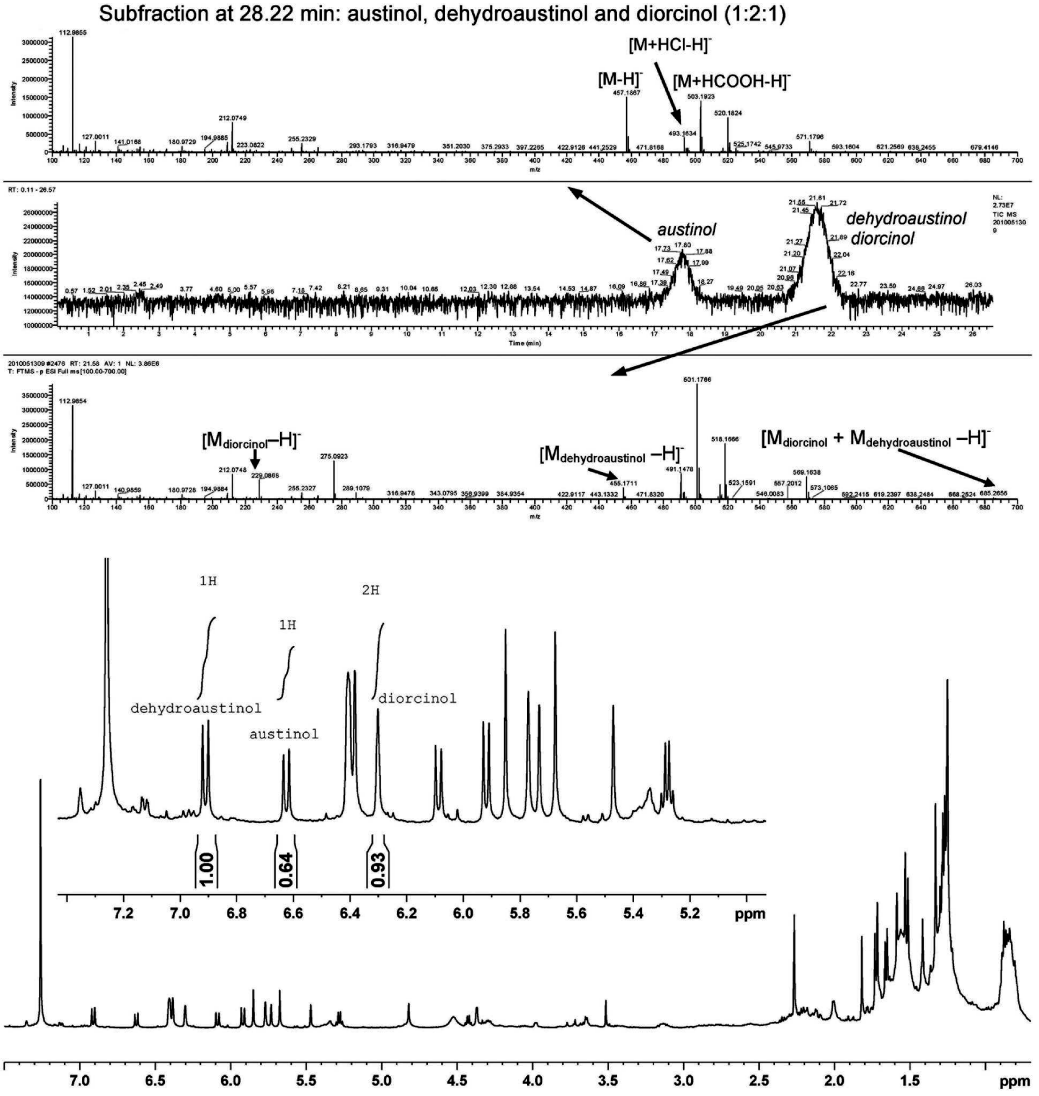

Figure 3. LC−MS chromatogram, HRESI mass spectra in the negative mode, and ^{1}H NMR spectra of the active subfraction obtained from the isocratic NP-HPLC at stage 4 (see Figure 2a). Chromatographic conditions: LTQ Orbitrap Thermo Fisher Sci spectrometer, Phenomenex Luna C18 column 4.6 mm × 150 mm, 5 μm. Temperature at 35 °C. Mobile phase: isocratic H_2O/acetonitrile (61:39) in 40 min, flow rate 0.6 mL/min.

The calculated *P* values, which represent the partition coefficient between octanol and water, for the austin derivatives diorcinol and orcinol were examined in order to evaluate the hydrophobic character of these compounds. Comparison of the calculated *P* values (Table 2) revealed that dehydroaustin and dehydroaustinol have the highest values and these represent octanol/water partition coefficients that are approximately 1 order of magnitude higher than those of the inactive austin derivatives. Moreover, diorcinol has a partition coefficient in octanol/water (*P*) that is 3 orders of magnitude greater than that of dehydroaustinol and orcinol. These values suggest that the dehydroaustinol-diorcinol adduct would have an increased hydrophobic character, which would in turn favor its access to the plasma membrane.

The prolific production of secondary metabolites by microorganisms has puzzled chemical biologists for decades and various hypotheses have emerged to explain such biosynthetic versatility.[16,17] In some instances, the signaling compounds are packaged and delivered with the aid of other molecules. A notable example is the encapsulation of the quorum signaling compound 2-heptyl-3-hydroxy-4-quinolone into membrane vesicles.[18] In the present study, the adduct of two secondary metabolites ensures that the signal molecule remains in suspension at the hypha/air interface, where rapid evaporation of the aqueous film and concomitant solute concentration may result in crystal formation. Moreover, the calculated P values for the adduct suggest that it is capable of accessing hydrophobic sites and/or crossing the plasma membrane barrier. The putative receptor and signal transduction mechanism remains to be clarified.

The positive identification of the FluG signaling molecule as a meroterpenoid with conformational characteristics that are common to dehydroaustinol and dehydroaustin should entail that any mutation resulting in the loss of function of any enzyme that participates in the biosynthesis of these compounds should yield non-sporulating fluffy mutants. In agreement with this postulate, studies in which the PPTase-encoding gene *cfwA* was mutated blocked the production of dehydroaustinol and a range of other metabolites and yielded non-sporulating colonies.[11] However, a recent genome wide analysis of polyketide synthases (PKS), which identified *ausA* (AN8383) as the gene encoding a PKS producing 3,5-dimethyl

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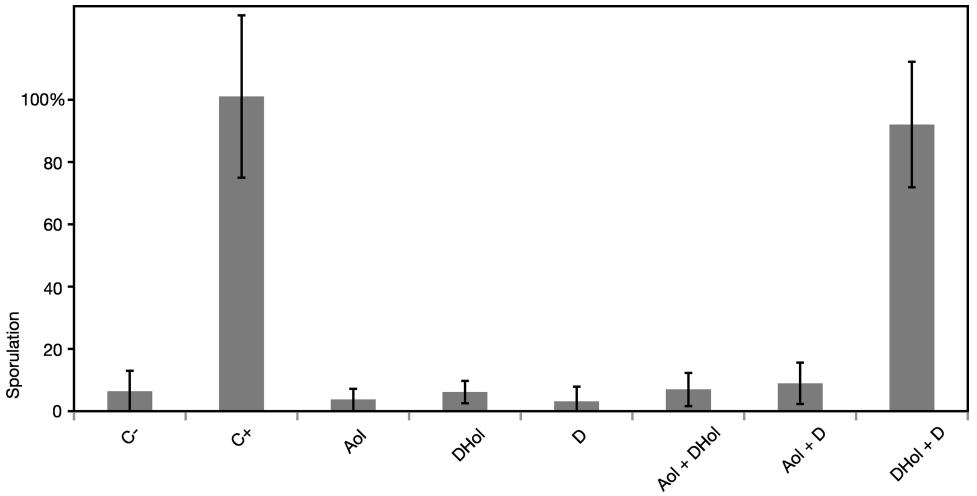

Figure 4. Sporulation induction measured in a Δ*flu*G strain on addition of dehydroaustinol (10 μg), diorcinol (5 μg), and austinol (5 μg) in the proportion (2:1:1) found in the active fraction (Figure 2d). C−, control with methanol; C+, active extract from stage 2 (Figure 2a,10 μg); DHol, dehydroaustinol (10 μg); D, diorcinol (5 μg); Aol, austinol (5 μg). Results are presented as percentage of the positive control ((2.9 ± 0.9) × 10^4 spore mm^{-2}). The results are the mean of six separate experiments and bars indicate standard error of the mean.

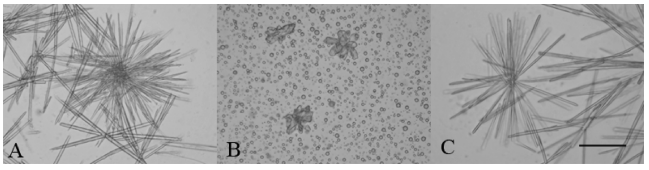

Figure 5. Diorcinol suppresses dehydroaustinol crystal formation in an aqueous environment. (A) Dehydroaustinol. (B) Dehydroaustinol + Diorcinol (2:1). (C) Dehydroaustinol + Orcinol (2:1). Bar = 0.05 mm.

orsellinic acid, the first intermediate in the complex biosynthesis of austinol and dehydroaustinol, has produced a contrasting result.[19] The phenotype observed for all single PKS mutants in that study (including Δ*ausA*) was of sporulating colonies. Moreover, austinol and dehydroaustinol were not produced in all of the media tested in the study, and this variability did not reportedly alter the sporulating phenotype of the colonies. Our current interpretation of all the results taken together is that the FluG signal may involve a group of metabolites that includes dehydroaustinol and dehydroaustin-like molecules, as well as other unidentified compounds. All would require the early biosynthetic step involving CfwA/NpgA for their synthesis. However, the later PKS activity of AusA would affect dehydroaustinol and dehydroaustin, but not the other putative signaling compounds. This hypothesis is currently under investigation.

In this study it was determined that diorcinol is a necessary accompanying compound of dehydroaustinol in order to exert sporulating activity in Δ*fluG* colonies. Diorcinol overproduction has been reported in mutants that display sporulation defects,[20] and some of these mutants involve subunits of the COP9 signalosome (CSN).[21−23] However, the synthetic route for diorcinol, or its regulation, along with that of dehydroaustinol need to be clarified before any hypotheses emerge on their possible regulatory connections.

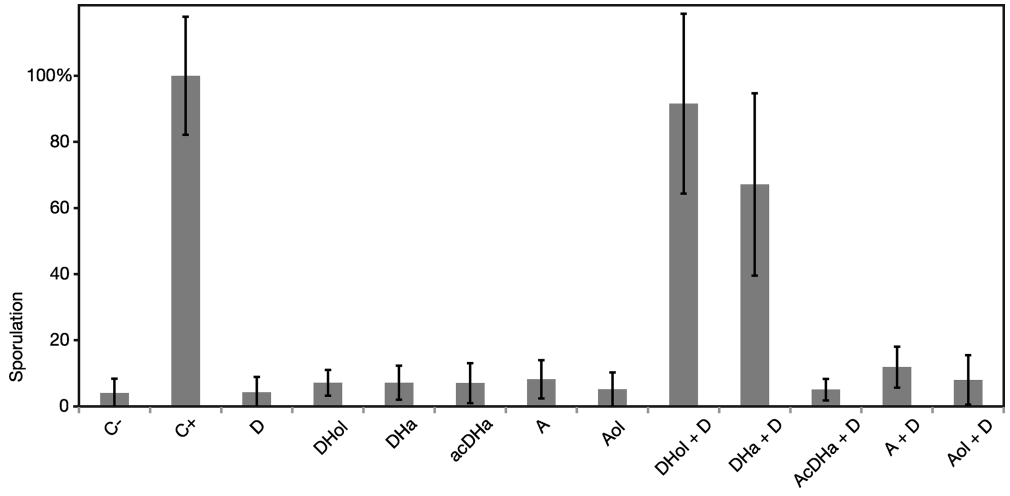

Figure 6. Effect of austin derivatives on sporulation in surface cultures of Δ*fluG* colonies. C−, control with methanol; C+, active extract from stage 2 (Figure 2a,10 μg); D, diorcinol; DHol, dehydroaustinol; DHa, dehydroaustin; AcDHa, acetoxydehydroaustin; A, austin; Aol, austinol. Results are presented as percentage of the positive control ((2.9 ± 0.9) × 10^4 spore mm^{-2}). The results are the mean of five separate experiments, and bars indicate standard error of the mean.

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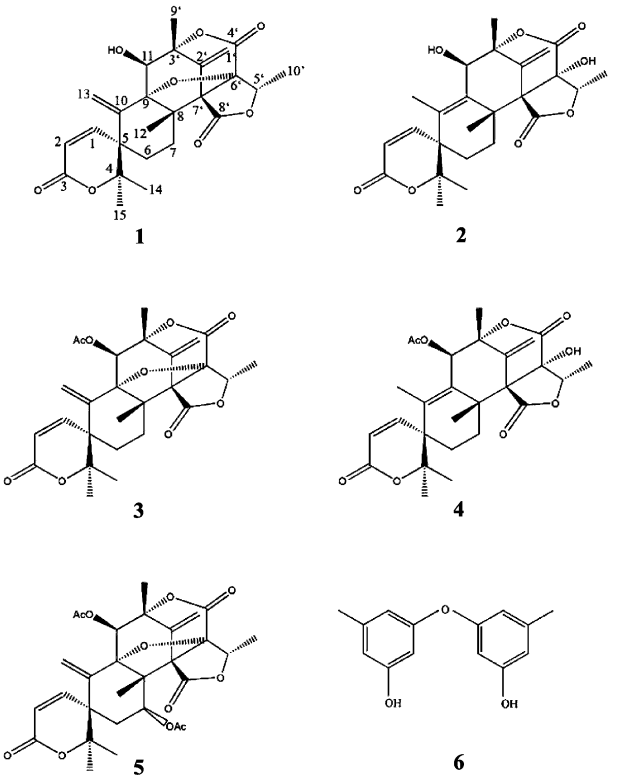

Figure 7. Chemical structures of austin derivatives and diorcinol: dehydroaustinol (**1**), austinol (**2**), dehydroaustin (**3**), austin (**4**), acetoxydehydroaustin (**5**) and diorcinol (**6**).

Table 2. Calculated log *P* values (*P* = Partition Coefficient) of Austin Derivatives Diorcinol and Orcinol in an Octanol/Water Two-Phase System at 25 °C (Source: SciFinder Database)

compound	log *P*
austin	0.140 ± 0.846
austinol	−0.098 ± 0.843
dehydroaustin	1.092 ± 0.872
dehydroaustinol	1.042 ± 0.869
acetoxydehydroaustin	0.458 ± 0.876
diorcinol	4.603 ± 0.349
orcinol	1.279 ± 0.207

A novel aspect revealed in this study is the chemical interaction linking dehydroaustinol and diorcinol and the functional implications of this structure. Initial attempts to separate these two compounds by reverse phase chromatography proved unsuccessful, but normal phase chromatography (Figure 2d) showed that the two compounds interacted to form an adduct with a significantly higher Rt. Changes in this parameter have been used to characterize intermolecular adduct formation[24] and yielded results qualitatively similar to those found in this preliminary study.

The dehydroaustinol-diorcinol adduct would not only serve the purpose of inhibiting dehydroaustinol crystal formation at the hypha/air interface, but the calculated log *P* values support the view that the adduct may also facilitate delivery of dehydroaustinol into or across membrane barriers to a putative receptor. We therefore postulate that the reported interaction is likely to be part of a built-in functional design, and this represents another elegant example of the complex biology and chemistry of secondary metabolism.

METHODS

Proton and carbon NMR spectra were recorded on Varian Mercury Plus 200, Bruker Avance 300 and 500 MHz spectrometers using $CDCl_3$ and CD_3OD as the solvents. Multiplicities of ^{13}C signals were obtained by DEPT-135 experiments. High resolution electrospray ionization mass spectra (HRESIMS) were measured on an Elite spectrometer. LC−MS were run on an Applied Biosystems Q-q-TOF QSTAR spectrometer or an Applied Biosystem API 3200 system connected to Agilent 1200 HPLC chromatographs. Also for LC−MS/MS experiments, a Thermo Fisher Scientific LTQ Orbitrap system connected to an Accela U-HPLC was used. HPLC separations were run on a Shimadzu-6A instrument.

Microscopic observations were carried out with Nikon Optiphot and Nikon SMZ800 stereoscopic microscopes. Images were taken with a Nikon Coolpix 8400 camera.

Strains and Culture Conditions. The *A. nidulans* strains used in this study are listed in Table 1. The sporulation signaling compounds were obtained from 7 day-old static liquid cultures of the wild-type FGSC26 strain, grown in 150 mm diameter Petri dishes containing 100 mL of medium, consisting of malt extract (3%) and peptone (0.5%) in distilled water (pH = 5.4),[25] with an inoculum load of 10^6 spore mL^{-1}.

Bioassays were carried out in glucose minimal nitrate medium (MMA),[26] adequately supplemented in case of auxotrophy. Agar (1.5%) was added to produce solid medium. All the cultures were performed at 37 °C.

Bioassays for Sporulating Activity. A sporulation induction bioassay was carried out in shaken flask cultures. Erlenmeyer flasks (100 mL) previously siliconized[27] and containing 25 mL of fresh MMA with 0.1 M sodium phosphate buffer adjusted to pH 6.5 and Tween 20 0.05% (w/v) were supplemented with lyophilized 7-day mature medium (medium on which growth has already taken place).[12] Flasks were inoculated with 10^4 spore mL^{-1}, and cultures were grown at 37 °C in a rotary incubator at 200 rpm. The presence of sporulation-inducing activity was determined (by microscopic observation of mycelium pellets taken at different times) according to the shortening of the period required for *A. nidulans* to sporulate in a fresh medium. Sporulation time was considered to be the time of the appearance of the first three conidiophores per pellet.

Solid agar culture bioassays were performed with the non-sporulating Δ*fluG* mutant strain (BD85). Mature medium methanolic extracts from strain FGSC26 were assayed by culturing the Δ*fluG* mutant for 23 h in MMA supplemented with yeast extract 0.025% (w/v) to avoid spontaneous sporulation. The methanolic extract solution (10 μL) was then added to the colony. The colonies were then observed with a binocular microscope 17 h after the addition of the extract. A spore count was also carried out by scraping of the colony surface with 1 mL of Tween 20 (0.02%, w/v). Spores were counted using a Thoma counting chamber.

Purification and Identification of Active Compounds. The malt extract mature medium cultured for 7 days (15 × 1 L batches) was separated from the mycelium and extracted with ethyl acetate. This extract was evaporated to dryness to give 1.235 g of residue. This residue was dissolved in methanol, and water was added to give a concentration of 5% MeOH in water. This solution was subjected to solid phase extraction (SPE) through a 10 g Supelclean LC-18 column (Supelco) that was eluted with a methanol/water gradient. After elution with 50 mL of 50% aqueous methanol, the retained activity was eluted with 100 mL of 60% aqueous methanol, which gave after evaporation and lyophilization 488 mg of residue. This material was dissolved in 1 mL of methanol and further purified by reverse-phase semipreparative HPLC on a Hypersil octadecylsilane column (10 mm × 250 mm; 5 μm diameter particle size) with a mobile phase consisting of a 40 min linear gradient from 0 to 100% acetonitrile/water (v/v) at a flow rate of 1.8 mL min^{-1} and UV detection at λ 210 nm. The HPLC fraction with a retention time of 36.5 min showed

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sporulation-inducing activity. This fraction was evaporated to dryness to give 12.1 mg of residue, which was further purified by normal phase semipreparative HPLC on a Kromasil SIL column (8 mm × 250 mm; 10 μm diameter particle size) using an isocratic mixture of hexane/ethyl acetate (6:4, v/v) as the mobile phase at a flow rate of 1.8 mL min^{-1} and UV detection at λ 254 nm. The process yielded 4.2 mg of an active subfraction (Supplemental Figure 1; Supplemental Table 2).

Experimental Design and Statistical Treatment of Results. Experiments were carried out at least three times each, with at least 3 replicates per experiment. Values are expressed as the mean ± standard deviation. When the standard deviation is not shown, it was less than 10% of the value.

■ ASSOCIATED CONTENT

Supporting Information

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