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Discovery of an extended austinoid biosynthetic pathway in *Aspergillus calidoustus*

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Abbreviations: NP, natural product; NRPS, non–ribosomal peptide synthetase; PKS, polyketide synthase

Running title: Austinoids in *Aspergillus calidoustus*

Key words: *Aspergillus*, meroterpenoids, austinoids, genome mining and natural products

ABSTRACT

Filamentous fungi produce a wide range of natural products that are commonly used in various industrial contexts (*e.g.* pharmaceuticals and insecticides). Meroterpenoids are natural products of interest because of their various biological activities. Among the meroterpenoids, there is a group of insecticidal compounds known as the austinoids. These compounds have also been studied because of their intriguing spiro–lactone ring formation along with various modifications. Here we present an extension of the original austinol/dehydroaustinol biosynthesis pathway from *Aspergillus nidulans*, in the recently identified filamentous fungus *Aspergillus calidoustus*. Besides the discovery and elucidation of further derivatives, genome mining led to the discovery of new putative biosynthetic genes. The genes involved in the biosynthesis of later austinoid products were characterized and amongst them was a second polyketide synthase gene in the *A. calidoustus* cluster that was unusual because it was a noninterative polyketide synthase producing a diketide. This diketide product was then loaded onto the austinoid backbone resulting in a new insecticidal derivative, calidodehydroaustin.

INTRODUCTION

Filamentous fungi have the capacity to produce a copious number of chemical structures that can help in the discovery of important bioactive molecules[1]. In particular, many of these fungus–derived compounds have found important applications such as in the clinically used antibiotics penicillin and cephalosporin, the immunosuppressant cyclosporine, to even crop protectants like the strobilurins[2, 3].

Genes involved in the biosynthesis of fungal secondary metabolites, or also called natural products (NPs), tend to be clustered in the genome[4]. These clusters usually contain one or several central biosynthetic genes encoding multidomain proteins belonging to the polyketide synthase (PKS) or non–ribosomal peptide synthetase (NRPS) protein families[5-7]. Occasionally, the structural diversity generated by PKS is further expanded by prenyltransferase–mediated incorporation of terpenoid moieties. The resulting compounds are referred to as meroterpenoids[8].

The meroterpenoids are a prominent class of NPs and have been extensively studied, with particular emphasis on fungal compounds derived from 3,5–dimethylorsellinic acid[9, 10]. Biosynthetic clusters of meroterpenoids derived from 3,5–dimethylorsellinic acid have been previously reported, including terretonin from *Aspergillus terreus*[11], andrastins from *Penicillium chrysogenum*[12], anditomin from *Aspergillus variecolor*[13], paraherquonin from *Penicillium brasilianum* and austinol from *Aspergillus nidulans*[14, 15] (Figure 1). The biosynthesis of these compounds is very similar in the first step with the formation of the polyketide product 3,5–dimethylorsellinic acid. The following steps usually proceed by farnesylation, methylation, epoxidation and then subsequent cyclization for the biosynthesis[10]. Interestingly, for anditomin the proceeding steps after polyketide formation differ by formation of a phthalide prior to farnesylation, followed by epoxidation and then cyclization[16]. These compounds are also characterized by the cyclizations catalyzed by their terpene cyclases. Each example varies slightly resulting in a change in the main chemical scaffold[9]. Moreover, steps post–cyclization also vary amongst the different compounds. These are the small nuances, which distinguish these similar yet complex molecules.

Among this class, the austinoids have attracted interest due to their intriguing spiro–lactone scaffolds and insecticidal activities[17, 18]. Austinoids were mainly identified in *Aspergillus* and *Penicillium* species[8], and, since their discovery, these NPs have been the topic of labeling studies[19-22] and pathway characterizations[14, 15, 23] (Figure 1). The enzymatic characterization studies demonstrated the chemical complexity each individual enzyme can catalyze within the austinoid biosynthetic pathway (Supplementary Figure S1). For example, the characterization of the ketoglutarate dependent dioxygenase, AusE, provided insights into the formation of the spiro–lactone ring. AusE demonstrated an unprecedented way in which a dioxygenase not only acted in an iterative way, but also led to the formation of the spiro–lactone ring[23].

This study presents a continuation of the original austinol/dehydroaustinol biosynthetic pathway. Interestingly, this continuation was not seen in *A. nidulans*, but in a recently described species, *Aspergillus calidoustus*[24]. This apparent extension of the pathway in *A. calidoustus* led to the discovery of new derivatives, of which, one had insecticidal activity. Moreover, genome mining of the *A. calidoustus* genome revealed a putative austinoid biosynthetic gene cluster, which led to subsequent characterization of the genes involved beyond austinol/dehydroaustinol biosynthesis. Among these genes was a second PKS, which turned out to be noniterative, in fact it is a diketide synthase. This is highly unusual as the majority of fungal PKS are iterative in nature[5].

RESULTS AND DISCUSSION

Identification and characterization of novel austinoid derivatives

Meroterpenoids derived from 3,5–dimethylorsellinic acid have been studied extensively over the past 40 years[10, 25]. This includes the austinoids, with austin being the first derivative discovered in 1976[26]. The full biosynthetic characterization at the enzyme level has been completed not only for first part of the austinol biosynthesis in *A. nidulans*[14, 23], but also the andrastins from *P. chrysogenum*[12], terretonin from *A. terreus*[11, 27] and anditomin from *A. variecolor*[16] (Figure 1). Additionally, a partial biosynthesis of paraherquonin from *P. brasilianum* was also characterized up until berkeleydione[28].

The first austinoid to be discovered was austin **2** from the filamentous fungus *Aspergillus ustus*[26] (Figure 2). Other austinoids discovered from *A. nidulans* were **1** and **8**[29]. Additionally, *P. brasilianum* was reported to produce dehydroaustin **3**, and compounds **1**, **2**, **8**, and acetoxydehydroaustin **10**[28, 30] (Figure 2). Additionally, because austinoids have been shown to be active against different insects such as cockroaches[18] and the mosquito *Aedes aegypti*[17], this prompted us to search for additional austinoid producers to determine the chemical diversity, specifically for the austinoid compound class.

With this aim we analyzed a variety of fungal species from the Jena Microbial Resource Collection (JMRC) (Supplementary Table S1) and found that *Aspergillus calidoustus* was the most promising candidate, producing a vast array of austinoid compounds compared to *A. nidulans*. Many of these compounds were isolated, purified and structurally elucidated by NMR. These include **3**, 7–hydroxydehydroaustin **4**, precalidodehydroaustin **6**, calidodehydroaustin **7** and 1,2–dihydro–2–hydroxyacetoxydehydroaustin **12** (Figures 2, Supplementary Figures S2–S11, and Supplementary Tables S2–S6). NMR data for **3** and **4** are in accordance with previous studies [30, 31, 32]. Interestingly, *A. calidoustus* was only a recently described species, which was previously assigned as *A. ustus*. Hence, the production of austinoids was observed in *A. calidoustus* strains, but not in *A. ustus* making it unlikely that the latter species has the capability to produce austinoids (Supplementary Figure S12). Moreover, analysis of the available *A. ustus* genome (PMID:25706180)[33] did not reveal any potential austinoids' biosynthesis cluster.

Because of their previously known insecticidal activity for some of the austinoid derivatives, the novel *A. calidoustus* austinoid derivatives were tested on different insect families. Among the tested derivatives, **3** was the most active, showing a broad action against *Homoptera* and *Coleoptera* species, confirming previous reports of its insecticidal activity (Table 1)[17, 18]. A newly identified derivative, **7**, showed comparable activity to **3** against the green peach aphid *Myzus persicae* (sucking/piercing insect), but no activity against the other tested species. This was also shown for **2**, where only activity was seen against the green peach aphid. These results indicated that the isolated derivatives show differential activity against diverse insect families.

Identification of a putative austinoids' gene cluster in *A. calidoustus*

Since the genome sequence of *A. calidoustus* was recently released [24], the biosynthetic gene cluster for austinoids could be found by genome mining. The putative cluster encodes for approximately 25 different genes (Figure 3). In comparison to *A. nidulans*, the *A. calidoustus* cluster is not split on two separate chromosomes, but is present on a single scaffold. Additionally, the putative *A. calidoustus* austinoid biosynthetic gene cluster possesses five genes not present in *A. nidulans*. Of these five genes, one encodes a second putative PKS (Figure 3 and Table 2).

Characterization of the calidodehydroaustin pathway

Because of the variety of austinoids detected and the apparent extension of the original pathway from **1** and **8** (Figure 3), the enzymatic characterization of the putative novel and unknown genes was undertaken. In order to study the biosynthesis a classical method for determining the activity of each enzyme was employed. This required the creation of individual gene deletion mutants to show a particular enzyme is responsible for each biosynthetic step. Additionally, since *A. calidoustus* is not a filamentous fungal model organism, a transformation protocol had to be developed in order to characterize the newly annotated genes in the austinoid biosynthetic gene cluster. Furthermore, for the first deletion mutant, a special *A. calidoustus* strain was created by deletion of the non–homologous end–joining repair mechanism, which coded for the AkuB DNA helicase. For other fungi, this strategy has been shown to improve targeted transformation efficiency as much as 80 %[34]. The *akuB* knock–out mutant of *A. calidoustus* generated here was used as the recipient to obtain further deletion strains (Supplementary Figure S13 and Supplementary Table S7). Each step of the biosynthetic pathway was then verified by the deletion of the different genes in the cluster and linking them to the various austinoid derivatives (Figure 3).

Since the biosynthetic pathway up to austinol **1** has already been elucidated[11, 14, 15, 23] (Supplementary Figure S1), deletion of genes *ausA* (PKS) and *ausN* (prenyltransferase) was performed in order to confirm that the identified pathway in *A. calidoustus* was correctly annotated. As expected, the Δ*ausA* mutant strain showed a null chemotype, *i.e.*, no production of austinoids was observed, while the Δ*ausN* strain only produced the PKS product, 3,5–dimethylorsellinic acid (Figures 3 and Supplementary Figure S14). According to the obtained results, we focused our attention on the *A.*

calidoustus genes, whose orthologue genes were not previously characterized for austinol **1** biosynthesis in *A. nidulans* (Table 2 and Figure 3).

The biosynthesis from **1** to **2** involves an acetylation of the hydroxyl group on carbon–11. Remarkably, the austinoid gene cluster encodes two neighboring putative O–acetyltransferases, AusP and AusQ. Deletion of *ausP* showed complete loss of **2** production (Figure 4), confirming that this enzyme, not present in *A. nidulans*, uses **1** as a substrate. Moreover, this was also shown in the *ausQ* deletion mutant that can produce **2** and **3** (Figure 4). It was already hypothesized that the conversion from **1** to **8** could possibly be a spontaneous reaction[14]. As well as for **8**, the presence of **3** was always detected when **2** was produced in *A. calidoustus* pathway mutants. This means that the ether bridge between carbon–9 and –6' of austin is either due to a spontaneous reaction *in vivo*, as previously suggested[14], or catalyzed by an enzyme whose gene is located outside of the biosynthetic gene cluster.

The deletion of *ausR*, coding for a P450 monoxygenase, resulted in loss of 7–hydroxydehydroaustin **4** and yielded a new product, 1,2–dihydro–dehydroaustin **9**, explaining the double peak for *m/z* 501.21 $[M + H]^+$ (Figure 4). The hydroxylation catalyzed by AusR permitted the second O–acetyltransferase AusQ to add an additional acetyl group to the molecule, leading to the formation of **10**. As shown in Figure 4, AusQ not only catalyzes an acetylation reaction, but also the addition of the PKS product forming 1,2–dehydro–precalidodehydroaustin **13**. Altogether, AusQ and the progesterone 5–beta–reductase–like protein AusT, which catalyzes the reduction of the double bond present between carbon atoms –1 and –2, promoted a series of shunt products, including the formation of **13**. Of course, all these derivatives were only detected in *A. calidoustus*, being that *A. nidulans* does not possess the *ausT* gene (Figure 3 and Table 2).

As mentioned before, the *A. calidoustus* biosynthetic gene cluster contains an additional PKS–coding gene *ausV*. Domain analysis revealed that while AusA can be classified as a non–reducing PKS, AusV contained additional domains for dehydratase, enoyl–reductase and keto–reductase activity, categorizing it as highly–reducing PKS. This in turn would explain the alternate side chain observed in further products of the pathway, such as precalidodehydroaustin **6**, **7** and **13** (Figures 2 and 4).

Deletion of *ausV* confirmed that these derivatives were adorned by this specific PKS product (Figure 4).

A phylogenetic analysis of AusV suggested that this diketide synthase behaves as an outlier (Figure 5). Blasting the AusV-deduced amino acid sequence in the UniProt Database, the obtained hits are putative PKSs having a quite low similarity score (~30 %). We performed the same analysis using only the putative KS domain from AusV as blasting probe. This approach permitted to identify some PKSs having a KS domain similar to the one from AusV (>50 %). However, the phylogenetic analysis of the obtained entries showed that AusV remains not clustered, highlighting the uniqueness of AusV.

This PKS–derived side chain resembled another polyketide product from lovastatin biosynthesis (LovF) in *A. terreus*, where two distinct PKSs participate in the formation of the same molecule[35]. This was also shown for the similar lovastatin derivative ML–236B (compactin) from *Penicillium citrinum*[36]. However, LovF and AusV share a very low amino acid identity (33 %) and AusV appears to have a dysfunctional enoyl–reductase domain thus creating a product less reduced than the lovastatin side chain. For example LovF synthesizes the fully reduced diketide, α–*S*–methylbutyrate side chain[37, 38], whereas AusV synthesizes a lesser reduced diketide product, (E)–2–methyl–2–butenate (tiglate). Regarding the tiglate side chain produced from AusV, this modification has also been observed in different plant NPs[39, 40], including meteloidine from the plant *Datura metelcides* where the biosynthesis of tiglic acid was found to be derived from isoleucine[39]. Additionally, it has also been shown that the carabid beetle, *Pterostichus* (*Hypherpes*) *californicus* also produces isoleucine *via* 2–methylbutyric acid and is used as a defensive compound by the insect[41]. Not only is this side chain derived from a different biosynthetic route in higher eukaryotes, but it has also been shown to be a defense compound. This could explain why the addition of tiglic acid is seen in the austinoid biosynthesis.

The last elucidated biosynthetic step entailed a dioxygenase. The *A. calidoustus* austinoid gene cluster encoded two putative dioxygenases, AusO and AusU. Deletion of *ausO* showed loss of **7** (Figure 4), meaning that this enzyme promotes hydroxylation of carbon–2. Interestingly, AusO can catalyze another hydroxylation from 1,2–dihydro–acetoxydehydroaustin **11** to form 1,2–dihydro–2–hydroxyl–

acetoxydehydroaustin **12** (Figure 2). As reported here, there were still putative genes part of the cluster whose function could not be assigned, such as *ausS*, *ausU, ausW* and *ausX* (Figure 4). Interestingly, the *ausS* deletion led to a complete loss of austinoid production in *A. calidoustus*, suggesting its possible function as a regulator. However, the analysis of the AusS protein did not reveal any amino acid motif characteristic of known regulators. By contrast, the knock out of the *ausS* orthologue in *A. nidulans* showed opposing results, *i.e.*, austinoid production was not affected, as previously reported[14] (Supplementary Figures S13 and S15). Supplementary gene knock outs were also employed to establish the bordering genes of the cluster (Supplementary Figures S13 and S16) confirming that additional neighboring genes were not involved in any biosynthetic process.

CONCLUSIONS

The present study shows a continuation of the expanding austinoid pathway with an additional 6 genes discovered to be involved in the biosynthesis. This continuation, along with was previously studied in *A. nidulans* comprises an extremely large biosynthetic gene cluster with 25 different genes of which 22 were determined to be involved. Particularly, two enzymes from *A. calidoustus,* including the here discovered promiscuous acyltransferase, AusQ, and the diketide synthase, AusV, are of interest. They play a major role in producing further austinoid products with insecticidal activity. As mentioned above, the acyltransferase AusQ it promiscuous in its substrate specificity, transferring not only the acetyl group but it also appears to be the first example of a polyketide being directly loaded onto a meroterpenoid structure. A similar case was also seen with the β–*trans*–bergamotene where a polyketide is loaded onto a terpene during fumagillin biosynthesis in *Aspergillus fumigatus*[42].

Regarding the insect testing, the newly discovered end product, **7**, along with **2** and **3**, demonstrated insecticidal activities against different insect families, *i.e.*, *Homoptera* and *Coleoptera*. One of the main goals of agricultural research is the development of insecticides that can limit insect pest population without harming the surrounding fauna including beneficial insects[43]. In this perspective, due to their specificity, austinoids could be promising candidates. In particular, for *Homoptera* the activities were even species–specific further supporting the ecological speciation of austinoid

derivatives (Table 1). This example with austinoid biosynthesis in *A. calidoustus* has shown the capacity of bioprospecting for similar compounds produced by different strains.

Materials and Methods

Fungal strains and cultivation Fungal species tested for austinoid production are reported in the Supplementary Table S1, and were deposited in the Jena Microbial Resource Collection (www.uni-jena.de/Pilz__Referenz_Zentrum.html). Three different media were prepared: *Aspergillus* minimal medium (AMM)[44], Yeast Peptone Dextrose (YPD) broth (Carl Roth) and complex medium (1% (w/v) glucose, 0.2% (w/v) peptone, 0.1% (w/v) yeast extract, 0.1% (w/v) casamino acids, 10.8 mM MgSO4, 17.4 mM KCl, 28.7 mM KH_2PO_4, 0.1% (v/v) trace elements [44], 0.1% (v/v) vitamin solution (Stock: 0.07 mM p–aminobenzoic acid, 0.30 mM pyridoxine HCl, 0.008 mM biotin and 0.09 mM pantothenic acid) and 0.33 µM riboflavin. Depending on cultivation methods 1.5% (w/v) agar was added for agar plates.

For austinoid natural product production, cultures were grown in complex medium and inoculated with fresh spores with a concentration of $5x10^6$ spores mL^{-1}. Static cultures were grown at 30 °C for three days.

Fungal molecular biology techniques All polymerase chain reactions (PCR) were completed using 2x high fidelity Phusion master mix (Life Technologies), unless otherwise mentioned. For targeted gene knock outs in *A. calidoustus* and *A. nidulans*, a 3–fragment PCR was carried out as previously described[45] using primers from Supplementary Table S8. Two different selectable markers were used and amplified from the pSK275 plasmid holding the pyrithiamine resistance cassette[46] and the pUC–hph plasmid containing the hygromycin resistance cassette[47]. *A. calidoustus* transformations were carried out according to standard protocols[48], with the exception of the protoplast–forming solution, which was supplemented with 250 µL 12 mg mL^{-1} bovine serum albumin, 0.5 U of β–glucanase from *Aspergillus niger* (Sigma–Aldrich) and 2.5 U chitinase from *Trichoderma viride* (Sigma–Aldrich). Transformants were verified for correct deletions by Southern blot analysis (Supplementary Figures S7 and S13, and Supplementary Tables S7 and S9)[49].

Phylogeny of AusV The putative KS domain identified from the AusV deduced amino acidic sequence was used for BlastP analysis in the UniProt database. Entries were retrieved and analyzed using MEGA6 suite using the Neighbor–Joining method. The protein entries are depicted in Figure 5.

Extraction and HPLC–HRMS analysis Austinoids were extracted from *A. calidoustus*, *A. nidulans* and *P. brasilianum* cultures. The entire culture was homogenized with an Ultra–turrax T25 (IKA–Labortechnik) and extracted twice with equal v/v ethylacetate (Carl Roth). The organic phase was then dried with anhydrous sodium sulfate (Carl Roth) and concentrated with a rotary evaporator. Samples were resuspended in methanol (Carl Roth) and filtered through a 0.2 µm PTFE filter (Carl Roth).

High–resolution mass spectrometry (HRMS) was conducted on a Thermo Fischer Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer with an electrospray ion source and an Accela HPLC system (Thermo Fisher Scientific) equipped with a C18 column (Accucore 2.6 µm 150 x 2.1 mm). Mobile phase 0.2 ml min^{-1}: Water (0.1 % (v/v) formic acid): acetonitrile (0.1 % (v/v) formic acid), start 95:5 then to 2:98 in 10 minutes, four minutes 2:98, 0.1 minutes 95:5, 6.9 95:5; 20 minutes total run.

Structure elucidation by NMR and HRMS 1D and 2D NMR spectra were recorded at 300 K on Bruker Avance III spectrometers (600 MHz, equipped with a cryo platform) using CDCl3 as solvent. All spectra were referenced to the residual solvent signals (resonances at δH = 7.24 ppm and δC = 77.0 ppm). ^{1}H and ^{13}C NMR data was collected for dehydroaustin **3** see Supplementary Figures S2 and S3, and Supplementary Table S2, 7–hydroxydehydroaustin **4** see Supplementary Figures S4 and S5, and Supplementary Table S3, precalidodehydroaustin **6** see Supplementary Figures S6 and S7, and Supplementary Table S4, calidodehydroaustin **7** see Supplementary Figures S8 and S9, and Supplementary Table S5 and 1,2–dihydro–2–hydroxyl–acetoxydehydroaustin **12** see Supplementary Figures S10 and S11, and Supplementary Table S6. HRMS data is shown in Table S10 for all proposed austinoid products. Details about isolation of the compounds can be found in the supporting information.

ASSOCIATED CONTENT

Supporting information

The Supporting Information is available free of charge *via* the Internet.

Experimental details, supplementary figures and tables can be found in the supporting information. This includes all NMR and HRMS data for compounds that were isolated along with additional supporting information regarding insect testing and compound purification.

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Competing interest

The authors declare competing financial interests: part of this work was filed for patent under EP15200500.5.

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FIGURE LEGENDS

Figure 1. Structures of different meroterpenoids derived from 3,5–dimethylorsellinic acid. This includes anditomin from *A. variecolor*, terretonin from *A. terreus*, austinol from *A. nidulans* and andrastin A from *P. chrysogenum*. Bonds indicated in green represent where the polyketide, 3,5–dimethylorsellinic acid, is incorporated[25].

Figure 2. Proposed biosynthetic pathway for austinoids. Black arrows (➔) depict the main metabolic pathway, while all the identified shunt pathways are depicted in blue (➔). Pathway begins with austinol **1** and dehydroaustinol **8,** which are the final products identified in the *A. nidulans* austinoid pathway. Early pathway is shown in Supplementary Figure S1. Structures 5, 9, 10, 11, and 13 are proposed structures based on HRMS data, while structures 3, 4, 6, 7, and 12 were elucidated by NMR.

Figure 3. Austinoid gene clusters from *A. nidulans* and *A. calidoustus*. Genes colored in black have been characterized and the function shown during austinoid biosynthesis. Genes in grey are within the clusters, but have no apparent function, while genes in white are not part of the cluster. Striped genes in the *A. nidulans* cluster represent genes characterized in *A. calidoustus*, but have no apparent function in *A. nidulans*.

Figure 4. Verification of the austinoid biosynthetic gene cluster in *A. calidoustus*. High resolution mass spectrometry data using overlays of EIC of the various biosynthetic pathway deletion mutants of *A. calidoustus* in comparison with the wild type. Blue chromatograms refer to shunt products. Each peak corresponds to the following *m/z*: **1**, *m/z* 459.20 $[M + H]^+$; **2**, *m/z* 501.21 $[M + H]^+$; **3**, *m/z* 499.19 $[M + H]^+$; **4**, *m/z* 515.19 $[M + H]^+$; **5**, *m/z* 517.20 $[M + H]^+$; **6**, *m/z* 599.24 $[M + H]^+$; **7**, *m/z* 615.24 $[M + H]^+$; **8**, *m/z* 457.18 $[M + H]^+$; **9**, *m/z* 501.21 $[M + H]^+$; **10**, *m/z* 557.20 $[M + H]^+$; **11**, *m/z* 559.21 $[M + H]^+$; **12**, *m/z* 575.21 $[M + H]^+$; **13**, *m/z* 597.23 $[M + H]^+$.

Figure 5 Phylogenetic tree of the diketide synthase AusV.

The tree reports distances among PKSs identified by BlastP analysis using the KS domain from AusV as probe. The figure highlighted that the AusV gene product (in bold) is unique among the ones reported in databases. UniProt accession numbers are also reported.

TABLES

Table 1. Insect tests. Austinoid derivatives were purified and tested against different insect families. Concentrations for each compound was 2500 ppm (w/v) and the values indicate the average percentage killed in the assays. All assays were performed at least twice. Methods for insect testing can be found in the supporting materials.

Common name	green peach aphid	vetch aphid	tobacco budworm	boll weevil	Mediterr. fruit–fly	yellow–fever mosquito
Linnaean name	*Myzus persicae*	*Megoura viciae*	*Heliothis virescens*	*Anthonomus grandis*	*Ceratitis capitata*	*Aedes aegypti*
Family	*Homoptera*	*Homoptera*	*Lepidoptera*	*Coleoptera*	*Diptera*	*Diptera*
	Killing (%)	Killing (%)	Killing (%)	Killing (%)	Killing (%)	Killing (%)
Austin **2**	75	0	0	0	0	0
Dehydroaustin **3**	100	100	0	50	0	0
Calidodehydroaustin **7**	100	0	0	0	0	0

Table 2. Genes identified in the *A. calidoustus* austinoid gene cluster. These genes are shown with their orthologues in *A. nidulans* along with their putative function.

Name	Gene ID *A. calidoustus*	Orthologue in *A. nidulans*	Putative Function
	ASPCAL14352	–	Ribonuclease H (RNase H)
AusO	ASPCAL14353	–	Dioxygenase and part of the 2OG–Fe(II) oxygenase superfamily
AusE	ASPCAL14354	AN9246	Non–heme iron–dependent dioxygenase [23]
AusF	ASPCAL14355	AN9247	Hypothetical protein[14]
AusG	ASPCAL14356	AN9248	Cytochrome P450 monooxygenase[14]
AusH	ASPCAL14357	AN9249	Hypothetical protein[14]
AusP	ASPCAL14358	–	O–actetyltransferase with a condensation domain
AusQ	ASPCAL14359	AN9250	O–actetyltransferase with a condensation domain
AusR	ASPCAL14360	AN9251	Cytochrome P450 monooxygenase
AusS	ASPCAL14361	AN9252	Hypothetical protein
AusT	ASPCAL14362	–	Progesterone 5–beta–reductase–like protein
AusI	ASPCAL14363	AN9253	Cytochrome P450 monooxygenase[14]
AusU	ASPCAL14364	AN9254	Dioxygenase and part of the 2OG–Fe(II) oxygenase superfamily
AusV	ASPCAL14365	–	Highly reducing polyketide synthase
AusJ	ASPCAL14366	AN11214	Acid–catalyzed keto–rearrangement and ring contraction[14]
AusK	ASPCAL14367	AN11205	Aldo–keto reductase[14]
AusD	ASPCAL14368	AN8384	Putative methyltransferase[11]
AusA	ASPCAL14369	AN8383	Non–reducing polyketide synthase[14]
AusW	ASPCAL14370	–	Hypothetical protein
AusC	ASPCAL14371	AN8381	Baeyer–Villiger monooxygenase (Flavin monooxygenase)[23]
AusB	ASPCAL14372	AN8379	5'–hydroxylase (Flavin monooxygenase)[23]
AusX	ASPCAL14373	AN12376	Putative short–chain dehydrogenase/reductase (SDR)
AusL	ASPCAL14374	AN9257	Terpene cyclase[14]
AusY	ASPCAL14375	AN11217	Transporter (major facilitator superfamily)
AusM	ASPCAL14376	AN11206	Epoxidase[14]
AusN	ASPCAL14377	AN9259	Prenyltransferase[14]
	ASPCAL14378	–	Hypothetical protein

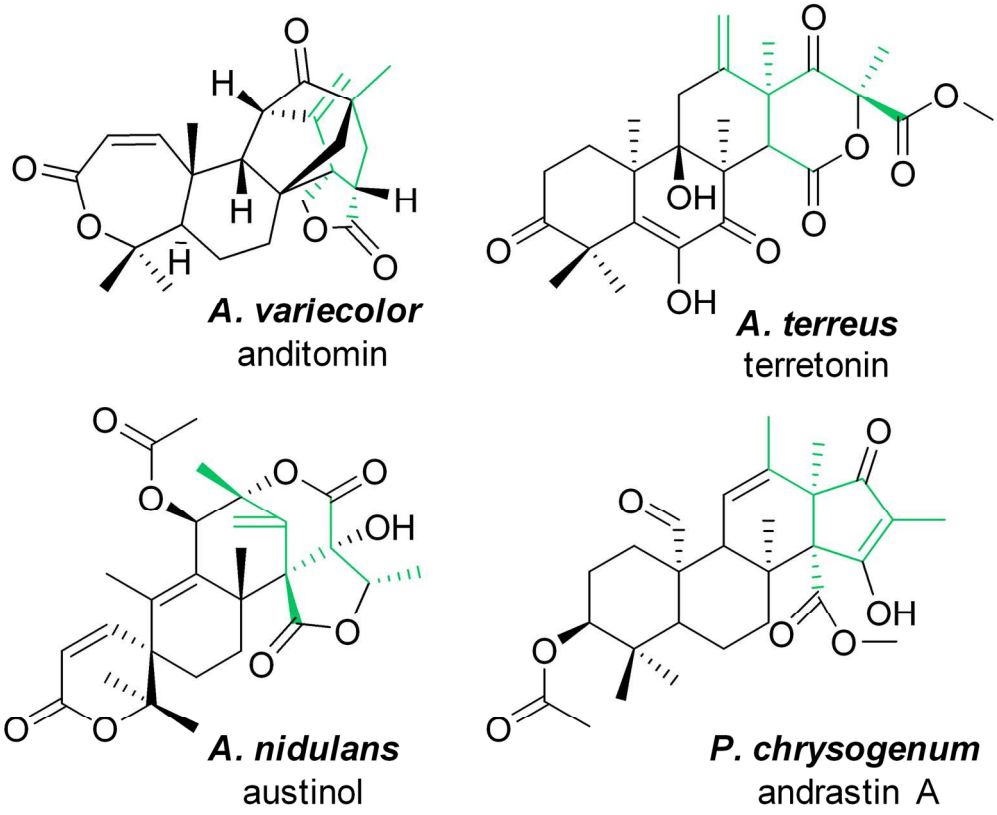


Figure 1. Structures of different meroterpenoids derived from 3,5–dimethylorsellinic acid.
Figure 1
80x66mm (600 x 600 DPI)

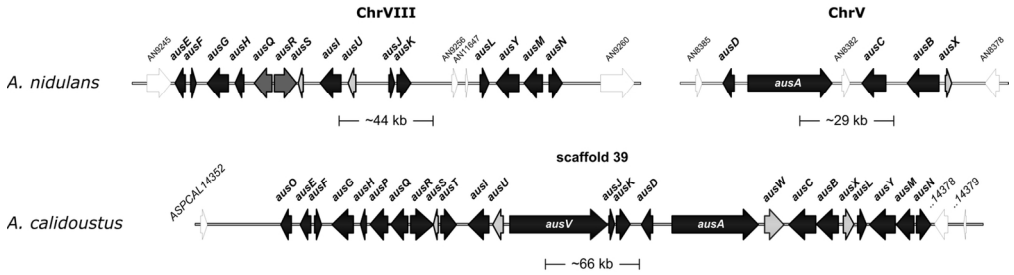


Figure 2. Proposed biosynthetic pathway for austinoids.
Figure 2
68x18mm (600 x 600 DPI)

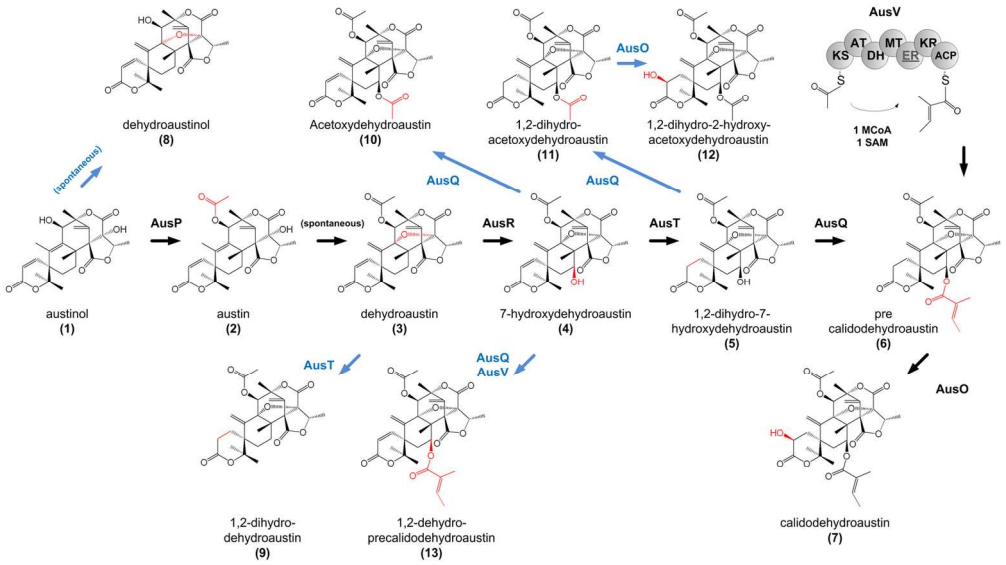


Figure 3. Austinoid gene clusters from A. nidulans and A. calidoustus.
Figure 3
74x41mm (600 x 600 DPI)

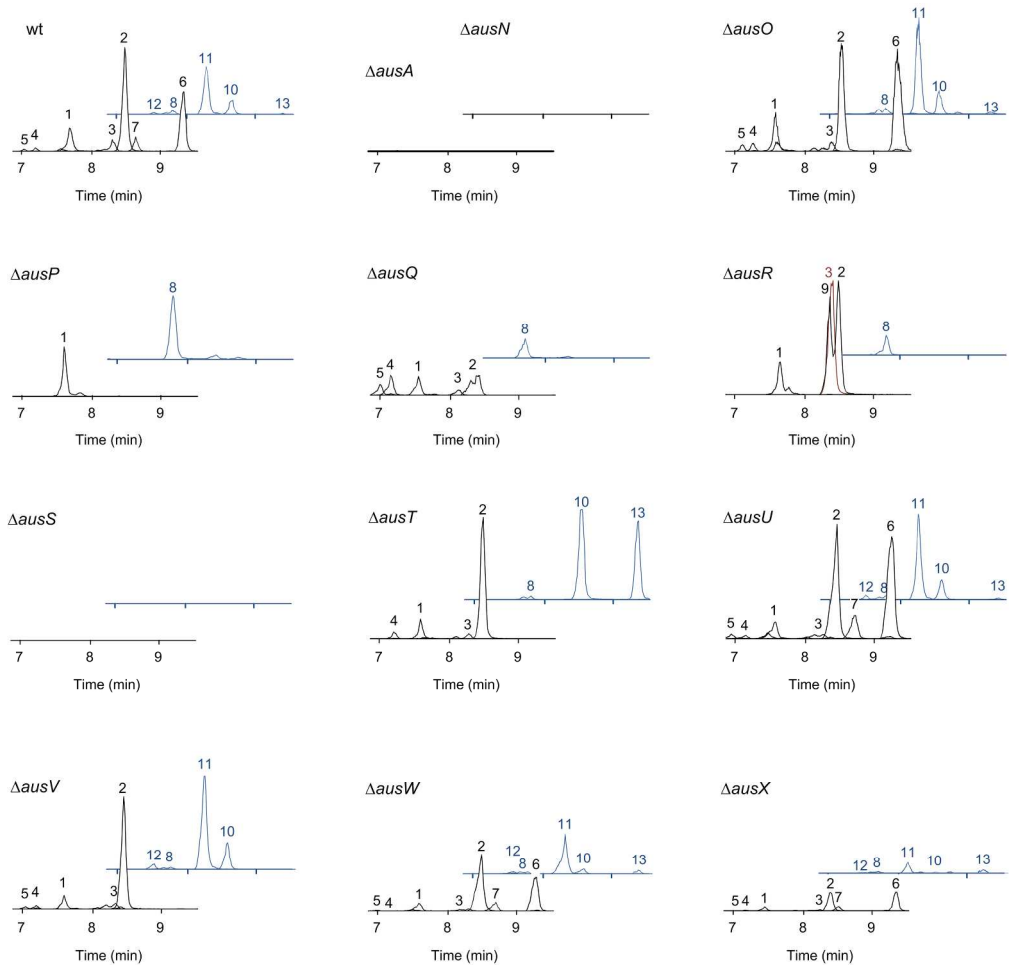


Figure 4. Verification of the austinoid biosynthetic gene cluster in A. calidoustus.
Figure 4
120x116mm (600 x 600 DPI)

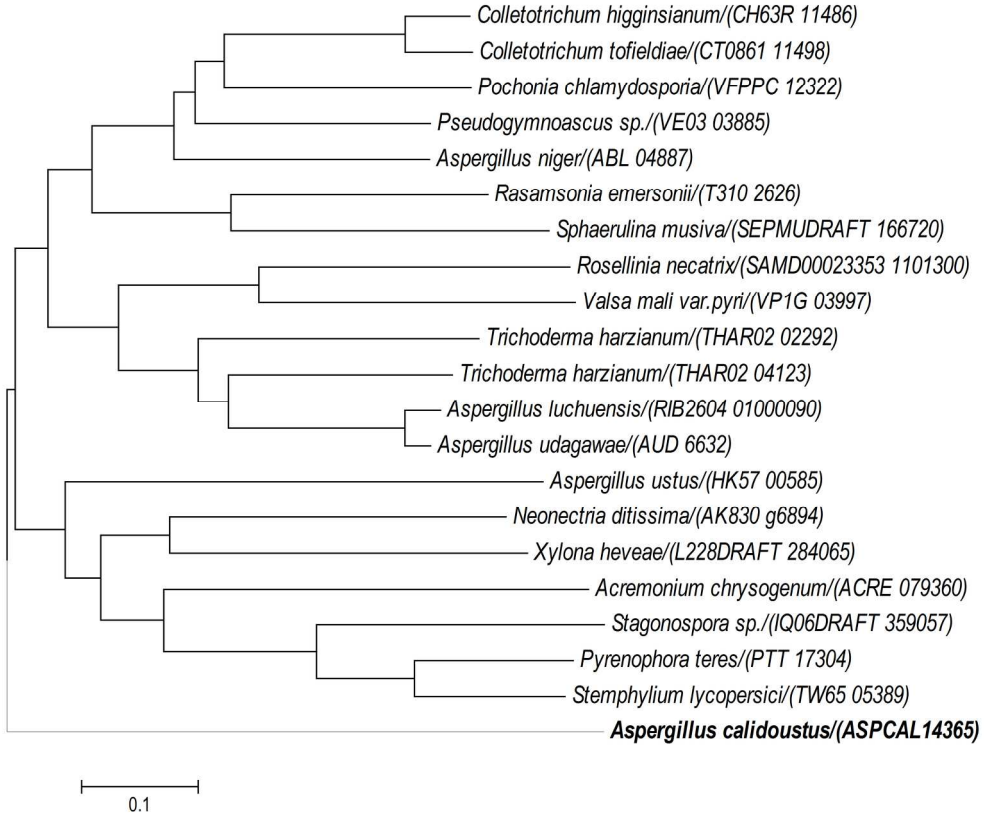
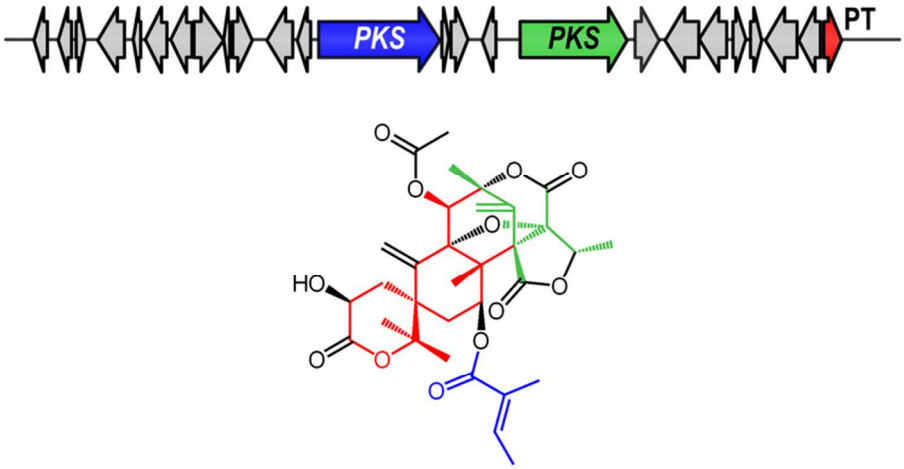


Figure 5. Phylogenetic tree of the diketide synthase AusV.
Figure 5
99x83mm (600 x 600 DPI)



Toc graphic
Toc graphic
39x19mm (600 x 600 DPI)