Elucidation of cladofulvin biosynthesis reveals a cytochrome P450 monooxygenase required for anthraquinone dimerization

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Anthraquinones are a large family of secondary metabolites (SMs) that are extensively studied for their diverse biological activities. These activities are determined by functional group decorations and the formation of dimers from anthraquinone monomers. Despite their numerous medicinal qualities, very few anthraquinone biosynthetic pathways have been elucidated so far, including the enzymatic dimerization steps. In this study, we report the elucidation of the biosynthesis of cladofulvin, an asymmetrical homodimer of natale-emodin produced by the fungus Cladosporium fulvum. A gene cluster of 10 genes controls cladofulvin biosynthesis, which begins with the production of atrochrysone carboxylic acid by the polyketide synthase ClaG and the β-lactamase ClaF. This compound is decarboxylated by ClaH to yield emodin, which is then converted to chrysophanol hydroquinone by the reductase ClaC and the dehydratase ClaB. We show that the predicted cytochrome P450 ClaM catalyzes the dimerization of natale-emodin to cladofulvin. Remarkably, such dimerization dramatically increases natale-emodin cytotoxicity against mammalian cell lines. These findings shed light on the enzymatic mechanisms involved in anthraquinone dimerization. Future characterization of the ClaM enzyme should facilitate engineering the biosynthesis of novel, potent, dimeric anthraquinones and structurally related compound families.

Significance

Anthraquinones are potent secondary metabolites produced by many fungi and plants used in traditional Chinese and Indian medicine. Many display useful biological properties, including antineoplastic, antiinflammatory, antifungal, or antiarthritic activities. The chemical structure of anthraquinones is very diverse, with many occurring as homo- and heterodimers. Anthraquinone biosynthetic pathways must be elucidated before novel structurally complex chemicals with new or enhanced biological activity can be engineered. In this study, we identified an enzyme involved in asymmetrical dimerization of natale-emodin, which results in increased cytotoxicity toward a range of cancer cell lines. Mastering the substrate specificity of this enzyme (and other similar enzymes) could lead to the dimerization of anthraquinone-related compounds with medicinal activities.

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through aryl–aryl bond formation is assumed to be catalyzed enzymatically, no responsible enzymes have so far been identified in fungi.

The bianthraquinone cladofulvin 1 is a homodimer composed of two natale-emodin moieties linked by an aryl–aryl bond, and it is the sole detectable SM produced by the fungal plant pathogen Cladosporium fulvum during growth on artificial media (15). However, no biological activities or functions have so far been determined (15). From 23 predicted core SM genes identified in the genome of C. fulvum, only the PKS6 gene is consistently and highly expressed during cladofulvin production (15). Extensive synteny was reported between the PKS6 locus and the monodictyphenone gene cluster in A. nidulans but with clear signs of divergence (Fig. L4) (15). Notably, the PKS6 locus does not contain homologs of mdpH, mdpL, or mdpC, genes that are essential for the production of monodictyphenone (7, 15). Conversely, the PKS6 locus contains two genes that are not present in the monodictyphenone gene cluster, genes that encode a predicted cytochrome P450 monoxygenase and a predicted short-chain dehydrogenase/reductase (15). A homologous gene cluster appears to be present in Aspergillus terreus (Fig. L4 and SI Appendix, Table S1), which contains genes that encode enzymes for the production of atrochrysone 2 (14).

In this study, we aimed to elucidate the cladofulvin biosynthetic pathway and identify the enzyme(s) involved in the dimerization of natale-emodin to yield cladofulvin. Using C. fulvum deletion mutants and heterologous expression of presumed cladofulvin biosynthetic genes in Aspergillus oryzae, we propose a complete route to cladofulvin biosynthesis, including the final enzymatic dimerization step. We also report that dimerization of natale-emodin increases the ability of this compound to inhibit the growth of mammalian cell lines.

Results

1) Nine Genes at the clag Locus and claf Are Coregulated During Cladofulvin Production and Define the Putative Cladofulvin Gene Cluster. We first wanted to ascertain which genes define the predicted cladofulvin gene cluster. For this purpose, we compared the expressions of the cladofulvin (cla) genes predicted to be involved in cladofulvin 1 production in WT C. fulvum and genetically modified strains of this fungus in which cladofulvin 1 production is abolished. Indeed, C. fulvum deletion mutants of the global transcriptional regulator Wor1 and the overexpression OE.CJWor1 transformant do not produce cladofulvin 1, consistent with down-regulation of the PKS6 gene in both genetic backgrounds (16). Deletion of the histone deacetylase gene, CJHdaA, in C. fulvum also abolished cladofulvin production (17). Available RNA-sequencing (RNA-seq) data from the Δclag deletion mutants showed that, relative to the WT, with the exception of claf, all of the predicted cla genes are significantly down-regulated, whereas the β-tubulin control gene and clafu184401 border gene are not (Fig. 1B). In the OE.CJWor1 transformant, only genes from the predicted cladofulvin gene cluster, including claf, are significantly down-regulated at the clag locus (Fig. 1B). Similarly, in the ΔclagΔaa deletion mutants, clafB, clafC, clag, and clafM are significantly down-regulated compared with the WT, with the other genes also showing a clear decrease in expression (Fig. 1B). In addition to genes at the clag locus, the clafu190728 and clafu192728 genes were included, because their products bear high similarity to MdpH and MdpL, respectively, in A. nidulans (SI Appendix, Table S1). The putative claH gene (clafu190728) is significantly down-regulated in all three genetic backgrounds compared with the WT, but the expression of clafu192728 was unchanged (Fig. 1B). These results show that claH and genes at the clag locus are coregulated and likely belong to the same biosynthetic pathway.

2) Biosynthesis of Cladofulvin Depends on the Megasynthetic Clag. To validate the hypothesis that the nonreducing polyketide synthase (nPKS) (18) Clag is responsible for cladofulvin 1 production, we replaced the cla gene in C. fulvum WT through homologous recombination. Two confirmed independent deletion mutants and an ectopic transformant were selected for additional analysis (SI Appendix, Fig. S1). Both clag deletion mutants appeared to be gray in color than the ectopic transformant and the WT, but otherwise, they showed no obvious phenotypic abnormalities (Fig. 2A). Ethyl acetate extracts from cultures of two independent ∆clag deletion mutants, WT and ectopic transformant, were analyzed by HPLC alongside purified cladofulvin 1 as a standard (15). Cladofulvin 1 was detected in the controls as previously reported (15) but not in the ∆clag deletion mutants (Fig. 2B), confirming that clag, indeed, encodes the responsible PKS. The other coregulated cla genes described above might, therefore, play a role in the regulation or production of cladofulvin 1 in C. fulvum.

3) Concerted Activity of ClaG and ClaF Results in Atrochrysone Biosynthesis. To characterize the early steps of cladofulvin 1 biosynthesis, we heterologously expressed selected cla genes in A. oryzae M-2-3, a fungal strain with a silent SM profile and a proven track record in expressing heterologous SM genes (14, 19).
alone did not produce eight compounds assigned to atrochrysone in standard product. Pure cladofulvin was used as a standard. (ES 7 min; UV maximum |A. nidulans 5.8 min; UV maximum |A. oryzae 5.6 min; UV maximum |A. fulvum genome, the gene is coregulated with the other genes in the genome, the deletion mutants. Pure cladofulvin no. 25 [RT 225, 221, 288, and 440; m/z (electrospray; ES) 273 [M–H–]− was assigned to endocrocin anthrone and emodin anthrone, respectively, by comparing their UV and MS data with those published (18). Its exact mass was confirmed with high-resolution MS analysis (m/z (ES+) 275.0919 [M+H]+) (SI Appendix, Fig. S7). Product 3 [RT = 5.5 min; UV maximum = 224, 287, and 442 nm; m/z (ES+) 313 [M–H–]−] was assigned to endocrocin by comparing its UV and MS data with those published (7) (SI Appendix, Figs. S3–S6). Product 4 [RT = 8 min; UV maximum = 226 and 351 nm; m/z (ES+) 255 [M–H–]−] and 5 [RT = 8.1 min; UV maximum = 224, 288, and 440 nm; m/z (ES+) 269 [M–H–]−] are likely emodin anthrone and emodin, respectively, by comparing their UV and MS data with those published (7, 14) (SI Appendix, Figs. S3–S6). Previously, coexpression of ACAS and ACTE enzymes in A. oryzae yielded diastereomers of homodimers and heterodimers of endocrocin anthrone and emodin anthrone in addition to products 2–4 (14). Based on UV and MS data from this study, the minor products 6 [RT = 5.6 min; UV maximum = 224 and 361 nm; m/z (ES+) 597 [M–H–]−], 7 [RT = 5.8 min; UV maximum = 224 and 359; m/z (ES+) 597 [M–H–]−], and 8 [RT = 7 min; UV maximum = 255 and 361 nm; m/z (ES+) 553 [M–H–]−] likely correspond to such dimers, but they were not further investigated. An evaporative light scattering detector chromatogram showed that ClaG and ClaF produced atrochrynse 2 as the major product in A. oryzae (SI Appendix, Fig. S8).

(4) ClaH Is a Decarboxylase That Yields Emodin. In A. nidulans, biosynthesis of monocytidinephenone involves atrochrynse 2 and emodin 5 as intermediate compounds (7). It was hypothesized that mdpH is required for the biosynthesis of these intermediates, because endocrocin alone accumulated in ΔmdpH deletion mutants (7). In contrast, deletion of any other tailoring gene resulted in the production of emodin and other emodin-related shunt products, which lack a carboxyl group (7). In the C. fulvum genome, the claH gene is coregulated with the other cla genes (Fig. 1) and encodes a predicted protein bearing 64% identity to the N-terminal region of MdpH (SI Appendix, Fig. S9 and Table S1). To address the role of ClaH in cladofulvin biosynthesis, we coexpressed claH with claF and claG in A. oryzae. The resulting transformants mainly produced product 5, which was confirmed to be emodin from its comparison with the RT, UV, and MS data of a commercial emodin standard (Fig. 3 C and D and SI Appendix, Figs. S3–S6). This result suggests that claH and mdpH are functional decarboxylase homologs and that emodin is the second stable intermediate in cladofulvin biosynthesis.
MdpH shares homology with HypC and EncC, which are involved in the production of aflatoxin and endocrocin, respectively (10, 22). HypC is an aniline oxidase that converts norsorionic acid anthrone to norsorionic acid (23). EncC was also hypothesized to catalyze the oxidation of endocrocin anthrone to endocrocin (22). The three enzymes contain the conserved domain DUF1772, but MdpH also contains the additional conserved domain EthD (SI Appendix, Fig. S9). ClaH shares this EthD domain only and does not share any similarity with HypC or EncC (SI Appendix, Fig. S9). The results of the work by Chiang et al. (7) and our study suggest that MdpH and ClaH are decarboxylases. In contrast, HypC and EncC clearly do not have such activity as shown by the oxidation of norsorionic acid anthrone (23) and the absence of emodin in Aspergillus fumigatus (22), respectively. Altogether, it suggests that the EthD domain is responsible for the observed decarboxylase activity. A gene encoding a protein that carries an EthD domain is present at the ACAS-ACTE locus and should be tested for a similar decarboxylation activity (SI Appendix, Table S1).

(5) ClaC and ClaB Are Responsible for the Conversion of Emodin to Chrysophanol. Simpson (8) proposed that, during monodictyphenone biosynthesis, emodin 5 is converted to chrysophanol 9 by reduction and dehydration and catalyzed successively by the trihydroxyanthraquinone reductase MdpC and sctalone dehydratase MdpB. It was subsequently shown that MdpC is, indeed, capable of reducing emodin hydroquinone to 3-hydroxy-3,4-dihydroanthracen-1(2H)-one (24). To test the involvement of claC and claB in converting emodin 5 to chrysophanol 9, both genes were coexpressed in A. oryzae. Induced cultures of these transformants, in addition to untransformed A. oryzae, were supplemented with emodin. Liquid chromatography (LC)–MS examination of organic extracts from the untransformed A. oryzae contained emodin 5 only (Fig. 5D). Extracts from transformants coexpressing claB and claC contained not only emodin 5 but also, traces of product 10 (ES+ 269 [M+H]+), which was assigned to chrysophanol by comparing its RT, UV, and MS spectra with those of a commercial standard (Fig. 3E and F and SI Appendix, Figs. S3–S6). These results suggest that ClaC and ClaB convert emodin 5 to chrysophanol 9, presumably through sequential reduction and dehydration.

(6) Cytochrome P450 ClaM Is Responsible for Dimerization of Nataloe-Emodin. Targeted deletion of claM was performed in C. fulvum to determine the role of this cytochrome P450 in cladofulvin 1 biosynthesis. Two confirmed independent deletion mutants and an ectopic transformant were selected for additional analysis (SI Appendix, Fig. S1). The ΔclaM deletion mutants were more gray-brown in color compared with WT and ectopic controls (Fig. 4A). A dark brown compound accumulated in the cytoplasm of fungal cells and diffused into the agar (Fig. 4A and B). LC-MS analysis of ethyl acetate extracts confirmed that cladofulvin 1 was present in the controls but not in the ΔclaM deletion mutants (Fig. 4C). The ΔclaM deletion mutants produced several compounds, and the major species were isolated by mass-directed HPLC and examined by NMR. The first eluted compound 10 [RT = 7.3 min; UV maximum = 231, 259, and 430 min; m/z (ES+) 269 [M+H]+] was purified, and comparison of its 1H NMR data with a literature standard proved it to be nataloe-emodin (SI Appendix, Figs. S3–S5 and S10 and Table S3), an SM previously unknown in fungi. The next eluted product was emodin 5, also identified by 1H NMR (SI Appendix, Figs. S3–S5 and S10 and Table S2). Exact masses of both compounds were also confirmed with high-resolution MS analysis [product 5: m/z (ES−) 269.0449 [M−H]−; product 10: m/z (ES−) 269.0448 [M−H]−] (SI Appendix, Fig. S7). Later eluted products were not further investigated, because they were present in traces and rapidly degraded on purification, which prevented structural elucidation. These results confirm that nataloe-emodin 10 is, indeed, the immediate precursor to cladofulvin and most importantly, that its dimerization involves the cytochrome P450 ClaM.

(7) Cytotoxicity of Cladofulvin and Its Precursors. Nataloe-emodin 10 potently inhibits the growth of immortalized mammalian cell lines (25), but no biological activities have been reported for cladofulvin 1 so far (15). To test if it exhibits a similar cytotoxic activity, cladofulvin 1 was applied to diverse nataloe-emodin–sensitive mammalian cell lines (25), including nontumorigenic 3T3 mouse embryo cells and diverse human tumor cell lines (Table 1). In addition, emodin 5 and chrysophanol 9 were also tested on the same cell lines. Emodin 5 inhibited the growth of cell lines with significantly lower potency than nataloe-emodin 10 [multiple t tests corrected for multiple comparisons with the Holm–Sidak method; α = 0.025; P values ranging from 7.5e-6 (H460) to 0.012 (3T3)], except for M-14 and K562 cell lines, against which emodin 5 showed higher (P value = 0.005) and equal

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**Table 1. Toxicity of cladofulvin and its precursors against animal cell lines**

| Compound          | 3T3          | H460         | HuTu80       | DU145        | MCF-7        | M-14        | HT-29       | K562        |
|-------------------|--------------|--------------|--------------|--------------|--------------|-------------|-------------|-------------|
| Emodin            | 15.329 (0.193) | 9.928 (0.182) | 7.831 (0.308) | 13.973 (0.831) | 8.178 (0.138) | 11.802 (0.072) | 17.007 (0.293) | 8.094 (0.197) |
| Chrysophanol*     | >200         | >200         | 46.972 (1.153)| >200         | >200         | >200        | >200        | >200        |
| Nataloe-emodin†   | 10.345 (1.975) | 4.800 (0.236) | na           | 7.928 (0.608) | 7.324 (0.087) | 13.640 (0.557) | 6.545 (1.194) | 12.499 (3.887) |
| Cladofulvin       | 0.060 (0.002) | 0.037 (0.001) | 0.097 (0.005) | 0.055 (0.002) | 0.036 (0.001) | 0.097 (0.003) | 0.275 (0.015) | 0.039 (0.001) |

G50 (50% growth inhibition) (micrograms per milliliter−1) of each compound against different cell lines was determined. Numbers in parentheses indicate the SD of at least three technical replicates.

*The maximum concentration tested was 200 μg mL−1.

†Data published in ref. 32.
The formation of endocrocin 3 and emodin 5 might occur spontaneously. All oxidation ([O]) steps could be spontaneous in air. Conversion of chrysophanol 9 to nataloe-emodin 10 is the only step without any support and might involve ClaN and ClaK. Compounds in between brackets were not identified in this study. ACP, acyl carrier protein; AT, acyl transferase; KS, keto synthase; PT, product template; SAT, starter unit acyl carrier protein transacylase.

(P value = 0.121) inhibition, respectively (Table 1). In contrast, chrysophanol 9 weakly inhibited the growth of HuTu-80 and K562 cells only [GI₅₀ (50% growth inhibition) = 47 and 132 μg mL⁻¹, respectively; P values = 5.7e-7 and 0.001 compared with emodin, respectively] (Table 1). These results suggest that the C3 or C2 hydroxyl group in emodin 5 and nataloe-emodin 10, respectively, is essential for the cytotoxic activity of these compounds, but the C2 position seems to provide significantly higher potency. Strikingly, compared with nataloe-emodin 10, cladofulvin 1 was significantly more active toward each target cell line, ranging from a 24-fold increase for HT-29 to a 320-fold increase for K562 [P values ranging from 1.2e-10 (MCF-7) to 0.001 (K562)] (Table 1). These results show that dimerization of nataloe-emodin dramatically enhanced its cytotoxicity.

Discussion

(1) Atrochrysone Carboxylic Acid Is Likely the Raw Polyketide Produced by ClaF and ClaG. Based on the metabolic profiles of C. fulvum deletion mutants and A. oryzae transformants expressing putative cladofulvin 1 biosynthetic genes, we propose a biosynthetic route to cladofulvin 1 production (Fig. 5). Consistent with the SM profile of A. oryzae expressing ACAS and ACTE (14), the coexpression of claF and claG in the same host yielded the same compounds, apart from endocrocin anthrone. Spontaneous conversion of endocrocin 3 to emodin 5 is considered to be thermodynamically unfavorable (7, 14). Thus, to account for the co-detection of endocrocin 3, emodin 5, and atrochrysone 2, the highly unstable compound atrochrysone carboxylic acid was proposed as the raw polyketide released from ACAS by ACTE through a hydrolysis mechanism (14). We propose the same mechanism in the cladofulvin 1 biosynthetic pathway with atrochrysone carboxylic acid as the first unstable intermediate, which preferentially degrades to form atrochrysone 2 and endocrocin 3 (Fig. 5). Atrochrysone carboxylic acid is likely the first intermediate produced by any other orthologous pairs, including MdpG/MdpF (7) and EncA/EncB (22).

The sole presence of emodin 5 in transformants coexpressing claH, claG, and claF suggests that ClaH significantly accelerates decarboxylation of atrochrysone carboxylic acid, consistent with the absence of endocrocin 3. When transformants expressing claG and claF only were grown for 2 additional days, endocrocin 3 became the major product produced instead of atrochrysone 2 (SI Appendix, Fig. S8). This result is consistent with the decarboxylase activity of ClaH and suggests that spontaneous decarboxylation of atrochrysone carboxylic acid is slower than its spontaneous dehydration. The trace amounts of emodin anthrone 4 and emodin 5 detected in A. oryzae transformants expressing claF and claG are consistent with successive spontaneous dehydration and oxidation of atrochrysone 2 and emodin anthrone 4, respectively. The latter step yielding emodin 5 was, indeed, shown to occur spontaneously and may bypass the activity of anthrone oxidases (7, 14, 23).

(2) Conversion of Emodin into Nataelo-Emodin Likely Involves Chrysophanol Hydroquinone as an Intermediate. Heterologous expression of claC and claB in A. oryzae yielded traces of chrysophanol 9 after the respective cultures were supplemented with emodin 5. The very limited yield of this reaction might be explained by the substrate provided to ClaC and ClaB. Indeed, it was shown that the substrate of MdpC is emodin hydroquinone, which is expected to be the prevalent form in vivo (24). Thus, we propose that ClaC may preferentially use emodin hydroquinone as a substrate, which implies that the product of ClaB might be chrysophanol hydroquinone (Fig. 5). We hypothesized that ClaK and ClaN might be involved in the next step toward the formation of nataelo-emodin 10. However, coexpression of claK and claN in A. oryzae did not yield any new compound when cultures of the respective transformants were supplemented with chrysophanol 9. This result suggests that ClaK/ClaN might oxidize the intermediate chrysophanol hydroquinone rather than chrysophanol 9. Such a route between emodin 5 and nataelo- emodin 10 is in agreement with findings by Schätzle et al. (24) and the absence of chrysophanol 9 in ΔclaM deletion mutants. Additional investigations are needed to ascertain this part of the proposed route to cladofulvin 1.

(3) Dimerization of Nataelo-Emodin by the Cytochrome P450 ClaM. Despite the large number of known dimeric anthranoids and xanthonoids occurring in nature (5, 13), only one dimerizing enzyme from Streptomyces coelicolor A3(2), ActVA-ORF4, has been reported (26). This enzyme, which shares similarities with dihydroflavonol-4-reductases of plants (27), catalyzes the dimerization of hydroxylated dihydrokalafungin to produce the benzoisochromanoneactinorhodin (26). A number of dimeric anthrones have been isolated from fungi (7, 14, 28), with strong evidence that their formation is also enzymatically catalyzed (14). However, no fungal enzyme involved in catalyzing their formation has yet been reported. Our results show for the first time, to our knowledge, that a cytochrome P450 is involved in anthraquinone dimerization. ClaM is required for the production of the bianthraquinone 9.
cladofulvin I through the C5–C7 linkage of nataleoin-10 (Fig. 5). However, cladofulvin I was not produced when A. oryzae transformants expressing claM were supplemented with nataleoin-10. Therefore, it cannot be excluded that the reduced form of nataleoin-10 is the actual substrate for dimerization by ClaM.

The involvement of cytochrome P450s in the dimerization of naphthoquinones, coumarins, and diketopiperazine alkaloid was reported in bacteria, Aspergillus niger, and Aspergillus flavus, respectively (29–31). In S. coelicolor A3(2), flavinoid is dimerized by a cytochrome P450 (30), and a similar enzyme in Streptomyces griseus dimerizes tetrahydroxynaphthalene into 1,4,6,7,9,12-hexahydroxyperylene-3,10-quinone (32). In A. niger, the cytochrome P450 KtnC dimerizes dimethyl siderin into kotanin (29). The cytochrome P450 DtpC in A. flavus is responsible for the concomitant pyrroloindole ring formation and homo- or heterodimerization of the diketopiperazine alkaloids ditytrtophenaline, dibrevianamide F, and tryptophenaline (31). Nataleoin-10 dimers with alternative aryl–aryl bonds have not been observed in C. fulvum, and they do not have dimeric forms of emodin 5, which suggests that ClaM might be highly selective for both substrate and type of linkage that it catalyzes. In contrast, Alternaria species produce a large variety of altorperriols, which are heterodimers of macrpsorin and altersolanol exhibiting various couplings, including C5–C5′, C1–C7, C7–C5′, and C2–C2′ bonds (28, 33). Homodimers of macrpsorin or altersolanol were also isolated and show C4–C4′ and C4–C8′ bonds (28). The diversity of dimers for a given anthraquinone likely depends on both substrates and specificity of enzymes involved in dimerization. The former will determine the positions at which radicals or cations can react, while the latter may be caused by multiple dimerizing enzymes or fewer but less selective enzymes that catalyze multiple reactions.

The production of so many different alterperriols by the former will determine the positions at which radicals or cations can react, while the latter may be caused by multiple dimerizing enzymes or fewer but less selective enzymes that catalyze multiple reactions. Dimerization and the type of bond that links monomers profoundly affect the biological activities of SMs. As we showed for nataleoin-10 and cladofulvin I, dimerization can strengthen an existing activity. Additional investigation is needed to determine the exact dimerization mechanism catalyzed by ClaM and assess the substrate specificity of this enzyme. The identification of ClaM undoubtedly help accelerate the in silico discovery of functionally similar enzymes in other fungal species, although only a limited number of close homologs were found by BLAST analysis using current publically available databases (SI Appendix, Fig. S11). A future challenge will be to assess the potential utility of ClaM and related cytochrome P450s as biocatalysts for the conversion of natural monomers into novel dimeric compounds with enhanced or novel biological activities.

Materials and Methods

Heterologous expression in A. oryzae and construction of C. fulvum deletion mutants were conducted as previously described (14, 16, 17, 19). Strains were grown in standard media, and SMs were extracted using ethyl acetate. Extracts were analyzed by UV-HPLC and LC-MS. Standard chromatographic methods were used, and peaks were detected either between 200 and 800 nm or between 200 and 400 nm. Analyses were performed simultaneously in ES+ and ES− modes between 100 and 650 m/z. NMR analyses were performed using a mass-directed chromatography system (15). Mammalian cell cytotoxicity bio-assays were performed as previously described (25). Full experimental procedures are described in SI Appendix.

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