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Genome mining for fungal polyketide-diterpenoid hybrids: discovery of key terpene cyclases and multifunctional P450s for structural diversification†

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A biosynthetic gene cluster for chevalone E (**1**) and its oxidized derivatives have been identified within the genome of the endophytic fungus *Aspergillus versicolor* 0312, by a mining strategy targeting a polyketide-diterpenoid hybrid molecule. The biosynthetic pathway has been successfully reconstituted in the heterologous fungus *Aspergillus oryzae*. Interestingly, two P450 monooxygenases, Cle2 and Cle4, were found to transform **1** into seven new analogues including **7** and **8** that possess a unique five-membered lactone ring. Furthermore, the replacement of the terpene cyclase gene with that from another fungus led to the production of sartorypyrone D (**11**), which has a monocyclic terpenoid moiety. Finally, some of the compounds obtained in this study synergistically enhanced the cytotoxicity of doxorubicin (DOX) in breast cancer cells.

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Introduction

Natural products are one of the essential resources for drug discovery. Not only do they directly provide many bioactive small molecules, but they also provide inspiration for synthetic drug development. Meroterpenoids are naturally occurring organic compounds that in part originate from the terpenoid pathway, among which those found in filamentous fungi exhibit significant diversity in their biological activities and molecular architectures.[1,2] The fungal meroterpenoids have thus received broad attention from natural product researchers, and the biosynthesis and total syntheses of fungal meroterpenoids have been intensively studied in recent years.[2,3] The majority of the fungal meroterpenoids have a polyketide motif as a non-terpenoid moiety,[1] but biosynthetic studies on these hybrid natural products at genetic/enzymatic levels have mainly focused on those harboring a relatively small terpenoid moiety (C_{15} or less), and only a few biosynthetic gene clusters involved in the biosynthesis of fungal polyketide-diterpenoids (PK-DTs) have been identified to date.[4,5] Therefore, mining and utilization of unexploited fungal biosynthetic gene clusters (BCGs) for PK-DTs should accelerate the discovery of new molecules and provide fascinating insight into the biosynthetic logic governing the occurrence of fungal meroterpenoids.

As an experimental strategy, heterologous expression of biosynthetic genes offers an efficient way to enable genome mining, especially when the target genes either are silent in the host organism or are difficult to be activated. The fungus *Aspergillus oryzae* is a particularly robust platform developed for heterologous expression,[6–8] since the metabolite(s) derived from the expression of the introduced genes can generally be obtained in a good yield and are easily purifiable due to a low background. Thus, the *A. oryzae* system has been utilized for successful genome mining of several fungal biosynthetic genes, and this led to the discovery of several natural products with new scaffolds.[9–12] However, only pathways involving a few biosynthetic genes have been so far reconstituted in *A. oryzae* for the sake of genome mining, although it is known that >10 genes can be readily expressed in the fungus.[8,13] Thus, it

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would be interesting to see whether *A. oryzae* can serve as a useful tool to investigate the function of a biosynthetic gene cluster comprising a relatively large number of genes found by genome mining.

In this report, we describe the discovery and characterization of the gene cluster responsible for the biosynthesis of the fungal PK-DT chevalone E (**1**)[14] and its oxidized derivatives (**2–8**) (Fig. 1A), and demonstrate the successful reconstitution of the biosynthetic pathway in *A. oryzae*. In addition, we discovered another gene cluster similar to that for chevalones, and the terpene cyclase encoded by this cluster interestingly accepts the same substrate as that for chevalone E synthesis but produces a differently cyclized product sartorypyrone D (**11**) (Fig. 1A). Finally, we report that compounds **1**, **2**, and **5–8** and doxorubicin (DOX) have a synergistic effect on breast cancer cell growth inhibition.

Results and discussion

To find a BGC suitable for our genome mining study, we initially searched for a genomic region that encodes both a polyketide synthase (PKS) and a geranylgeranyl pyrophosphate (GGPP) synthase (GGPS) in the several fungal genome sequences available to us, since it is well known that BGCs for fungal meroditerpenoids, including indole-diterpenoids, encode a GGPS to provide the substrate for a prenyltransferase.[2,15] Consequently, we focused on a gene cluster of seven genes, derived from the endophytic fungus *Aspergillus versicolor* 0312 isolated from *Paris polyphylla* var. *yunnanensis*. The gene cluster, designated as the *cle* cluster, encodes a non-reducing PKS (Cle1), a prenyltransferase (Cle5), a GGPS (Cle6), and a terpene cyclase (Cle7) (Fig. 1B and Table S1;† DDBJ/EMBL/GenBank accession number: LC422695), and therefore implies that the *cle* cluster is involved in the biosynthesis of a PK-DT meroterpenoid. Importantly, the *cle* cluster is significantly different from the known clusters for fungal PK-DTs,[4,5] and therefore, it was expected that the gene cluster would be responsible for the biosynthesis of a new natural product.

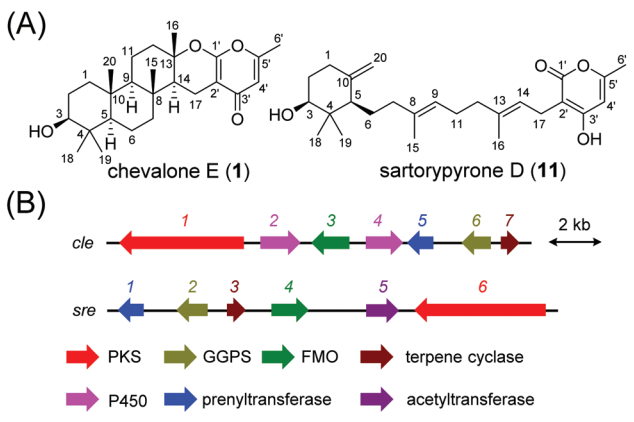

Fig. 1 (A) The structures of chevalone E (**1**) and sartorypyrone D (**11**). (B) Schematic representations of the *cle* and *sre* clusters. PKS: polyketide synthase; GGPS: geranylgeranyl pyrophosphate synthase; FMO: FAD-dependent monooxygenase.

On the basis of the predicted functions of each proteins encoded by the *cle* cluster, the biosynthetic pathway for the metabolite synthesized by the cluster can be proposed by analogy to the known pathways for the biosynthesis of other fungal meroterpenoids.[2] Initially, the PKS Cle1 yields a polyketide product, which is then geranylgeranylated by the prenyltransferase Cle5 utilizing GGPP synthesized by the GGPS Cle6. The next step would involve the epoxidation of one of the double bonds in the geranylgeranyl moiety, catalyzed by the FAD-dependent monooxygenase (FMO) Cle3. Subsequently, the terpene cyclase Cle7 would catalyze the cyclization of the prenyl group initiated by protonation and ring-opening of the epoxide. The two cytochrome P450 monooxygenases (Cle2 and Cle4) would decorate the backbone structure generated by the above-mentioned five enzymes to provide the pathway end product.

To analyze the function of each gene in the *cle* cluster and to obtain the metabolite produced by the cluster, the *cle* genes were heterologously expressed in the *A. oryzae* NSAR1 strain[16] according to the proposed pathway. Initially, the PKS gene *cle1* was solely expressed in *A. oryzae*, but unfortunately, no new product was detected as compared with the negative control strain without any *cle* genes (Fig. 2A, lanes i and ii). Nevertheless, further introduction of the prenyltransferase and GGPS genes, *cle5* and *cle6*, resulted in the production of a hydrophobic molecule **9** (Fig. 2A, lane iii), whose molecular formula was determined to be $C_{26}H_{38}O_3$ by the HR-MS analysis. Following large-scale cultivation, **9** was isolated in sufficient amounts for structural characterization, with NMR analysis revealing that **9** is a geranylgeranylated form of triacetic acid lactone (TAL) at the C-3 position (Fig. 2B). Although it is unclear why the single expression of the PKS gene did not generate TAL, the observation strongly suggests that the PKS Cle1 is a TAL synthase, which is consistent with the domain organization of Cle1 (SAT-KS-AT-PT-ACP-ACP). Coexpression of the FMO gene *cle3* in the transformant producing **9** then generated a new compound **10** (Fig. 2A, lane iv), which is much more hydrophilic than **9**. Compound **10** possesses a similar structure to that of **9**, but it has two additional hydroxyl groups at the terminus of the geranylgeranyl chain (Fig. 2B). The diol moiety should be the consequence of the hydrolysis of the epoxide ring synthesized by the FMO Cle3. Finally, the five-gene expression system including the terpene cyclase gene *cle7* afforded the cyclized product **1** (Fig. 2A, lane v), which was identified as the known natural product chevalone E (Fig. 2B).[14]

Given the production of chevalone E (**1**) by the five enzymes for the backbone structure synthesis, the two P450s Cle2 and Cle4 would decorate the chevalone E scaffold into further oxidized metabolites. To investigate the order of involvement of the P450s, *cle2* and *cle4* were individually introduced into the transformant that synthesizes **1**, and both transformants interestingly produced new compounds. The transformant with *cle2* yielded two new metabolites **2** and **3** (Fig. 2A, lane vi),

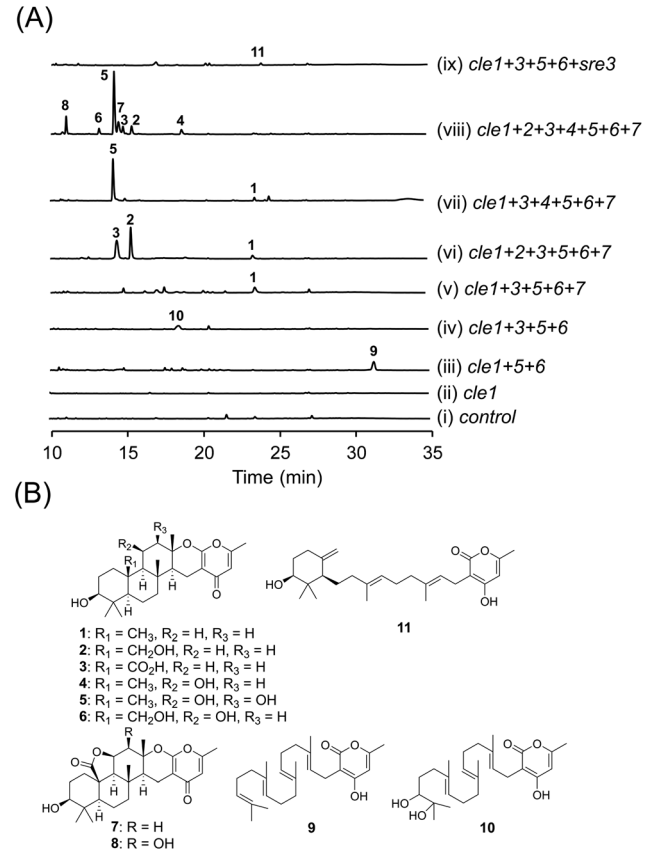

Fig. 2 (A) HPLC profiles of culture supernatant extracts from *A. oryzae* NSAR1 transformants. Transformants (i) with only empty vectors; (ii) with *cle1*; (iii) with *cle1 + 5 + 6*; (iv) with *cle1 + 3 + 5 + 6*; (v) with *cle1 + 3 + 5 + 6 + 7*; (vi) with *cle1 + 2 + 3 + 5 + 6 + 7*; (vii) with *cle1 + 3 + 4 + 5 + 6 + 7*; (viii) with *cle1 + 2 + 3 + 4 + 5 + 6 + 7*; and (ix) with *cle1 + 3 + 5 + 6 + 7 + sre3*. The chromatograms were monitored at 230 nm. (B) Structures of the metabolites isolated in this study.

both of which are oxidized at the C-20 methyl group; compounds **2** and **3** have hydroxyl and carboxyl groups at this position, respectively (Fig. 2B). Thus, Cle2 could accept **1** as a substrate to perform three consecutive oxidations at C-20 to transform the methyl group into carboxylate. On the other hand, the separate expression of the other P450 gene *cle4* generated a single new product **5** (Fig. 2A, lane vii) with two β-hydroxyl groups at C-11 and C-12 (Fig. 2B), suggesting that Cle4 can oxidize the two adjacent carbon atoms. Finally, coexpression of *cle2* and *cle4* with the other five genes provided a much more complicated metabolite profile with four additional new molecules (**4** and **6–8**). Among these compounds, **4** was found to be a less oxidized analogue of **5**, containing only the C-11 alcohol, while **6** is an isomer of **5** and harbors two hydroxyl groups at C-11 and C-20 (Fig. 2B). Interestingly, **7** has a unique five-membered lactone ring, and **8** is an oxidized analogue of **7** with one additional hydroxyl group at C-12 and seems to be the end product of the pathway (Fig. 2B).

In order to more deeply explore the functions of the two P450s Cle2 and Cle4, we then performed a substrate-feeding experiment to verify the metabolites resulting from the actions of the P450s as true pathway intermediates. To this end, **4**, **5**, and **6** were fed to the transformant only lacking *cle4*, while **2**, **3**, **6**, and **7** were incubated with the strain without *cle2* (Fig. S2†). The result revealed that **4**, **5**, and **6** are all further oxidized into downstream metabolites by Cle2 to eventually yield the products with the five-membered lactone, **7** or **8** (Fig. 3A), indicating that Cle2 has a broad substrate specificity and oxidizes the C-20 position of all the tested compounds into carboxylate. On the other hand, the substrate specificity of Cle4 appears to be stricter than that of Cle2 as evidenced by the observation that **3** and **6** remained unchanged in the presence of Cle4 (Fig. 3B, lanes iii and vi). Meanwhile, **2** and **7** underwent a single round of oxidation by Cle4 to afford **6** and **8**, respectively (Fig. 3B, lanes ii and v).

On the basis of the structures obtained from the heterologous reconstitution and substrate feeding experiments, plausible pathways leading to the predicted end product **8** can be proposed as follows (Fig. 4). First, the non-reducing PKS Cle1 synthesizes the α-pyrone TAL from one molecule of acetyl-CoA and two molecules of malonyl-CoA. The membrane-bound

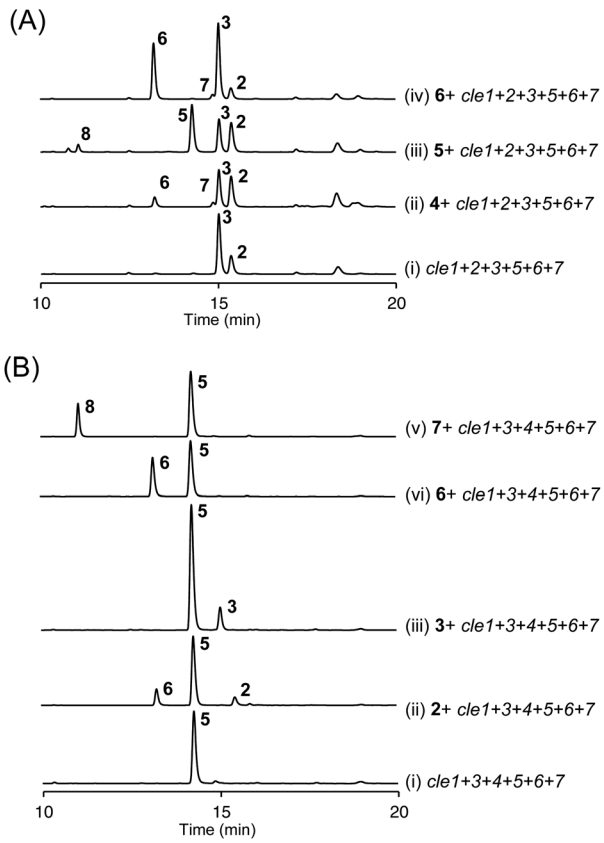

Fig. 3 Functional analyses of Cle2 and Cle4. (A) HPLC profiles of culture supernatant extracts from the *A. oryzae* transformant harboring *cle1 + 2 + 3 + 5 + 6 + 7* incubated with (i) only culture media; (ii) **4**; (iii) **5**; and (iv) **6**. (B) HPLC profiles of culture supernatant extracts from the *A. oryzae* transformant harboring *cle1 + 3 + 4 + 5 + 6 + 7* incubated with (i) only culture media; (ii) **2**; (iii) **3**; (iv) **6**; and (v) **7**. The chromatograms were monitored at 254 nm.

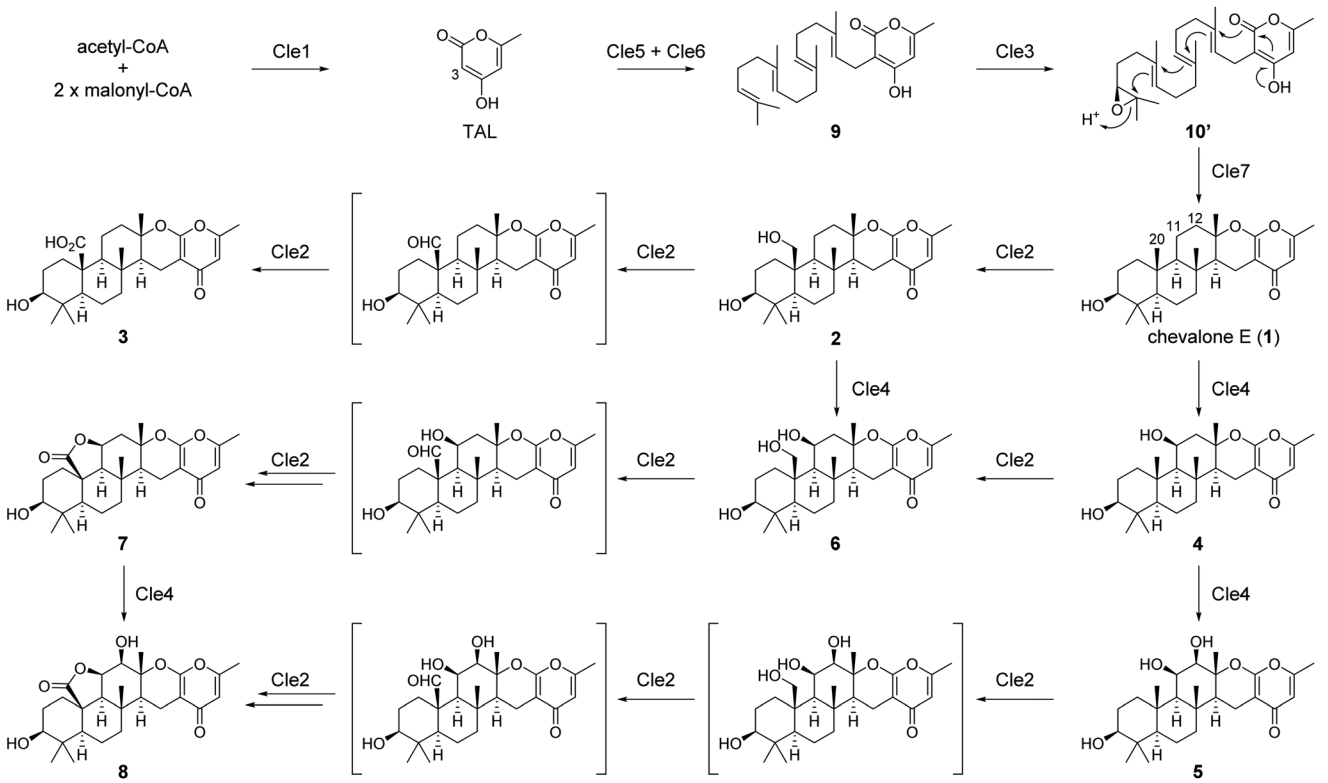

Fig. 4 Proposed biosynthetic pathway leading to **8**.

prenyltransferase Cle5 then accepts TAL as its substrate to perform a C-3 geranylgeranylation reaction to afford **9**, in which the pathway-dedicated GGPS Cle6 is required to provide GGPP, the other substrate of Cle5. Subsequently, the FMO Cle3 forms an (*S*)-epoxide ring at the terminal olefin of the geranylgeranyl group to provide **10′**, which is then protonated by the terpene cyclase Cle7 to undergo the cyclization reaction that yields the pentacyclic pathway intermediate chevalone E (**1**). Importantly, the α-pyrone seen in **9** is transformed into γ-pyrone after the cyclization event, which could be attributed to the occurrence of the tautomerization of the TAL-derived moiety during the terpene cyclization. After the generation of the cyclized product **1**, **1** could follow several possible pathways to be converted into oxidized derivatives. In the absence of the action of one of the P450s Cle4, **1** is subjected to oxidation at C-20 by Cle2 to give the alcohol **2** and the carboxylate **3**. On the other hand, if Cle2 is missing from the pathway, **1** is initially oxidized at C-11 by Cle4 to yield **4**, which is further oxidized at C-12 by the same enzyme to afford **5**.

In the coexistence of Cle2 and Cle4, the end product **8** could be achieved by several routes. In this process, the characteristic lactone ring seems to be spontaneously formed after the placement of the carboxyl group at C-20 and the β-hydroxyl group at C-11, or alternatively, it could also be generated by the oxidation of lactol that would occur after alcohol dehydrogenation to aldehyde. Collectively, all of the chevalone E derivatives isolated in this study except for **3** serve as the pathway intermediate for **8**. It should be noted that the precyclized intermediate **9** appears to be widely involved in the biosynthesis of many fungal meroterpenoids. For example, the fungus *Eurotium chevalieri* produces chevalone A, a close analogue of **1** with the α-pyrone, as well as the acetylated derivative of **1** with the γ-pyrone moiety.[17] The fact that no such cyclized metabolite carrying the α-pyrone was obtained in our study suggests that the terpene cyclase Cle7 has a stricter product selectivity. Furthermore, partially cyclized analogues of **1** with mono- or tricyclic terpenoid moieties, such as aszonapyrones[17,18] and sartorypyrones,[19–21] have been discovered in other fungi. These observations indicate that there are several different terpene cyclases that can accept the same substrate **10′** but then produce differently cyclized products, as seen in the biosynthesis of fungal meroterpenoids derived from 3,5-dimethylorsellinic acid.[22,23] To investigate whether our *A. oryzae* expression system can be readily utilized to obtain analogous natural products of chevalones with a different terpenoid moiety, we searched for a gene cluster that is similar to the *cle* cluster but is involved in the biosynthesis of a different natural product. As a result, a gene cluster found in *Aspergillus felis* 0260 was selected and hereby named the *sre* cluster (Fig. 1B and Table S1;† DDBJ/EMBL/GenBank accession number: LC428283). The terpene cyclase gene in the *sre* cluster, *sre3*, was coexpressed with *cle1*, *cle5*, *cle6*, and *cle3*, which yielded **11**, an isomer of chevalone E (**1**) (Fig. 2A, lane ix). The cyclized product **11** was identified to be sartorypyrone D,[21] demonstrat-

ing that Sre3 (38% protein sequence identity with Cle7) also accepts **10′** as Cle7 but performs a distinct cyclization reaction. Given that the *sre* cluster encodes the acetyltransferase Sre5, the end product of the *sre* pathway would be sartorypyrone A, which is the acetylated form of **11** (Fig. 5).

It is reported that chevalone analogues inhibit the growth of a variety of malignant solid cancer cells with IC_{50} values (half maximal inhibitory concentrations) from 2.9 to 100 μM.[17,24] Therefore, we evaluated the cytotoxicity of selected compounds (**1**, **2**, and **5–8**) against two breast cancer lines (MDA-MB-231 and MCF-7) but found that all of the tested compounds did not show inhibitory activity even at a concentration of 40 μM. Although DOX (1.25 μM) treatment alone for 48 h caused an inhibition of about 50% decrease of cell viability in MDA-MB-231 (Fig. 6) and MCF-7 cells (Fig. S2†), co-treatment of DOX with the indicated compounds resulted in an enhanced effect on the reduction of cell viability in both MDA-MB-231 and MCF-7 cells. To characterize the interactions between the DOX and the indicated compounds, the drug combination index (CI) was further calculated using the CompuSyn software.[25] We observed that all of the tested compounds synergistically enhanced the cytotoxicity of DOX in both MDA-MB-231 (Fig. 6G) and MCF-7 cells (Fig. S2G†), as all of the CI values calculated were less than 1.

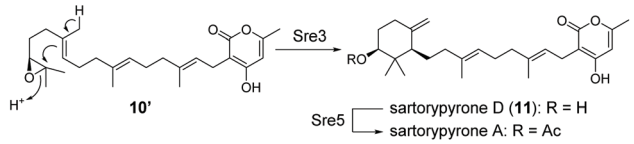

Fig. 5 Proposed biosynthetic pathway leading to sartorypyrone A.

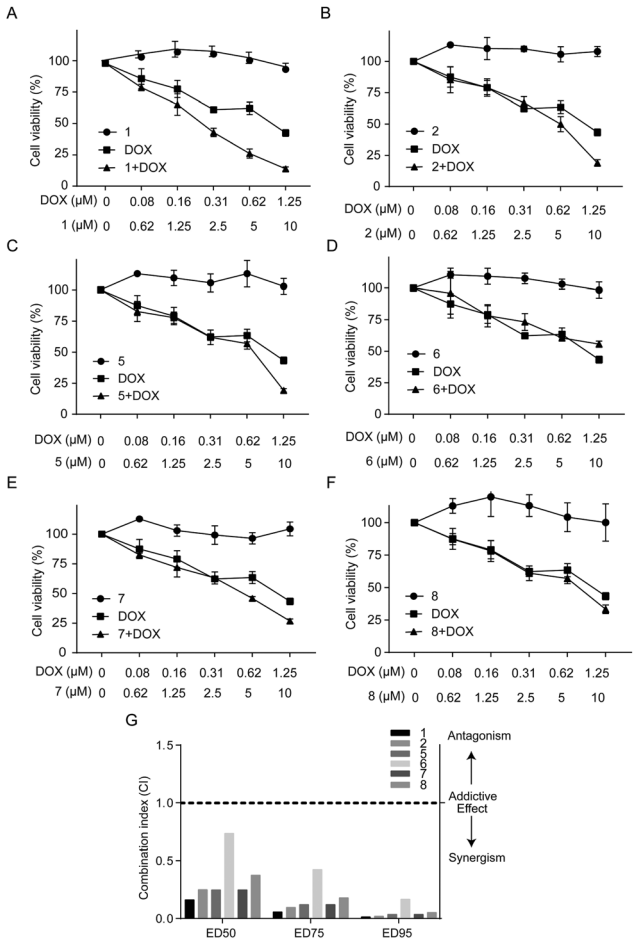

Fig. 6 Compounds **1**, **2**, and **5–8** synergistically enhance the cytotoxicity of DOX in MDA-MB-231 breast cancer cells. (A–F) MDA-MB-231 cells were treated with DOX, compounds **1**, **2**, **5–8**, or the combination of both agents at a 1 : 8 M ratio. After 48 h, cell viability was determined using the colorimetric MTT assay. (G) Combination index values were calculated using CompuSyn software to determine drug interaction. CI < 1 indicates synergism, CI = 1 indicates additive effects, and CI > 1 indicates antagonism. The data are presented as mean ± standard deviation of three independent experiments.

Conclusions

In summary, we have discovered a gene cluster responsible for the biosynthesis of novel chevalone E analogues, by a genome mining approach that targeted a PK-DT hybrid, and we unambiguously characterized the functions of each enzyme encoded by the *cle* cluster. Our result provides the first example in which the complete biosynthesis of a fungal PK-DT hybrid has been successfully reconstituted in a heterologous host and indicates the usefulness of the *A. oryzae* system for obtaining new natural products. Furthermore, we have obtained another PK-DT, sartorypyrone D (**11**), which is synthesized by a cyclization mode distinct from that for **1**. Since geranylgeranyl-TAL-derived meroterpenoids are widespread in fungi, our finding further facilitates future biosynthetic studies on compounds that are structurally related to chevalone E (**1**) and sartorypyrone A. Importantly, we have found two P450s, Cle2 and Cle4, which can derivatize **1** into seven new analogues (**2–8**). Interestingly, two of the new analogues, **7** and **8**, possess a characteristic five-membered lactone ring, which has never been found in the structures of fungal meroterpenoids and are only found in a few natural products.[26,27] We have thus demonstrated that the genome mining study focusing on fungal PK-DTs is a promising way to discover new bioactive natural products. Future studies will involve the identification of gene clusters similar to the *cle* cluster, and the combinatorial biosynthesis utilizing biosynthetic genes obtained from more different TAL-derived meroterpenoid pathways to create new molecules with desired functions.

Experimental section

General experimental procedures

Solvents and chemicals were purchased from Merck KGaA Ltd (Darmstadt, Germany). Oligonucleotide primers were purchased from Tsingke (Beijing, China) and are listed in

Table S2.† PCR was performed using an Applied Biosystems® SimpliAmp™ Thermal Cycler (Thermo Fisher) with Phanta Max Super-Fidelity DNA Polymerase (Vazyme Biotech Co., Ltd). Analytical and preparative HPLC were performed on an Agilent 1260 system, using a ZORBAX SB-C18 column (4.6 mm i.d. × 250 mm, Agilent Co. Ltd) and a ZORBAX SB-C18 column (9.4 mm i.d. × 250 mm, Agilent Co. Ltd), respectively. Silica gel column chromatography was performed using Synthware C189463. NMR spectra were obtained at 800 MHz (^{1}H) and 200 MHz (^{13}C) with a Bruker Avance III HD 800 MHz NMR spectrometer at Kunming Institute of Botany, Chinese Academy of Sciences. Samples for LC-HRMS analysis were injected into a Thermo Scientific Dionex Ultimate 3000 UHPLC system equipped with a Thermo high resolution Q Exactive focus mass spectrometer; chromatographic column: Thermo Scientific Hypersil GOLD C18 (2.1 × 100 mm, 1.9 μm); flow rate: 0.2 mL min^{-1}; column temperature: 25 °C.

Strains and media

Aspergillus versicolor 0312 and *Aspergillus felis* 0260 were isolated from the healthy tissue of *Paris polyphylla* var. *yunnanensis* collected from Kunming, Yunnan province. These two strains were cultivated at 30 °C, 200 rpm, in DPY medium (2% dextrin, 1% hipolypepton (Nihon Pharmaceutical Co., Ltd), 0.5% yeast extract (Oxoid), 0.5% KH_2PO_4, and 0.05% $MgSO_4{\cdot}7H_2O$) for 3 days, and used as a source for whole genome sequencing and the cloning of each gene in the *cle and sre* clusters, respectively.

Aspergillus oryzae NSAR1 (*niaD*$^-$, *sC*$^-$, *ΔargB*, *adeA*$^-$)[16] was used as the heterologous fungal host. The *A. oryzae* transformants were grown in shaking cultures in DPY medium for three days at 30 °C and at 200 rpm for the production of the metabolites derived from the introduced genes.

Standard DNA engineering experiments were carried out using *Escherichia coli* DH5α (Takara Bio Inc.). *E. coli* cells harboring each plasmid were cultivated in Luria–Bertani (LB) medium and were selected with ampicillin.

Whole genome sequencing and analysis

Genome sequencing of *A. versicolor* 0312 and *A. felis* 0260 was performed by Novogene Co., Ltd (Beijing, China) with an Illumina Novaseq system.

For the genome sequence of *A. versicolor* 0312, we generated 6.3 Gb of HiSeq reads to estimate the genome features with the GCE[28] (v1.0.0) software. Given the unique *k*-mer depth of 74 under 15-mers, the genome size was calculated to be 36.1 Mb. A total of 5.1 Gb of SMRT reads were produced on the PacBio Sequel system, and the subreads were corrected, trimmed, and assembled using CANU[29] (v1.6) with the parameters "genome size = 36 m". The consensus sequences were corrected by Arrow(v2.2.1) with SMRT subreads and polished by Pilon[30] (v1.22) with the Illumina short reads. Lastly, we obtained 17 contigs with N_{50} of 3.96 Mb that constitute 95.6% (34.5 Mb) of the genome. The BUSCO[31] (v3.0.2) analysis indicated that 98.9% of the 290 fungal conserved genes were present.

For the genome sequence of *A. felis* 0260, the 6.7 Gb of HiSeq raw reads was treated with NGSQCToolkit[32] (v2.3.3), fastuniq[33] (v1.1), and BLESS[34] (v1.02) software. Sequence assembly was performed with platanus[35] (v1.2.4) to yield 54 scaffolds with N_{50} of 1.62 Mb covering approximately 32.6 Mb.

Gene structure annotation was obtained by using AUGUSTUS (http://bioinf.uni-greifswald.de/webaugustus) with the *Aspergillus fumigatus* gene model used as the training set. All of the predicted gene products were used to construct the database to perform the local BLAST search.

Construction of fungal expression plasmids

To construct one-gene containing fungal transformation plasmids, each gene in the *cle* cluster or the *sre* cluster was first amplified from the *A. versicolor* 0312 and *A. felis* 0260 genomic DNA, with the primers listed in Tables S2 and S3.† The full-length *cle* and *sre* genes were purified and then introduced into the *Sma*I-digested pTAex3[36] vector using an In-Fusion® HD Cloning Kit (Clontech Laboratories, Inc.) according to the manufacturer's protocol (Table S3†). For the introduction of *cle2*, *cle3*, or *cle4* into pBARI,[8] a DNA fragment with the amyB promoter (*PamyB*) and the amyB terminator (*TamyB*) was amplified from the pTAex3-based plasmids constructed above and ligated into the *Hin*dIII-digested vector (Table S3†). To construct the co-expression plasmid of *cle2* and *cle4*, *cle5* and *cle6*, *cle3* and *cle7*, and *cle3* and *sre3* two different gene fragments containing *PamyB* and/or *TamyB* with *cle2*, *cle3*, *cle4*, *cle5*, *cle6*, *cle7*, or *sre3* were amplified from the pTAex3-based plasmids and ligated into *Hin*dIII-digested pBARI, *Xba*I-digested pAdeA,[37] or *Sma*I-digested pUSA[38] using the In-Fusion® HD Cloning Kit, to generate pBarI-*cle2* + *4*, pAdeA-*cle5* + *6*, pUSA-*cle3* + *7*, and pUSA-*cle3* + *sre3*, respectively (Table S3†).

Transformation of *Aspergillus oryzae* NSAR1

Transformation of *A. oryzae* NSAR1 was performed by the previously reported protoplast–polyethylene glycol method.[39] To co-express *cle1*, *cle5*, and *cle6*, two plasmids, pTAex3-*cle*1 and pAdeA-*cle5* + *6*, were used for the transformation. To co-express *cle1*, *cle3*, *cle5*, and *cle6*, three plasmids, pTAex3-*cle1*, pBarI-*cle3*, and pAdeA-*cle5* + *6*, were used for the transformation. To co-express *cle1*, *cle3*, *cle5*, *cle6*, and *cle7*, three plasmids, pTAex3-*cle1*, pUSA-*cle3* + *7*, and pAdeA-*cle5* + *6*, were used for the transformation. To co-express *cle1*, *cle3*, *cle5*, *cle6*, and *sre3*, three plasmids, pTAex3-*cle1*, pUSA-*cle3* + *sre3*, and pAdeA-*cle5* + *6*, were used for the transformation. To co-express *cle1*, *cle2*, *cle3*, *cle5*, *cle6*, and *cle7*, four plasmids, pTAex3-*cle*1, pBarI-*cle2*, pUSA-*cle3* + *7*, and pAdeA-*cle5* + *6*, were used for the transformation. To co-express *cle1*, *cle3*, *cle4*, *cle5*, *cle6*, and *cle7*, four plasmids, pTAex3-*cle1*, pBarI-*cle4*, pUSA-*cle3* + *7*, and pAdeA-*cle5* + *6*, were used for the transformation. To co-express *cle1*, *cle2*, *cle3*, *cle4*, *cle5*, *cle6*, and *cle7*, four plasmids, pTAex3-*cle1*, pBarI-*cle2* + *4*, pUSA-*cle3* + *7*, and pAdeA-*cle5* + *6*, were used for the transformation. To construct negative control strains without one or more genes, the corres-

ponding empty vectors or plasmids harboring a single gene were used for the transformation.

HPLC analysis of each product

Metabolites from each *A. oryzae* transformant were analyzed by HPLC, with a solvent system of H_2O (solvent A) and acetonitrile (solvent B), at a flow rate of 1.0 ml min^{-1} and a column temperature of 35 °C. Separation was carried out with solvent B/solvent A using a linear gradient from 10 : 90 to 100 : 0 within 30 min, 100 : 0 for 5 additional min, and a linear gradient from 100 : 0 to 10 : 90 within the following 5 min. Products from the feeding experiments were analyzed with the same method described above except for the addition of 0.1% trifluoroacetic acid in solvent A.

Feeding experiment

The *A. oryzae* transformant harboring *cle1 + 2 + 3 + 5 + 6 + 7* was cultivated in the presence of 1 mg of either **4**, **5**, or **6**, while the *A. oryzae* transformant harboring *cle1 + 3 + 4 + 5 + 6 + 7* was cultivated in the presence of 1 mg of either **2**, **3**, **6**, or **7**. Both transformants were cultivated in 20 mL of DPY media at 30 °C, 200 rpm, for 72 h. After the incubation, each culture broth was extracted with ethyl acetate, and the mycelia were extracted with acetone at room temperature, concentrated, and reextracted with ethyl acetate. Both extracts were combined and further analyzed by HPLC using the same method described above.

Isolation and purification of each metabolite

To isolate each metabolite, the broth from one liter of the culture was extracted with ethyl acetate. Mycelia were extracted with acetone at ambient temperature overnight, concentrated *in vacuo*, and reextracted with ethyl acetate. Both extracts were then combined and subjected to silica-gel column chromatography and further purification by semi-preparative HPLC. The detailed purification methods for all of the isolated compounds are provided in the ESI.†

MTT assay

Two human breast cancer cell lines (MDA-MB-231 and MCF-7) were used in the cell viability assay, which were acquired from American Type Culture Collection (Rockville, MD, USA). All cells were routinely cultured in RPMI 1640 medium (Hyclone, Logan, UT, USA) complemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and antibiotics (100 units per mL of penicillin and 100 μg mL^{-1} of streptomycin) under a standard humidified atmosphere containing 5% CO_2 at 37 °C. Exponentially growing MDA-MB-231 and MCF-7 cells were seeded into 96-well plates at 5.0×10^3 cells per well and cultured overnight. Different concentrations of DOX (Sigma), the selected compounds (**1**, **2**, and **5–8**), and their combinations were then added as indicated. After 48 h of treatment, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; dissolved in PBS to 5 g L^{-1}) was added for 4 h, followed by dissolving the formazan crystals with dimethyl sulfoxide. The spectrophotometric absorbance at 570 nm was measured using a microplate reader (Tecan Infinite 200 Pro, Hombrechtikon, Switzerland) and presented as relative cell viability.

Conflicts of interest

The authors declare no competing financial interests.

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