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1 **Deletion of the epigenetic regulator GcnE in *Aspergillus niger* FGSC**

2 **A1279 activates the production of multiple polyketide metabolites**

3  
4 Bin Wang<sup>a,b,\*</sup>, Xuejie Li<sup>a,b,\*</sup>, Dou Yu<sup>a,b</sup>, Xiaoyi Chen<sup>a,b</sup>, Jioji Tabudravu<sup>c,d</sup>, Hai Deng<sup>a,b,c,#</sup>, Li  
5 Pan<sup>a,b,#</sup>

6 <sup>a</sup>School of Biology and Biological Engineering, South China University of Technology,  
7 Guangzhou Higher Education Mega Center, Guangzhou, 510006, China

8 <sup>b</sup>Guangdong Provincial Key Laboratory of Fermentation and Enzyme Engineering, South  
9 China University of Technology, Guangzhou Higher Education Mega Center, Guangzhou  
10 510006, China

11 <sup>c</sup>Marine Biodiscovery Centre, Department of Chemistry, University of Aberdeen, Meston  
12 Walk, Aberdeen AB24 3UE, Scotland, UK

13 <sup>d</sup>School of Forensics and Applied Sciences, Faculty of Science & Technology, University of  
14 Central Lancashire, Preston, Lancashire, PR1 2HE, UK

15  
16 **\* These authors contributed equally to this work.**

17  
18 **# Corresponding authors**

19 Hai Deng: h.deng@abdn.ac.uk

20 Li Pan: btlipan@scut.edu.cn

21  
22  
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## 25 **ABSTRACT**

26 Epigenetic modification is an important regulatory mechanism in the biosynthesis of  
27 secondary metabolites in *Aspergillus* species, which have been considered to be the treasure  
28 trove of new bioactive secondary metabolites. In this study, we reported that deletion of the  
29 epigenetic regulator *gcnE*, a histone acetyltransferase in the SAGA/ADA complex, resulted  
30 in the production of 12 polyketide secondary metabolites in *A. niger* FGSC A1279, which  
31 was previously not known to produce toxins or secondary metabolites. Chemical workup and  
32 structural elucidation by 1D/2D NMR and high resolution electrospray ionization mass (HR-  
33 ESIMS) yielded the novel compound nigerpyrone (**1**) and five known compounds:  
34 carbonarone A (**2**), pestalamide A (**3**), funalenone (**4**), aurasperone E (**5**), and aurasperone A  
35 (**6**). Based on chemical information and the literature, the biosynthetic gene clusters of  
36 funalenone (**4**), aurasperone E (**5**), and aurasperone A (**6**) were located on chromosomes of *A.*  
37 *niger* FGSC A1279. This study found that inactivation of GcnE activated the production of  
38 secondary metabolites in *A. niger*. The biosynthetic pathway for nigerpyrone and its  
39 derivatives was identified and characterized via gene knockout and complementation  
40 experiments. A biosynthetic model of this group of pyran-based fungal metabolites was  
41 proposed.

## 42 **Keywords**

43 Histone acetyltransferase GcnE, Secondary metabolite, Epigenetic regulator, Polyketide,  
44 Nigerpyrone

## 45 **1. Introduction**

46 The genus *Aspergillus* is a well-known producer of secondary metabolites (SMs) including  
47 polyketides, non-ribosomal peptides, indole terpenes and terpenes (Varga et al., 2003; Bok et  
48 al., 2006; Sanchez et al., 2012), which are important resources for new drug discovery  
49 (Rossano et al., 1999; Fisch et al., 2009). For example, bioinformatic analysis of four

50 available genomes of *A. niger* species (NRRL3, ATCC 9029) (Baker, 2006), ATCC1015  
51 (Andersen et al., 2011), CBS513.88 (Pel et al., 2007), and SH2 (Yin et al., 2014), indicates  
52 that *A. niger* encodes at least 81 putative SM biosynthetic gene clusters (Inglis et al., 2013)  
53 for 42 polyketides (PKS), 33 non-ribosomal peptides (NRPS), four PKS-NRPS hybrids and  
54 two indole alkaloids. The large number of putative SM biosynthetic gene clusters suggests  
55 that *A. niger* has the potential to produce abundant SM products. However, most of these  
56 putative gene clusters are silent in standard laboratory cultivation conditions (Fisch et al.,  
57 2009), and only limited SM products have been characterized from *A. niger* (Tanaka et al.,  
58 1966; Hiort et al., 2004; Serra et al., 2005; Nielsen et al., 2009; Sorensen et al., 2009; Chiang  
59 et al., 2011).

60 Epigenetic modification is an important regulatory mechanism in the biosynthesis of SM  
61 products in *Aspergilli* (Fisch et al., 2009), which could activate SM gene clusters and induce  
62 new SM products by changing the chromosomal region of SM gene clusters from a  
63 heterochromatic to a euchromatic state via histone demethylation and acetylation (Bayram et  
64 al., 2008; Fisch et al., 2009). For example, 10 novel SM compounds are induced by adding  
65 the epigenetic modification reagent 5-azacytidine (5-AZA) to *A. niger* ATCC1015 cultures  
66 (Fisch et al., 2009).

67 Spt-Ada-Gcn5-acetyltransferase (SAGA/ADA) complex is an epigenetic regulator in  
68 fungal secondary metabolism (Wu and Yu, 2015). GcnE (the Gcn5 homolog in *Aspergilli*) is  
69 a histone acetyltransferase (HAT) in SAGA/ADA complex that might regulate secondary  
70 metabolism by histone modification (Baker and Grant, 2007; Brakhage, 2013). Previous work  
71 illustrated that GcnE participates in increasing the acetylation level of histone H3 lysine K9  
72 in *Aspergilli* (Reyes-Dominguez et al., 2008), and affects the biosynthesis of secondary  
73 metabolites. For example, *A. flavus*  $\Delta gcnE$  mutant does not produce aflatoxin (Lan et al.,

74 2016). Deletion of the *gcnE* gene in *A. nidulans* decreases the production of orsellinic acid,  
75 sterigmatocystin, penicillin and terrequinone (Nutzmann et al., 2011).

76 *A. niger* strain FGSC A1279 (*kusA::DR-amdS-DR, pyrG<sup>-</sup>*), a derivative of industrial strain  
77 NRRL3 (ATCC9029) that is used for gluconic acid production (Baker, 2006; Yuan et al.,  
78 2008; Carvalho et al., 2010; Arentshorst et al., 2015), is an SM-silent strain and has zero level  
79 of SM production. The low background of SM production in FGSC A1279 makes it ideal for  
80 investigating the role of epigenetic regulators on SM production. Moreover, genome  
81 sequences of NRRL3 (ATCC 9029) (Baker, 2006) and FGSC A1279 (Wang et al., 2017) are  
82 available for detailed gene information of the host strain.

83 In this study, we reported that deletion of the epigenetic regulator *gcnE*, a histone  
84 acetyltransferase in the SAGA/ADA complex, resulted in the production of 12 polyketide  
85 secondary metabolites in *A. niger* FGSC A1279. Chemical workup and structural elucidation  
86 by 1D/2D NMR and high resolution electrospray ionization mass (HR-ESIMS) yielded the  
87 novel compound nigerpyrone (**1**) and five known compounds: carbonarone A (**2**),  
88 pestalamide A (**3**), funalenone (**4**), aurasperone E (**5**), and aurasperone A (**6**). Based on  
89 chemical information and literature searches, the biosynthetic gene clusters of funalenone (**4**),  
90 aurasperone E (**5**), and aurasperone A (**6**) were located on the chromosomes of *A. niger*  
91 FGSC A1279. This study showed that inactivation of GcnE activated the production of toxins  
92 and metabolites in *A. niger*. This is distinct from the role of GcnE in previous studies,  
93 suggesting that the role of GcnE may have diverse effects on regulating SMs among different  
94 *Aspergilli*. The biosynthetic pathway of nigerpyrone and its derivatives was identified via  
95 gene knockout and complementation experiments, and a biosynthetic model of this group of  
96 pyran-based fungal metabolites was proposed.

## 97 **2. Material and methods**

### 98 **2.1. Strains and culture conditions**

99 All strains used in this study are in Table S1. Strains were maintained on potato dextrose  
100 agar (PDA) medium (20 g dextrose, 15 g agar, infusion from 200 g potatoes per 1 L medium,  
101 pH6.0) at 30 °C for spore harvest. For genetic transformation, CD medium (0.3% NaNO<sub>3</sub>, 0.2%  
102 KCl, 0.05% MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.001% FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.1% K<sub>2</sub>HPO<sub>4</sub>, 2.0% glucose, pH5.5) was  
103 used. WATM, YES, and CYA media were used for SM profile analysis. WATM medium  
104 was composed of 2.0 g/l yeast extract, 3.0 g/l peptone, 2.0 g/l dextrose, 30.0 g/l sucrose, 5.0  
105 g/l corn steep solids, 2.0 g/l NaNO<sub>3</sub>, 1.0 g/l K<sub>2</sub>HPO<sub>4</sub>·3H<sub>2</sub>O, 0.5 g/l MgSO<sub>4</sub>, 0.2 g/l KCl, 0.01  
106 g/l FeSO<sub>4</sub>·7H<sub>2</sub>O, pH7.0. YES medium was composed of 20 g/l yeast extract, 150 g/l sucrose,  
107 pH 6.0. CYA medium was prepared on the basis of CD medium by adding 5 g/l yeast extract  
108 and 1 ml/l trace metal solution (1% ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.5% CuSO<sub>4</sub>·5H<sub>2</sub>O). For solid cultivation,  
109 15 g/l agar was added.

110 An *A. niger gcnE* deletion mutant ( $\Delta gcnE$ ) was constructed according to the method  
111 developed by Szewczyk *et al.* (Szewczyk *et al.*, 2006), based on homologous recombination  
112 using a PCR amplified deletion cassette containing the upstream flanking arm (1.4 kb) of  
113 *gcnE*, the selection marker *pyrG* (orotidine-5'-phosphate decarboxylase) and the downstream  
114 flanking arm of *gcnE*. The *pyrG* gene was used for auxotrophic selection. Uridine  
115 prototrophic transformants were selected and confirmed by PCR amplification (Fig. S1).  
116 Primers for deletion cassette construction and transformant identification are listed in Table  
117 S2. When required, 10 mM uridine was added to medium to maintain auxotrophy. The SM  
118 backbone gene *epaA* was knocked out using the same method (Table S3).

## 119 **2.2. Cultivation of *A. niger* $\Delta gcnE$ mutant for secondary metabolite collection**

120 To collect secondary metabolites, the *A. niger*  $\Delta gcnE$  mutant was cultivated for 7 days at  
121 25 °C in the dark on 10 Petri dishes with WATM medium. Cultures were extracted with ethyl  
122 acetate (EtOAc) plus 1% formic acid for 24 hours. The crude extract was filtered and dried  
123 on a rotary evaporator at 38 °C.

124 **2.3. SPE separation of *A. niger*  $\Delta$ gcnE crude SM extract**

125 A C18 solid-phase extraction (SPE) column (SPE C18-E Giga Tube 20 g/60 ml, Strata,  
126 Phenomenex, USA) was used to separate *A. niger*  $\Delta$ gcnE crude extracts. The SPE stationary  
127 phase was conditioned by sequential washing with 60 ml 100% methanol and 240 ml 100%  
128 deionized water. *A. niger*  $\Delta$ gcnE crude extract (4.0 g) was chromatographed with four  
129 subfractions (480 ml each) generated stepwise from deionized water to methanol: 25%  
130 methanol, 50% methanol, 100% methanol, and 100% methanol plus 0.1% trifluoroacetic acid  
131 (TFA). Subfraction 2 (50% methanol, 50 mg) and 3 (100% methanol, 410 mg) were further  
132 purified by semi-preparative HPLC (semi-prep HPLC).

133 **2.4. Semi-prep HPLC fractionation**

134 Semi-prep HPLC was used to purify subfractions obtained by SPE separation. A semi-prep  
135 HPLC column ACE C18-HL (250 mm  $\times$  10 mm i.d., Advanced Chromatography  
136 Technologies, UK) was connected to an Agilent 1200 series binary pump and monitored by  
137 an Agilent photodiode array detector. Detection was at 230, 254, 280, and 410 nm. Flow rate  
138 was 1.5-2.0 ml/min. Solvent A consists of 95% H<sub>2</sub>O, 5% methanol, and 0.05% TFA. Solvent  
139 B was 100% acetonitrile. Separations were done at room temperature (20-25°C). Optimal  
140 separation conditions were determined using analytical systems and then conditions were  
141 adapted to the semi-preparative scale.

142 **2.5. LC/HR-ESIMS assay of *A. niger* secondary metabolites**

143 For LC/HR-ESIMS analysis, each semi-prep HPLC subfraction was dissolved in  
144 MeOH:Milli-Q H<sub>2</sub>O (9:1), and centrifuged at 10,000 rpm for 10 min. LC/HR-ESIMS analysis  
145 was conducted as follows. High resolution mass spectrometric data were obtained using an  
146 LTQ XL/LTQ Orbitrap Discovery MS system (Thermo Fisher Scientific, Waltham, MA,  
147 USA) coupled to a Thermo Instruments HPLC system (Accela PDA detector, Accela PDA  
148 autosampler, and Accela pump, C18 SunFire 150  $\times$  4.6 mm Waters). Conditions used were:

149 capillary voltage 45 V, capillary temperature 320°C, auxiliary gas flow rate 10-20 arbitrary  
150 units, sheath gas flow rate 40-50 arbitrary units, spray voltage 4.5 kV, and mass range 100-  
151 2000 amu (maximum resolution 30,000). For LC/HR-ESIMS, a Waters SunFire C18  
152 analytical HPLC column (5 µm, 4.6 × 150 mm) was used with mobile phase of 0-100%  
153 MeOH over 30 min at a flow rate of 1 ml/min. LC/HR-ESIMS data were analyzed by  
154 Xcalibur software followed by dereplication using Antibase2012 database.

## 155 **2.6. NMR characterization of *A. niger* secondary metabolites**

156 NMR spectra were used to elucidate the structure of *A. niger* secondary metabolites. <sup>1</sup>H  
157 NMR, <sup>13</sup>C NMR, COSY, HSQC and HMBC spectra were recorded on a Bruker Daltonics  
158 Advance III HD 400 NMR spectrometer, using deuterated solvent methanol-d<sub>4</sub> (Cambridge  
159 Isotopes Laboratories, USA). Chemical shift values were reported in parts per million (ppm)  
160 with tetramethylsilane (TMS) as the external standard. Coupling constants were recorded in  
161 Hertz (Hz). When higher field was needed, NMR spectra were recorded on a Bruker  
162 Daltonics Advance III HD 600 NMR. NMR data were analyzed using MestReNova 9.0.1  
163 software and ACD/I-Lab (<http://ilab.cds.rsc.org/?cdsrdr=1>).

## 164 **2.7. Characterization of putative biosynthetic gene clusters for *A. niger* secondary 165 metabolites by gene knockout**

166 Gene knockout of putative cluster genes for identified *A. niger* secondary metabolites was  
167 done based on homologous recombination using a PCR-amplified deletion cassette  
168 containing the upstream and downstream flanking arms of the target gene and the *ptrA*  
169 (pyrithiamine (PT) resistance) selection marker. Primers for constructing deletion cassettes  
170 are listed in Table S3. 0.5 µg/ml pyrithiamine (PT) was used to screen transformants. The  
171 host strain for gene knockout was the *A. niger* FGSC A1279  $\Delta$ *gcnE* mutant. Gene deletion  
172 transformants were confirmed by PCR amplification using internal primers (Table S3 and Fig.  
173 S1).

### 174 **3. Results and discussion**

#### 175 **3.1. Chemical diversity of *A. niger* FGSC A1279 $\Delta$ *gcnE* mutant**

176 To investigate the effect of *gcnE* on *A. niger* secondary metabolism, we constructed a  
177  $\Delta$ *gcnE* mutant and a *gcnE* complementation (*gcnE*-com) strain using homologous  
178 recombination (Fig. S1). Three types of solid media (WATM, CYA and YES) commonly  
179 used for fungal secondary metabolite production were chosen (Frisvad and Thrane, 1987;  
180 Rank et al., 2012) to investigate metabolite changes in the  $\Delta$ *gcnE* mutant and *gcnE*-com  
181 strain. Crude extract from cultures was subjected to LC/HR-ESIMS analysis. No compounds  
182 were produced in *A. niger* FGSC A1279 cultures. Metabolite profiling by LC/HR-ESIMS  
183 analysis demonstrated that multiple SMs were produced in all of three  $\Delta$ *gcnE* mutant cultures  
184 and the SM profile of *gcnE*-com strain was similar with the wild strain (Fig. 1), suggesting  
185 that *gcnE* deletion activated the synthesis of SM products in *A. niger*. It has been reported  
186 that, in *Aspergilli*, histone acetylation usually correlates with transcriptional activation and  
187 chromatin rearrangement (Baker and Grant, 2007; Reyes-Dominguez et al., 2008). The *gcnE*  
188 gene is an epigenetic regulator that is a key histone acetyltransferase in the SAGA/ADA  
189 complex. In primary metabolism, GcnE increases the acetylation level of the proline  
190 utilization cluster, as shown by chromatin immunoprecipitation in *A. nidulans* (Reyes-  
191 Dominguez et al., 2008). However, the role of GcnE might vary for different secondary  
192 metabolite gene clusters in fungi. For example, inactivation of *gcnE* abolishes the production  
193 of aflatoxin in *A. flavus* (Lan et al., 2016) and reduces the production of four known  
194 metabolites (orsellinic acid, sterigmatocystin, penicillin and terrequinone) in *A. nidulans*,  
195 suggesting that *gcnE* is required for transcription activation in these two *Aspergillus* strains  
196 (Nutzmann et al., 2011). However, recent genome-wide ChIP-seq experiments revealed that  
197 most SM cluster genes in *A. nidulans* are poorly decorated with activating heterochromatic  
198 marks (histone demethylation and acetylation), even under inducing conditions (Connolly et

199 al., 2013; Gacek-Matthews et al., 2016), implying that *gcnE* may not be required for  
200 transcriptional activation of certain SM gene clusters. Therefore, GcnE may have diverse  
201 effects on regulating SMs among different *Aspergilli*. This result was in accordance with the  
202 effects of histone deacetylase HdaA in *A. nidulans*. Deletion of HdaA induces the  
203 biosynthesis of sterigmatocystin, penicillin, and norsolorinic acid in *A. nidulans*, but does not  
204 change the expression of terraquinone A (Shwab et al., 2007). A similar phenomenon was  
205 found in the plant pathogen *Fusarium graminearum*. Deletion of heterochromatin protein 1  
206 (*hep1*) in *F. graminearum* leads to the activation of the aurofusarin gene cluster while  
207 repressed the deoxynivalenol cluster (Gacek and Strauss, 2012).

### 208 **3.2. Dereplication of newly produced compounds in *A. niger* $\Delta$ *gcnE* mutant**

209 To further analyze the chemical diversity in the  $\Delta$ *gcnE* mutant, we used WATM medium  
210 since more SM products are likely to be produced on this medium (Fig. 1). Dereplication  
211 using the Antibase database (H, 2012) led to the identification of 11 SM products in the *A.*  
212 *niger*  $\Delta$ *gcnE* mutant (Fig. 2 and Table S4), all of which were known fungal metabolites,  
213 including 9 compounds discovered in *Aspergilli*. The ion peak with  $t_R$  (7.84) min was likely  
214 to be carbonarone A, originally isolated from the fungal strain *A. carbonarius* WZ-4-11  
215 (Zhang et al., 2007). Funalenone ( $t_R$  = 8.42 min) belongs to polyketide compound group of  
216 phenalenones which have diverse structures and biological activities (Gao et al., 2016). The  
217 biosynthetic gene cluster of funalenones has been fully characterized (Gao et al., 2016).  
218 Pestalamide A may have been eluted at  $t_R$  (10.10 min), which was isolated from fungal strain  
219 *Pestalotiopsis theae* W148 (Ding et al., 2008). The highly toxic metabolites fumonisin B2 ( $t_R$   
220 = 10.94 min) and B4 ( $t_R$  = 11.92 min) were detected in the *A. niger*  $\Delta$ *gcnE* mutant. *A. niger*  
221 NRRL3 (ATCC9029), the starting strain of FGSC A1279, is generally regarded as safe  
222 (GRAS) in the industry and should not produce any toxins or toxic compounds. This is  
223 particularly true for *A. niger* FGSC A1279 which is used for gluconic acid production (Baker,

224 2006). These results indicate that deletion of *gcnE* had an epigenetic impact on its SM  
225 production. Five polyketide derivatives (dimeric naphthopyrone family), aurasperone E ( $t_R =$   
226 13.70 min), fonsecinone B ( $t_R = 14.50$  min), isoaurasperone A ( $t_R = 14.77$  min), aurasperone  
227 A ( $t_R = 15.66$  min) and asperpyrone C ( $t_R = 16.33$  min) were also found in the extracts of the  
228 *A. niger*  $\Delta gcnE$  mutant (Fig. 2). Taken together, these results indicated that the newly  
229 identified compounds were likely to be polyketide metabolites.

### 230 3.3. Structural elucidation by HR-ESIMS and 1D/2D NMR

231 To verify the identity of the newly emerged metabolites in the  $\Delta gcnE$  mutant, the strain  
232 was cultivated on WATM medium at 2-L scale and crude extract was subjected to chemical  
233 workup and semi-prep HPLC purification, yielding six pure compounds **1** (3.7 mg), **2** (1.8  
234 mg), **3** (7.8 mg), **4** (9.2 mg), **5** (1.5 mg), and **6** (3.0 mg).

235 The molecular formula of nigerpyrone (**1**) was established as  $C_{12}H_{10}O_2$  by HR-ESIMS  
236 ( $187.07 [M+H]^+$ ). Dereplication using the Antibase database suggested that this might be a  
237 new compound. The molecular formula suggested eight degrees of unsaturation. The  $^{13}C$  and  
238 HSQC NMR data of **1** in  $CD_3OD$  (Fig. S2) revealed one benzene/aromatic ring ( $\delta_C$  128.38-  
239 136.30 ppm), one carbonyl ( $\delta_C$  178.14 ppm), and four olefinic carbons ( $\delta_C$  117.36-117.52  
240 ppm and 158.45-169.48 ppm), consistent with the presence of five double bonds and one ring  
241 system. This analysis accounted for seven double bonds, suggesting the presence of an  
242 additional ring in the structure of **1**. Analysis of the  $^1H$ - $^1H$  COSY spectrum revealed two  
243 contiguous spin systems, one consisting of H-10 through H-14, and the other consisting of H-  
244 2 to H-3 (Fig. 3 and Fig. S2). The connection of these two spin systems at C-6 was evident by  
245 the HMBC correlation of H-5 and H-8 to C-6 (Fig. 3), which was further confirmed by the  
246 correlation of H-8 to C-5 in the HMBC spectrum. The connection of methylene to the  
247 benzene ring was confirmed by the HMBC correlation of H-8 to C-9 (Fig. 3). The structure  
248 was further confirmed by comparing  $^{13}C$  experimental data with published data (Dai et al.,

249 2007) and predicted  $^{13}\text{C}$  NMR data (Elyashberg et al., 2010) (Fig. S2). The resulting  $r^2$  value  
250 of 0.9992 indicated that the proposed structure was correct (Bremser, 1978; Elyashberg et al.,  
251 2010). Inspection of the  $^1\text{H}$ ,  $^{13}\text{C}$ , and HR-ESIMS data of compounds **2** and **3** (Fig. S3 and S4)  
252 indicated that both **2** and **3** were known compounds (Zhang et al., 2007; Ding et al., 2008):  
253 carbonarone A (**2**) was isolated from *A. carbonarius* WZ-4-11 (Zhang et al., 2007) and  
254 pestalamide A (**3**) was isolated from *Pestalotiopsis theae* W148 (Ding et al., 2008).  
255 Comparison of  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of compounds **1**, **2** and **3** (Fig. S2, S3 and S4)  
256 confirmed the structure of the new compound **1**.

257 Inspection of the  $^1\text{H}$ ,  $^{13}\text{C}$  and HR-ESIMS data of compounds **4** (Fig. S5), **5** (Fig. S6) and **6**  
258 (Fig. S7), indicated that they were known. Compound 4 is produced by *A. niger* FO-5904)  
259 (Inokoshi et al., 1999), 5 by *A. niger* CMI-IMI 205879 (Priestap, 1984), and 6 by *A.*  
260 *fonsecaeus* NRRL 67, O 16-1 (Priestap, 1984).

### 261 **3.4. Putative biosynthetic gene clusters of compounds from the *ΔgcnE* mutant**

262 Homologous BLAST search and comparison of the literature allowed the identification of  
263 the putative biosynthetic gene clusters of known compounds discovered from the *ΔgcnE*  
264 mutant (Table S5). Genes for synthesizing funalenone (**4**) were identified in *Penicillium*  
265 *herquei* (Gao et al., 2016). By homologous search against the annotated genome of *A. niger*  
266 CBS513.88, we identified five homologous genes in *A. niger* responsible for the biosynthesis  
267 of funalenone (Fig. S8 and Table S5). The literature (Chiang et al., 2010) indicated that  
268 monodictyphenone might be the precursor of aurasperone E (**5**), aurasperone A (**6**) and other  
269 derivatives such as fonsecinone B, isoaurasperone A, and asperpyrone C. In *A. nidulans*, the  
270 key biosynthetic enzyme of monodictyphenone is proposed to be a non-reducing polyketide  
271 synthase (NR-PKS) *mdpG* (Chiang et al., 2010). We therefore performed a homologous  
272 search of the *A. niger* genome using *mdpG* as the reference query, and identified an open  
273 reading frame (ORF) An11g07310 located in contig 11, annotated as a NR-PKS with

274 multiple domains of KS-AT-PT-PP (Table S5). Other proposed biosynthetic genes involved  
275 in the biosynthesis of **5** and **6** were not located in the close proximity of An11g07310 and  
276 were scattered in other genomic loci. However, genes for secondary metabolites are generally  
277 tightly clustered.

### 278 **3.5. Characterization of key biosynthetic genes for pestalamide A**

279 Inspection of the structures of **1-3** led to speculation that their precursors contain one  
280 phenylacetate and two acetate units, suggesting that compounds **1-3** may be synthesized via a  
281 polyketide biosynthetic pathway. The incorporation of phenylacetate in fungal secondary  
282 metabolism is also observed in the biosynthesis of the  $\beta$ -lactam antibiotic penicillin G in  
283 *Penicillium chrysogenum* (Koetsier et al., 2009). *In vitro* assays show that the gene product  
284 PhlB is a phenylacetyl-CoA ligase responsible for the activation of phenylacetate into  
285 phenylacetyl-SCoA (Koetsier et al., 2009). We performed a homolog search of PhlB in *A.*  
286 *niger*, and identified ORF An09g01820, annotated as a ferulate:CoA ligase (AMP-forming)  
287 (Fig. 4A and Table S6), with moderate sequence identity (35%) to PhlB.

288 Analysis of genes in proximity to An09g01820 (*epaB*) identified a candidate NR-PKS gene  
289 cluster (*epa*) spanning a 23.9-kb genomic region (Fig. 4A and Table S6). The *epa* cluster  
290 possesses six ORFs (Table S6). *In silico* analysis indicated that *epaA* (An09g01860) encodes  
291 a typical Clade III NR-PKS (2,617 amino acids in length) with a domain organization of  
292 SAT-KS-AT-PT-ACP-Met-R domains, which shares high amino acid sequence identity (97%)  
293 with AzaA polyketide synthase (2,599 amino acids in length) in the biosynthesis of  
294 azaphilones from *A. niger* ATCC 1015 (Zabala et al., 2012). The gene product EpaC is  
295 annotated as an acyl-CoA transferase. Adjacent to *epaA*, there are three genes, orf1-3,  
296 encoding oxidoreductase, 3-hydroxybenzoate 4-hydroxylase, and salicylate hydroxylase  
297 (Table S6).

298 To determine if the *epa* cluster was responsible for synthesizing pestalamide A (**3**), we  
299 knocked out the PKS gene (An09g01860, *epaA*) by homologous recombination (Table S3  
300 and Fig. S1). Inactivation of the *epaA* gene abolished the production of **1-3** (Fig. 4B), and  
301 complementation strain (*epaA-com*) could produce compound **1-3** (Fig. S1 and Fig. 4B),  
302 suggesting that the identified biosynthetic genes were responsible for the synthesis of **1-3**. We  
303 also performed cell-free extract experiments, in which all biosynthetic enzymes and cofactors  
304 were available for the biosynthesis of **3**, to test if **1** and **2** were the biosynthetic precursors of  
305 **3** (data not shown). Feeding purified **1** and **2** into cell-free extracts of the  $\Delta$ *gcnE* mutant did  
306 not produce **3**, suggesting that **1** and **2** were by-products in the biosynthesis of **3**. Based on  
307 the genetic and cell-free extract experiments, a biosynthetic model for **1-3** is proposed in Fig.  
308 4C. We propose that the biosynthesis of **1-3** begins with the polyketide assembly by EpaA to  
309 form phenylacetyl triketide precursor from successive condensation of two malonyl-CoA,  
310 presumably with one phenylacetyl-CoA starter unit. For the nigerpyrone (**1**) biosynthesis, the  
311 reactive polyketide chain is released as an aldehyde (**8**) through the R-domain, in a manner  
312 similar to the previously characterized 3-methylorcinaldehyde synthase in *Acremonium*  
313 *strictum* (Bailey et al., 2007). The cyclization and dehydration of **8** may create nigerpyrone  
314 (**1**). This nonenzymatic rearrangement to form pyrone moiety from aldehyde was also  
315 observed in the plant metabolites, arabidopyl derivatives (Weng et al., 2012). For the  
316 biosynthesis of **2** and **3**, an extra methyl group will be added through the C-methyltransferase  
317 domain, followed by the reduction to generate **8a**, which undergoes oxidation and  
318 transamination to produce the intermediate **9**. The candidate gene products for this series of  
319 biotransformation could be orf1-3 (Table S6). The cyclization of **9** produces carbonarone A  
320 (**2**). We propose that EpaC (An09g01800), an acyl-CoA transferase, could catalyse the  
321 transfer of 2-methylsuccinyl-CoA, a common intermediate in the ethylmalonyl-CoA pathway  
322 (Erb et al., 2009), to generate the final product pestalamide A (**3**).

## 323 **4. Conclusions**

324 In conclusion, we confirmed that deletion of *gcnE* resulted in the production of 12  
325 polyketide metabolites in an *A. niger* FGSC A1279  $\Delta gcnE$  mutant. Chemical workup of  
326 organic extracts of the culture broth from the  $\Delta gcnE$  mutant identified six pure compounds,  
327 including one new compound nigerpyrone (**1**) and five known fungal polyketide metabolites  
328 (**2-6**). The structures of these compounds were confirmed by HR-ESIMS and 1D/2D NMR.  
329 Finally, the biosynthetic gene cluster for **1-3** was verified via gene knockout and  
330 complementation, and a biosynthetic model of this group of pyran-based fungal metabolites  
331 was proposed. To our best knowledge, this was the first time to confirm that the inactivation  
332 of *gcnE* resulted in activating the biosynthesis of polyketide metabolites in *A. niger* FGSC  
333 A1279.

334

## 335 **Declarations of interest**

336 None.

337

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345

## 346 **References**

347 Andersen MR, Salazar MP, Schaap PJ, van de Vondervoort PJ, Culley D, Thykaer J, Frisvad JC, Nielsen  
348 KF, Albang R, Albermann K, Berka RM, Braus GH, Braus-Stromeyer SA, Corrochano LM, Dai Z, van  
349 Dijck PW, Hofmann G, Lasure LL, Magnuson JK, Menke H, Meijer M, Meijer SL, Nielsen JB, Nielsen  
350 ML, van Ooyen AJ, Pel HJ, Poulsen L, Samson RA, Stam H, Tsang A, van den Brink JM, Atkins A,  
351 Aerts A, Shapiro H, Pangilinan J, Salamov A, Lou Y, Lindquist E, Lucas S, Grimwood J, Grigoriev IV,  
352 Kubicek CP, Martinez D, van Peij NN, Roubos JA, Nielsen J, Baker SE, 2011. Comparative genomics  
353 of citric-acid-producing *Aspergillus niger* ATCC 1015 versus enzyme-producing CBS 513.88.  
354 Genome Res. 21(6), 885-897.

355 Arentshorst M, Lagendijk EL, Ram AF, 2015. A new vector for efficient gene targeting to the pyrG  
356 locus in *Aspergillus niger*. Fungal Biol. Biotechnol. (2), 2.

357 Bailey AM, Cox RJ, Harley K, Lazarus CM, Simpson TJ, Skellam E, 2007. Characterisation of 3-  
358 methylorcinolaldehyde synthase (MOS) in *Acremonium strictum*: first observation of a reductive  
359 release mechanism during polyketide biosynthesis. Chem. Commun. (39), 4053-4055.

360 Baker SE, 2006. *Aspergillus niger* genomics: past, present and into the future. Med. Mycol. 44 Suppl  
361 1, S17-21.

362 Baker SP, Grant PA, 2007. The SAGA continues: expanding the cellular role of a transcriptional co-  
363 activator complex. Oncogene 26(37), 5329-5340.

364 Bayram O, Krappmann S, Ni M, Bok JW, Helmstaedt K, Valerius O, Braus-Stromeyer S, Kwon NJ,  
365 Keller NP, Yu JH, Braus GH, 2008. VelB/VeA/LaeA complex coordinates light signal with fungal  
366 development and secondary metabolism. Science 320(5882), 1504-1506.

367 Bok JW, Hoffmeister D, Maggio-Hall LA, Murillo R, Glasner JD, Keller NP, 2006. Genomic mining for  
368 *Aspergillus* natural products. Chem. Biol. 13(1), 31-37.

369 Brakhage AA, 2013. Regulation of fungal secondary metabolism. Nat. Rev. Microbiol. 11(1), 21-32.

370 Bremser W, 1978. Hse-a novel substructure code. Analytica Chimica Acta 103(4), 355-365.

371 Carvalho ND, Arentshorst M, Jin Kwon M, Meyer V, Ram AF, 2010. Expanding the ku70 toolbox for  
372 filamentous fungi: establishment of complementation vectors and recipient strains for advanced  
373 gene analyses. Appl. Microbiol. Biotechnol. 87(4), 1463-1473.

374 Chiang YM, Meyer KM, Praseuth M, Baker SE, Bruno KS, Wang CC, 2011. Characterization of a  
375 polyketide synthase in *Aspergillus niger* whose product is a precursor for both  
376 dihydroxynaphthalene (DHN) melanin and naphtho-gamma-pyrone. Fungal Genet. Biol. 48(4),  
377 430-437.

378 Chiang YM, Szewczyk E, Davidson AD, Entwistle R, Keller NP, Wang CC, Oakley BR, 2010.  
379 Characterization of the *Aspergillus nidulans* monodictyphenone gene cluster. Appl. Environ.  
380 Microbiol. 76(7), 2067-2074.

381 Connolly LR, Smith KM, Freitag M, 2013. The *Fusarium graminearum* histone H3 K27  
382 methyltransferase KMT6 regulates development and expression of secondary metabolite gene  
383 clusters. PLoS Genet. 9(10), e1003916.

384 Dai J, Liu Y, Jia H, Zhou YD, Nagle DG, 2007. Benzochromenones from the marine crinoid  
385 *Comantheria rotula* inhibit hypoxia-inducible factor-1 (HIF-1) in cell-based reporter assays and  
386 differentially suppress the growth of certain tumor cell lines. J. Nat. Prod. 70(9), 1462-1466.

387 Ding G, Jiang L, Guo L, Chen X, Zhang H, Che Y, 2008. Pestalazines and pestalamides, bioactive  
388 metabolites from the plant pathogenic fungus *Pestalotiopsis theae*. J. Nat. Prod. 71(11), 1861-  
389 1865.

390 Elyashberg M, Williams AJ, Blinov K, 2010. Structural revisions of natural products by Computer-  
391 Assisted Structure Elucidation (CASE) systems. Nat. Prod. Rep. 27(9), 1296-1328.

392 Erb TJ, Fuchs G, Alber BE, 2009. (2S)-Methylsuccinyl-CoA dehydrogenase closes the ethylmalonyl-  
393 CoA pathway for acetyl-CoA assimilation. Mol. Microbiol. 73(6), 992-1008.

394 Fisch KM, Gillaspay AF, Gipson M, Henrikson JC, Hoover AR, Jackson L, Najjar FZ, Wagele H, Cichewicz  
395 RH, 2009. Chemical induction of silent biosynthetic pathway transcription in *Aspergillus niger*. J.  
396 Ind. Microbiol. Biotechnol. 36(9), 1199-1213.

397 Frisvad JCThrane U, 1987. Standardized high-performance liquid chromatography of 182 mycotoxins  
398 and other fungal metabolites based on alkylphenone retention indices and UV-VIS spectra (diode  
399 array detection). *J. Chromatogr.* 404(1), 195-214.

400 Gacek-Matthews A, Berger H, Sasaki T, Wittstein K, Gruber C, Lewis ZA, Strauss J, 2016. KdmB, a  
401 Jumonji histone H3 demethylase, regulates genome-wide H3K4 trimethylation and is required for  
402 normal induction of secondary metabolism in *Aspergillus nidulans*. *PLoS Genet.* 12(8), e1006222.

403 Gacek AStrauss J, 2012. The chromatin code of fungal secondary metabolite gene clusters. *Appl.*  
404 *Microbiol. Biotechnol.* 95(6), 1389-1404.

405 Gao SS, Duan A, Xu W, Yu P, Hang L, Houk KN, Tang Y, 2016. Phenalenone polyketide cyclization  
406 catalyzed by fungal polyketide synthase and flavin-dependent monooxygenase. *J. Am. Chem. Soc.*  
407 138(12), 4249-4259.

408 Hiort J, Maksimenka K, Reichert M, Perovic-Ottstadt S, Lin WH, Wray V, Steube K, Schaumann K,  
409 Weber H, Proksch P, Ebel R, Muller WE, Bringmann G, 2004. New natural products from the  
410 sponge-derived fungus *Aspergillus niger*. *J. Nat. Prod.* 67(9), 1532-1543.

411 Inglis DO, Binkley J, Skrzypek MS, Arnaud MB, Cerqueira GC, Shah P, Wymore F, Wortman JR,  
412 Sherlock G, 2013. Comprehensive annotation of secondary metabolite biosynthetic genes and  
413 gene clusters of *Aspergillus nidulans*, *A. fumigatus*, *A. niger* and *A. oryzae*. *BMC Microbiol.* 13, 91.

414 Inokoshi J, Shiomi K, Masuma R, Tanaka H, Yamada H, Omura S, 1999. Funalenone, a novel  
415 collagenase inhibitor produced by *Aspergillus niger*. *J. Antibiot (Tokyo).* 52(12), 1095-1100.

416 Koetsier MJ, Jekel PA, van den Berg MA, Bovenberg RA, Janssen DB, 2009. Characterization of a  
417 phenylacetate-CoA ligase from *Penicillium chrysogenum*. *Biochem. J.* 417(2), 467-476.

418 Laatsch H, 2012. *AntiBase 2012 The natural compound identifier*. Wiley-VCH Verlag GmbH & Co,  
419 KGaA.

420 Lan H, Sun R, Fan K, Yang K, Zhang F, Nie XY, Wang X, Zhuang Z, Wang S, 2016. The *Aspergillus flavus*  
421 histone acetyltransferase aflGcnE regulates morphogenesis, aflatoxin biosynthesis, and  
422 pathogenicity. *Front. Microbiol.* 7, 1324.

423 Nielsen KF, Mogensen JM, Johansen M, Larsen TO, Frisvad JC, 2009. Review of secondary  
424 metabolites and mycotoxins from the *Aspergillus niger* group. *Anal. Bioanal. Chem.* 395(5), 1225-  
425 1242.

426 Nutzmann HW, Reyes-Dominguez Y, Scherlach K, Schroeckh V, Horn F, Gacek A, Schumann J,  
427 Hertweck C, Strauss J, Brakhage AA, 2011. Bacteria-induced natural product formation in the  
428 fungus *Aspergillus nidulans* requires Saga/Ada-mediated histone acetylation. *Proc. Natl. Acad. Sci.*  
429 *USA* 108(34), 14282-14287.

430 Pel HJ, de Winde JH, Archer DB, Dyer PS, Hofmann G, Schaap PJ, Turner G, de Vries RP, Albang R,  
431 Albermann K, Andersen MR, Bendtsen JD, Benen JA, van den Berg M, Breststraat S, Caddick MX,  
432 Contreras R, Cornell M, Coutinho PM, Danchin EG, Debets AJ, Dekker P, van Dijck PW, van Dijk A,  
433 Dijkhuizen L, Driessen AJ, d'Enfert C, Geysens S, Goosen C, Groot GS, de Groot PW, Guillemette T,  
434 Henrissat B, Herweijer M, van den Hombergh JP, van den Hondel CA, van der Heijden RT, van der  
435 Kaaij RM, Klis FM, Kools HJ, Kubicek CP, van Kuyk PA, Lauber J, Lu X, van der Maarel MJ,  
436 Meulenberg R, Menke H, Mortimer MA, Nielsen J, Oliver SG, Olsthoorn M, Pal K, van Peij NN,  
437 Ram AF, Rinas U, Roubos JA, Sagt CM, Schmoll M, Sun J, Ussery D, Varga J, Verweijen W, van de  
438 Vondervoort PJ, Wedler H, Wosten HA, Zeng AP, van Ooyen AJ, Visser J, Stam H, 2007. Genome  
439 sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. *Nat. Biotechnol.*  
440 25(2), 221-231.

441 Priestap HA, 1984. New naphthopyrones from *Aspergillus fonssecaeus*. *Tetrahedron* 40(19), 3617-  
442 3624.

443 Rank C, Klejnstrup ML, Petersen LM, Kildgaard S, Frisvad JC, Held Gotfredsen C, Ostenfeld Larsen T,  
444 2012. Comparative chemistry of *Aspergillus oryzae* (RIB40) and *A. flavus* (NRRL 3357).  
445 *Metabolites* 2(1), 39-56.

446 Reyes-Dominguez Y, Narendja F, Berger H, Gallmetzer A, Fernandez-Martin R, Garcia I, Scazzocchio C,  
447 Strauss J, 2008. Nucleosome positioning and histone H3 acetylation are independent processes  
448 in the *Aspergillus nidulans* prnD-prnB bidirectional promoter. Eukaryot. Cell 7(4), 656-663.  
449 Rossano F, Ortega De Luna L, Buommino E, Cusumano V, Losi E, Catania MR, 1999. Secondary  
450 metabolites of *Aspergillus* exert immunobiological effects on human monocytes. Res. Microbiol.  
451 150(1):13-19.  
452 Sanchez JF, Somoza AD, Keller NP, Wang CC, 2012. Advances in *Aspergillus* secondary metabolite  
453 research in the post-genomic era. Nat. Prod. Rep. 29(3), 351-371.  
454 Serra R, Braga A, Venancio A, 2005. Mycotoxin-producing and other fungi isolated from grapes for  
455 wine production, with particular emphasis on ochratoxin A. Res. Microbiol. 156(4), 515-521.  
456 Shwab EK, Bok JW, Tribus M, Galehr J, Graessle S, Keller NP, 2007. Histone deacetylase activity  
457 regulates chemical diversity in *Aspergillus*. Eukaryot. Cell 6(9), 1656-1664.  
458 Sorensen LM, Lametsch R, Andersen MR, Nielsen PV, Frisvad JC, 2009. Proteome analysis of  
459 *Aspergillus niger*: lactate added in starch-containing medium can increase production of the  
460 mycotoxin fumonisin B2 by modifying acetyl-CoA metabolism. BMC Microbiol. 9, 255.  
461 Szewczyk E, Nayak T, Oakley CE, Edgerton H, Xiong Y, Taheri-Talesh N, Osmani SA, Oakley BR, 2006.  
462 Fusion PCR and gene targeting in *Aspergillus nidulans*. Nat. Protoc. 1(6), 3111-3120.  
463 Tanaka H, Wang P-L, Yamada Late O, Tamura T, 1966. Yellow pigments of *Aspergillus niger* and *Asp.*  
464 *awamori*. Agric. Biol. Chem. 30(2), 107-113.  
465 Varga J, Rigo K, Kocsube S, Farkas B, Pal K, 2003. Diversity of polyketide synthase gene sequences in  
466 *Aspergillus* species. Res. Microbiol. 154(8), 593-600.  
467 Wang B, Lv Y, Li X, Lin Y, Deng H, Pan L, 2018. Profiling of secondary metabolite gene clusters  
468 regulated by LaeA in *Aspergillus niger* FGSC A1279 based on genome sequencing and  
469 transcriptome analysis. Res. Microbiol. 169, 67-77.  
470 Weng JK, Li Y, Mo H, Chapple C, 2012. Assembly of an evolutionarily new pathway for alpha-pyrone  
471 biosynthesis in *Arabidopsis*. Science 337(6097), 960-964.  
472 Wu M-YYu J-H, 2015. Epigenetics of fungal secondary metabolism related genes. In: Zeilinger S,  
473 Martín J-F, García-Estrada C editors. Biosynthesis and molecular genetics of fungal secondary  
474 metabolites, Volume 2. Springer New York, New York, pp. 29-42.  
475 Yin C, Wang B, He P, Lin Y, Pan L, 2014. Genomic analysis of the aconidial and high-performance  
476 protein producer, industrially relevant *Aspergillus niger* SH2 strain. Gene 541(2), 107-114.  
477 Yuan XL, van der Kaaij RM, van den Hondel CA, Punt PJ, van der Maarel MJ, Dijkhuizen L, Ram AF,  
478 2008. *Aspergillus niger* genome-wide analysis reveals a large number of novel alpha-glucan  
479 acting enzymes with unexpected expression profiles. Mol. Genet. Genomics 279(6), 545-561.  
480 Zabala AO, Xu W, Chooi YH, Tang Y, 2012. Discovery and characterization of a silent gene cluster that  
481 produces Azaphilones from *Aspergillus niger* ATCC 1015 reveal a hydroxylation-mediated pyran-  
482 ring formation. Chem. Biol. 19(8), 1049-1059.  
483 Zhang Y, Zhu T, Fang Y, Liu H, Gu Q, Zhu W, 2007. Carbonarones A and B, new bioactive gamma-  
484 pyrone and alpha-pyridone derivatives from the marine-derived fungus *Aspergillus carbonarius*. J.  
485 Antibiot (Tokyo). 60(2), 153-157.

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487

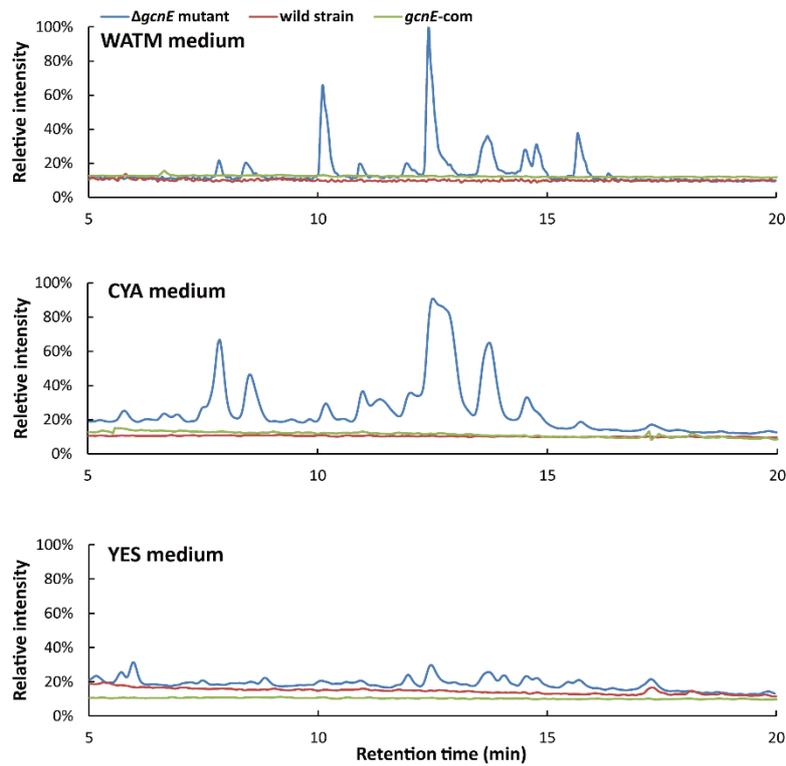
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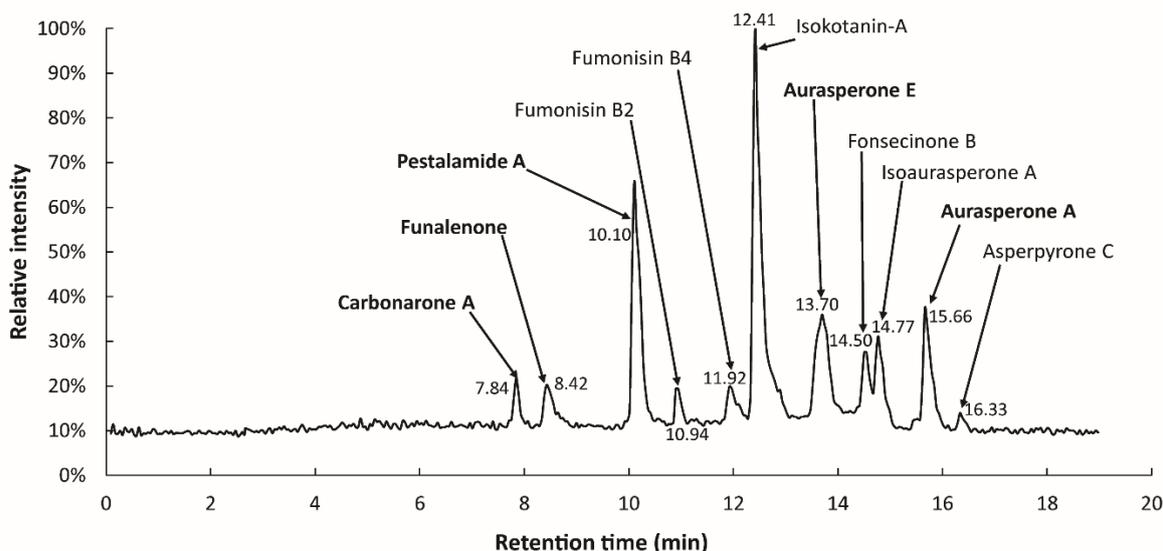
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492 **Figures**



493

494 **Figure 1. SM profile of *A. niger* FGSC A1279  $\Delta gcnE$  mutant cultivated on different**  
495 **media.** Blue curve,  $\Delta gcnE$  mutant; red curve, wild strain; green curve,  $gcnE$   
496 complementation strain ( $gcnE$ -com). To collect secondary metabolites, *A. niger* strains were  
497 cultivated for 7 days at 25 °C in the dark using different media. The crude extract was  
498 dissolved in MeOH:Milli-Q H<sub>2</sub>O (9:1) and centrifuged at 10,000 rpm for 10 min, before  
499 LC/MS analysis.



500

501 **Figure 2. SM products detected in *A. niger*  $\Delta$ gcnE mutant cultivated on WATM medium.**

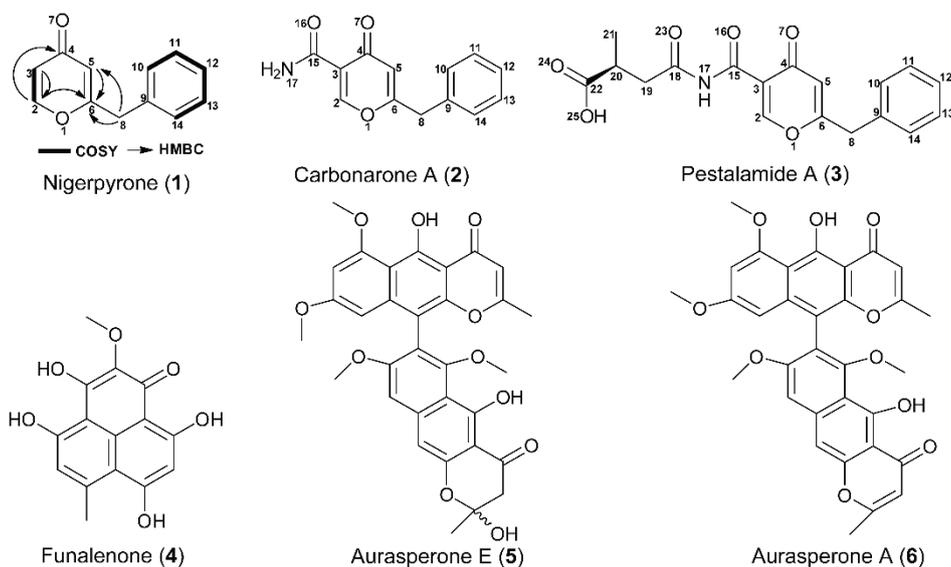
502 *A. niger*  $\Delta$ gcnE mutant was cultivated for 7 days at 25 °C in the dark on WATM medium.

503 High resolution mass spectrometric data were obtained using an LTQ XL/LTQ Orbitrap

504 Discovery MS system coupled to a Thermo Instruments HPLC system. LC/MS data were

505 analyzed by Xcalibur software with Antibase2012 database. Compounds that were purified

506 by chemical workup and characterized by spectroscopic analysis were marked in bold.



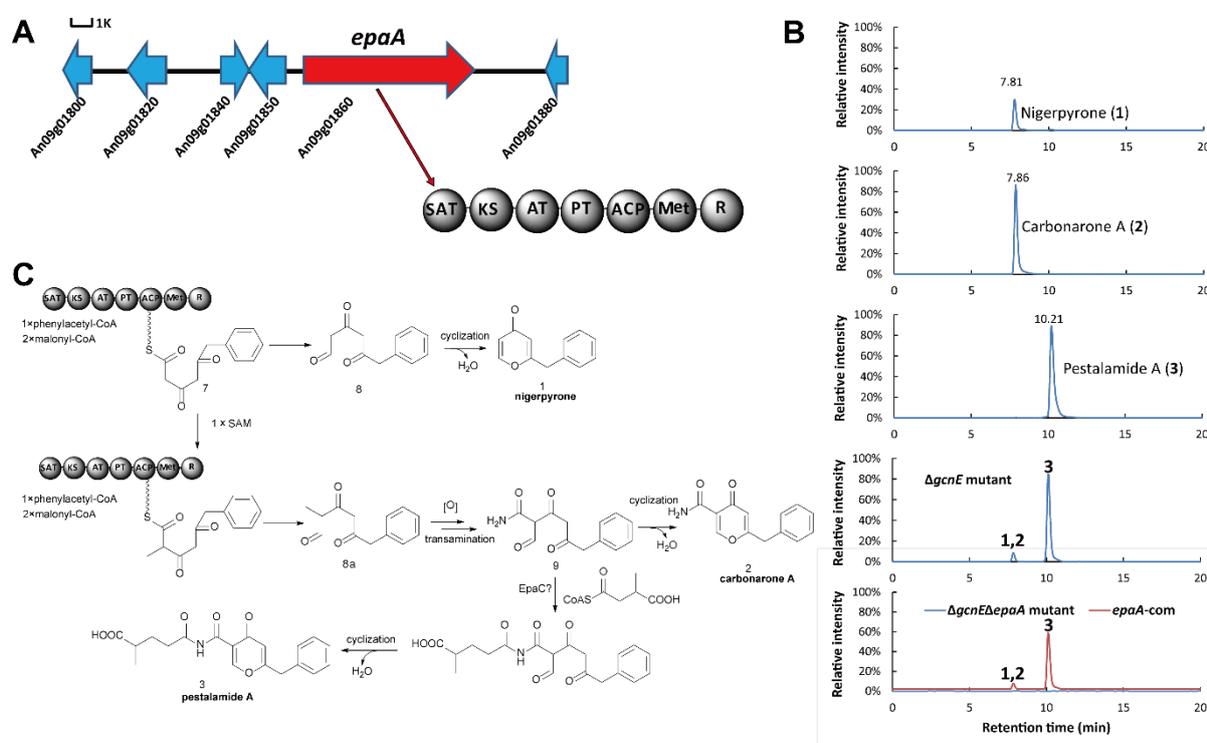
507

508 **Figure 3. Secondary metabolites isolated and characterized by HR-ESIMS and 1D/2D**

509 **NMR in *A. niger*  $\Delta$ gcnE mutant.** *A. niger*  $\Delta$ gcnE mutant was cultivated on WATM medium.

510  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR, COSY, HSQC and HMBC spectra were recorded on a Bruker Daltonics

511 Advance III HD 400 NMR spectrometer, using deuterated solvent methanol-d<sub>4</sub>. Chemical  
 512 shift values were reported in parts per million (ppm) with tetramethylsilane (TMS) as the  
 513 external standard. When higher field was needed, NMR spectra were recorded on a Bruker  
 514 Daltonics Advance III HD 600 NMR. NMR data were analyzed using MestReNova 9.0.1  
 515 software and ACD/I-Lab (<http://ilab.cds.rsc.org/?cdsrdr=1>). Compound structures were  
 516 drawn using ChemBioDraw software. Chemical formulas and exact masses are marked for  
 517 each compound. Dereplication data are in online supplemental file (Fig. S2-S7).  
 518



519  
 520 **Figure 4. Characterization of the biosynthetic cluster for pestalamide A.** (A) Putative  
 521 gene cluster of compounds **1-3**, which was identified by the homologous BLAST search of *P.*  
 522 *chrysogenum* PhIB gene. (B) LC/MS detection of compounds **1-3** in gene knockout and  
 523 complementation strains. Retention time for purified compounds **1-3** was determined by  
 524 LC/MS. *A. niger* strains were cultivated on WATM medium. (C) Proposed model of the  
 525 biosynthesis of nigerpyrone (**1**), carbonarone A (**2**) and pestalamide A (**3**).

526 **Supplemental materials**

527 **Table S1.** Strains and plasmids used in this study

528 **Table S2.** Primers used for *gcnE* deletion-cassette construction and transformant  
529 identification

530 **Table S3.** Primers used to knock out *epaA* (An09g01860) and verify mutants

531 **Table S4.** Putative compounds detected in *A. niger* FGSC A1279  $\Delta gcnE$  mutant based on  
532 LC/MS data and Antibase database

533 **Table S5.** Putative biosynthetic gene clusters for known compounds detected in the  $\Delta gcnE$   
534 mutant

535 **Table S6.** Putative biosynthetic gene cluster for pestalamide A.

536

537 **Figure S1.** Design of deletion cassettes, complementation cassettes, and PCR verification of  
538 *A. niger* mutants ( $\Delta gcnE$ ,  $\Delta gcnE\Delta epaA$ , *gcnE* complementation and *epaA* complementation).

539 **Figure S2.** LC/MS and NMR data for the novel compound nigerpyrone

540 **Figure S3.** LC/MS and NMR data of compound carbonarone A

541 **Figure S4.** LC/MS and NMR data of compound pestalamide A

542 **Figure S5.** LC/MS and NMR data of compound funalenone

543 **Figure S6.** LC/MS and NMR data of compound aurasperone E

544 **Figure S7.** LC/MS and NMR data of compound aurasperone A

545 **Figure S8.** Putative biosynthetic gene clusters of the known compounds in the  $\Delta gcnE$  mutant