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NOTE

Molecular epigenetic approach activates silent gene cluster producing dimeric bis-spiro-azaphilones in *Chaetomium globosum* CBS148.51

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Filamentous fungi have the potential capability to biosynthesize a wide range of structurally unique and biologically diverse secondary metabolites,[1,2] some of which have become the milestones in human medical history including the classical examples of penicillin and cyclosporin.[3] The recent large-scale genome sequencing and bioinformatic analysis revealed that filamentous fungi conserve many gene clusters which do not express in common culture condition. This implied that the metabolic potentiality of fungi may be underestimated significantly.[4] *Chaetomium globosum* has produced diverse secondary metabolites including cytochalasans, azaphilones and epidithiodioxopiperazines.[5,6] A wild-type (WT) *C. globosum* (CBS148.51) has the genome size of 34.3 Mb with a 55.6% GC content.[7] Bioinformatic analysis of the whole genome (Supplementary Figure S4) predicted that it has 40 gene clusters including 17 polyketike synthases (PKSs), 10 non-ribosomal peptide synthetases (NRPSs), 5 NRPS-PKSs hybrids, 5 terpene ones, 1 siderophone and 2 other gene clusters. This WT *C. globosum* (CBS148.51) only produced limited products with majority of them is flavipin under normal cultural condition (Supplementary Figure S8A). This does not match to its metabolic potentiality of its biosynthetic gene clusters, which implied that many secondary metabolite gene clusters only expressed at a low levels or remained silent in common lab conditions.

Negatively charged DNA and positively charged histones constitute nucleosome. Usually, the degrees of tightness or relaxation of nucleosome determine the expression or silence of a gene.[8] Acetylation of the lysine tail will decrease the positive charge of histones, and the electrostatic interaction of DNA and histone also attenuate. This will increase the degree of nucleosome relaxation. The relaxed nucleosome structure is then more accessible by replicative enzymes, and silent genes will be activated or low-expressed ones will be upregulated. Histone acetyltransferases (HATs) and histone deacetylase (HDAC) control the acetylation of histone.[8] Recently, examples of molecular epigenetic approaches by deleting HDAC or chemical epigenetic ways by inhibiting HDAC were introduced to enhance the expression of silent gene clusters,[9,10] whereas there is no report using overexpression of HATs method to activate or upregulate cryptic gene clusters in fungi. Considering the limited secondary metabolites produced by *C. globosum* (CBS148.51) in common lab conditions, using molecular epigenetic approach to overexpress *HATs* gene in this strain was first introduced to activate or upregulate the cryptic gene cluster. Cochliodone A (**1**) and cochliodone B (**2**), a pair of atropisomeric dimeric bis-spiro-azaphilones[11] that had not been obtained from *C. globosum* before were isolated together with six azaphilones chaetomugilin A (**3**),[12] chaetomugilin D (**4**),[13] chaetoviridin A (**5**),[14] 11-*epi*-chaetomugilin A (**6**),[15] chaetoviridin F (**7**)[11] and chaetoviridin H (**8**)[16] from *CgHAT* overexpression (*OE::CgHAT*) strain of *C. globosum* (CBS148.51) (Figure 1). The compounds were characterized by using 1D, 2D NMR and HR-MS analysis. Full details of the structural assignment can be found in the Supplementary Information.

The *CgHAT* gene was cloned from *C. globosum* (CBS148.51) with an open reading frame 1245 bp in size which encoding 414 amino acids. The MW and the isoelectric point of the target protein was 47.434 and 8.8 kDa, respectively, according to ProtParam prediction. BLAST analyses revealed that CgHAT belonged to HAT family which had 79, 78 and 76% sequence identity to TtHAT (*Thielavia terrestris*, XP_003648907), HcHAT (*Histoplasma capsulatum*, EEH09673.1) and MmHAT (*Madurella mycetomatis*, KXX81815), respectively (Supplementary Figure S5). Phylogenetic analyses indicated that CgHAT is close related to MmHAT (*Madurella mycetomatis*, KXX81815; Supplementary Figure S6).

Trpc gene promoter obtained from *Aspergillus nidulans* was introduced to construct a *CgHAT* overexpression cassette (Supplementary Figure S9A). The *OE::CgHAT* cassette was then transferred to *C. globosum* (CBS148.51) through $CaCl_2$/PEG method and screened

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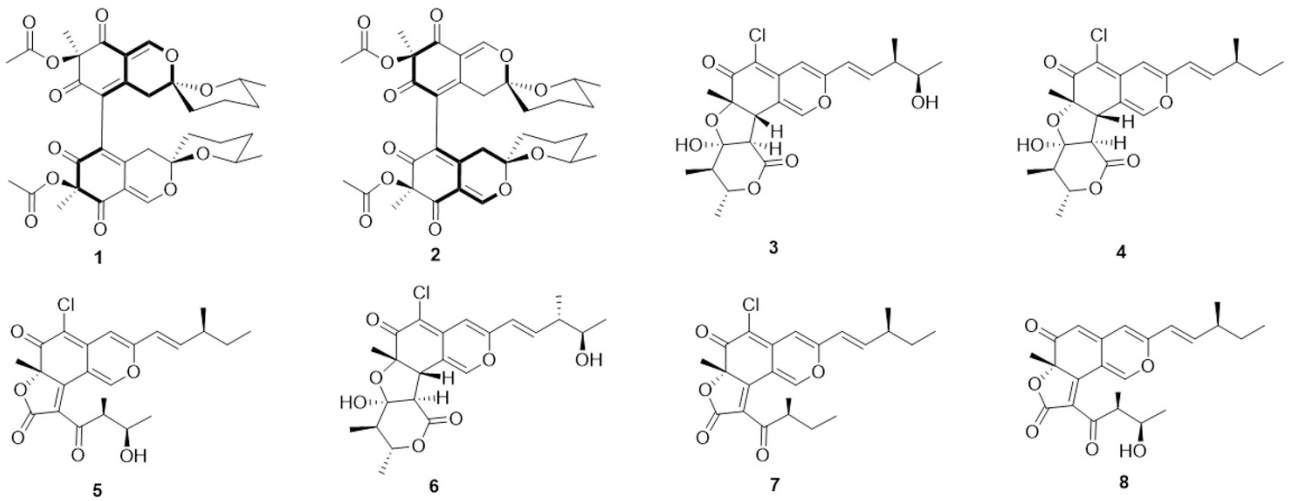

Figure 1 Structures of compounds **1**–**8**.

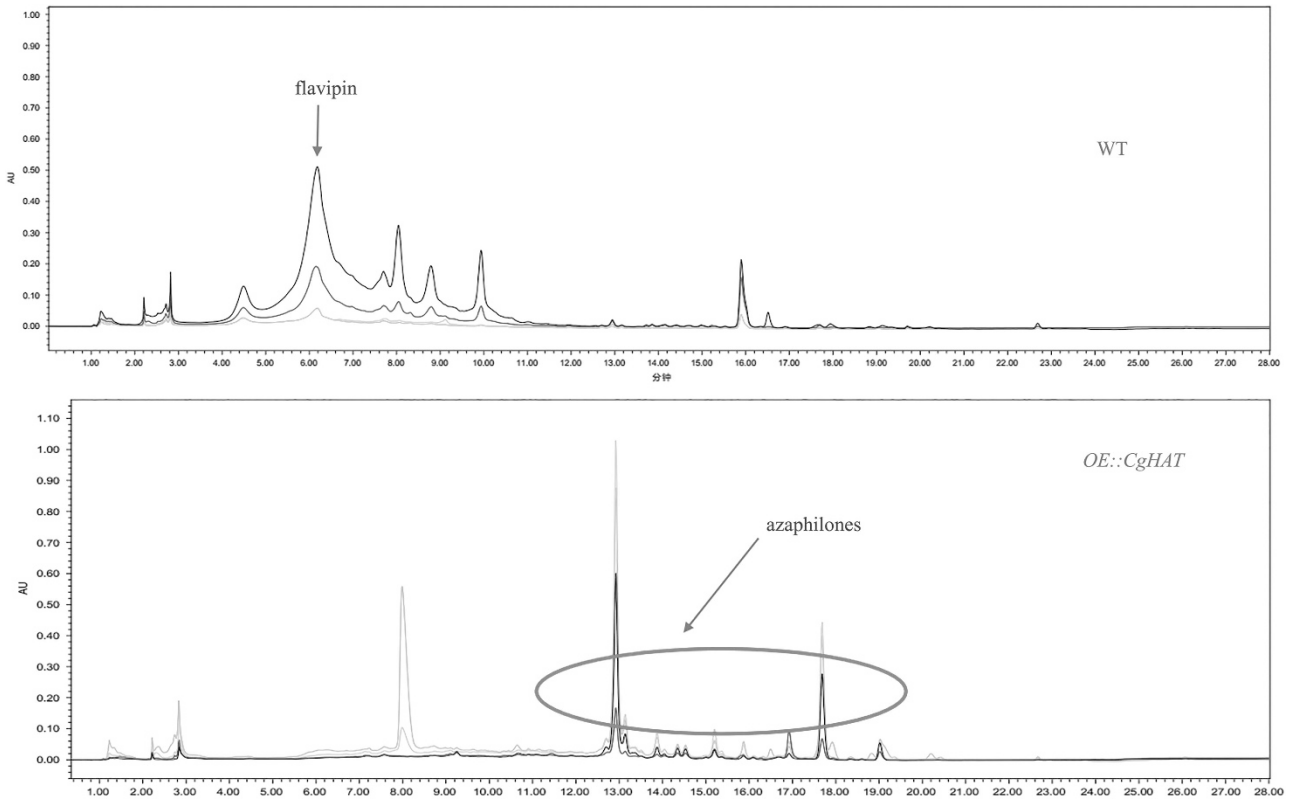

Figure 2 Metabolomic profiles of the wild type (WT) and *OE::CgHAT* strain. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

with 150 μg mg^{-1} G418 sulfate on the potato dextrose agar (PDA) media. Four *OE::CgHAT* transformants were obtained and confirmed by diagnostic PCR (Supplementary Figure S9B). One of them was chose for the subsequent experiments including quantitative real-time PCR and chemical work.

The metabolic profiles of transformants and wild strain were significant different (Figure 2). The main secondary metabolite from wild strain was flavipin, whereas azaphilones were the main ones in transformants. From the structural features of compounds **3–8**, it implied that there are two PKS synthases found in these compounds (Supplementary Figure S11). One PKS synthase should include ketosynthase (KS), ketoreductase (KR), dehydrase (DH), enoyl reductase (ER), together with some post-modification enzymes such as methyl transferase (MT), halogenase and oxidase, whereas the other PKS synthase should possess KS, KR and MT. Detailed analysis of the gene clusters in the genome of *C. globosum* (CBS148.51) revealed that

only one cluster (**cluster 24** in the genome of *C. globosum* CBS148.51) contained two PKS synthase matching with the above-mentioned genes especially the post-modified enzyme genes such as MT, halogenase and oxidase genes (Supplementary Figure S7).

Mutation analysis is an efficient tool to verify the function of targeted genes. However, the innate high nonhomologous random recombination activity of *C. globosum* against foreign DNA moleculars severely restricted the molecular genetic manipulation.[17] In fact, the gene knockout experiments were tried for many times in our lab while no trial gave a desired result.

To further support the conclusion that overexpression of HAT activates azaphilone gene cluster (**cluster 24**) to produce compounds **1–8**, the transcription level of some key genes in this biosynthetic gene cluster (**cluster 24**) including halogenase (CHGG_07641), ketoactyl synthase (CHGG_07645), acyl carrier protein (CHGG_07646), dehydratase (CHGG_07647), FAD linked oxidase domain protein (CHGG_07649) and reductase (CHGG_07650) were further analyzed in the WT and *OE::CgHAT* strains by quantitative reverse transcription PCR (qRT-PCR). The expression level of these genes in the *OE::CgHAT* strain were enhanced by 3–20 folds compared with the WT strain (Supplementary Figure S10). Therefore, overexpression of *CgHAT* indeed activated the expression level of azaphilone gene cluster.

It is an interesting and challenging job to identify the specific gene that is responsible for the formation of the dimer-type azaphilone. The biosynthetic cluster of mono-type azaphilones including chaetomugilins and chaetoviridins had been clarified before.[18] The bis-azaphilone are a small member of secondary metabolites, and few study investigated the biosynthesis of these dimmers. Watanabe *et al.* once proposed the dimerization of cochliodones that was catalyzed by a fungal laccase-like CHGG_10025, a putative multicopper oxidase.[17] However, the authentic function of CHGG_10025 had not examined by additional study.

Fortunately, we noticed that cytochrome P450 in *Aspergillus flavus* was determined to be the biosynthesis gene for the formation of a dimeric diketopiperazine alkaloid.[19] A similar process might occur during the dimerization of cochliodone A (**1**) and cochliodone B (**2**).

In the metabolome of WT, its crude extract contained abundant flavipin (Supplementary Figure S6C), whereas it nearly could not detected any flavipin in the *CgHAT* transformant of *C. globosum* (CBS148.51), although a large amount of additional peaks (azaphilones) were observed in the metabolic profile of the latter (Supplementary Figure S8A). This phenomenon implied that production of azaphilones in the transformant might inhibit the formation of flavipin directly, indirectly or both. From the structural features of flavipin, it displayed that this secondary metabolite was also originated from PKS biosynthetic pathway, in which the acetyl-CoA was the precursor same as that of secondary metabolites **1–8** (Supplementary Figure S11). In addition, a great amount of acetyl-CoA as substrates catalyzed by HATs was transferred to histones in the *CgHAT* transformant of *C. globosum* (CBS148.51). Thus substrate competition in these acetylation reactions might decrease the yield of flavipin in the *CgHAT* transformant, though the specific mechanism in the phenomena needs further investigation.

Azaphilones are a large family of fungal polyketides which possess a highly oxygenated pyranoquinone bicyclic core and a quaternary carbon center. Up to now, more than 400 azaphilones have been isolated from numerous fungi and many of them exhibit significant biological activities.[20] Among them, chaetomugilin A (**3**) and chaetomugilin D (**4**) have been reported to exhibit significant cytotoxicity against P388 and HL-60 cells.[12,13] Chaetoviridin F (**7**) shows antimycobacterial activity against *Mycobacterium tuberculosis* and cytotoxicity against the KB, BC1 and NCI-H187 cell lines.[11] To further exploit the biological potential of azaphilones, all the isolated compounds were evaluated against the A549, HepG 2 and HeLa cell lines by MTT colorimetric method with etoposide as positive controls (IC_{50} value 16.46 ± 1.22, 16.11 ± 0.10 and 15.00 ± 0.23 μM respectively). Unfortunately, none of these natural products showed significant cytotoxicity ($IC_{50} > 40$ μM) against the tested cancer cell lines.

In conclusion, HATwas overexpressed for the first time in *C. globosum* (CBS148.51). Chemical investigation and qRT-PCR analyses both revealed that one gene cluster in the transformant was activated, which produced a series of azaphilone analogs. A pair of atropisomeric dimeric bis-spiro-azaphilones cochliodone A (**1**) and cochliodone B (**2**) were first isolated from the transformant of *C. globosum* (CBS148.51). Based on bioinformatic analysis and possible biosynthetic pathways of flavipin and azaphilones, it implied that the substrate competition in the metabolic flux of acetyl-CoA was postulated to lead to the notable difference of secondary metabolism between the WT and *OE::CgHAT* strains, although the detailed mechanism needs to be investigated further. This was the first attempt to mine fungal secondary metabolites by molecular epigenetic approach overexpressing HAT to activate silent gene cluster, and it confirmed that this is an efficient method to excavate secondary metabolites from systematical chemically investigated fungal species.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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