ORIGINAL ARTICLE

A C G publications

Rec. Nat. Prod. 14:6 (2020) 387-394

records of natural products

Overexpression of Global Regulator *LaeA* Induced Secondary Metabolite Production in *Aspergillus versicolor* 0312

Ting He[1#], Yudan Wang[1,2#], Lianqiong Du[2], Fangru Li[1], Qiufen Hu[2], Guiguang Cheng[1*] and Weiguang Wang[2*]

[1]*Faculty of Agriculture and Food, Kunming University of Science and Technology, Kunming 650500, P. R. China*

[2]*Key Laboratory of Chemistry in Ethnic Medicinal Resources, State Ethnic Affairs Commission and Ministry of Education, Yunnan Minzu University, Kunming, 650031, P. R. China*

(Received March 29, 2020; Revised May 15, 2020; Accepted May 20, 2020)

Abstract: This experiment investigated the effect of the global regulator *laeA* on the secondary metabolites of *Aspergillus versicolor*. The *laeA* gene was cloned from *Aspergillus versicolor* 0312 to introduce *pSilent I* overexpression vector, and the recombinant plasmid was introduced into *Aspergillus versicolor* 0312 by PEG guided protoplast transformation. Then, the over-expressing strain 0312-*laeA* was screened and verified by metabolic level and gene level. A new compound versicolor A (**1**) was obtained by liquid fermentation of the over-expressing strain, together with four known compounds, acetylapoaranotin (**2**), acetylaranotin (**3**), diisobutyl phthalate (**4**) and ergosterol (**5**). The structure of the new compound was elucidated by using various spectroscopic techniques inclusive of HRESIMS, 1D- and 2D-NMR data. Compound **1** showed cytotoxicity against MOLT-4 cell lines with IC_{50} values of 29.6 μM, while **2** showed significant cytotoxicities against MOLT-4, MCF-7, and CaCo-2 cell lines with IC_{50} values of 7.8, 19.9, and 15.9 μM, respectively. The results further supported that *laeA* is a global regulator that could up-regulate and/or activate cryptic gene clusters to produce new secondary metabolites. This study provides an important basis for exploring the mechanism of *laeA* gene on the secondary metabolites of *Aspergillus versicolor*.

Keywords: Natural products; *Aspergillus versicolor*; *LaeA*; biosynthetic gene clusters; secondary metabolites.

1. Introduction

Natural products, produced by bacteria, fungi and plants, provide important resources for the development of new drugs [1-4]. Filamentous fungi can secrete secondary metabolites with biological activity. Some important clinical drug molecules are derived from filamentous fungi, such as penicillin (antibiotic) and cyclosporine (immunosuppressant) [5]. However, in the past two decades, it has been more and more difficult to find novel active substances from filamentous fungi, especially the

# These authors contributed equally to this work
* Corresponding author: E-Mail: ggcheng@kmust.edu.cn; wwg@live.cn; Phone: +86-871-65920586

The article was published by ACG Publications
http://www.acgpubs.org/journal/records-of-natural-products November-December 2020 EISSN:1307-6167
DOI: http://doi.org/10.25135/rnp.183.20.03.1593

compounds with a new backbone, by traditional separation methods. Recently, genome sequencing technology and bioinformatics research, researchers [6-7] have drawn more and more attention in the discovery of bioactive natural molecules, but also in the development of synthetic drug. However, many biosynthetic gene clusters are not expressed in the host organism, or not activated in the laboratory cultural conditions. Therefore, activating the silent gene clusters provide a promising strategy to yield novel or therapeutic metabolites [8-10]. *LaeA* gene is a global regulator in filamentous fungi, and could regulate expression of a large number of secondary metabolite gene clusters. Previous studies reveals that overexpression of the *laeA* regulator in *Aspergillus nidulans* increases the production of terrequinone A [11]; deletion of *laeA* in *Aspergillus flavus* inhibits aflatoxin gene transcription [12].

Aspergillus versicolor 0312, a filamentous fungus, was isolated from the stems of *Paris polyphylla var. yunnanensis*. The whole genome sequencing analysis of the target strain showed that the strain contained a large number of natural product biosynthetic gene clusters. However, in our preliminary cultural experiment on potato glucose medium, chase medium, and DPY medium, limited number of secondary metabolites were produced in *A. versicolor* 0312, as shown by UPLC analysis (Figure 1), implying that many of a large number of natural product biosynthetic genes or gene clusters in this fungus were not, or were only minimally, expressed. A heteroditerpenoid biosynthetic gene cluster in *A. versicolor* 0312 gene clusters was successfully introduced into *Aspergillus oryzae* NSAR1 and a series of novel heteroditerpenoids were discovered [13]. Therefore, overexpression of the global regulator *laeA* in *A. versicolor* 0312 was performed in order to activate or up-regulate the silent gene clusters and produce novel compounds. In this study, overexpression of the global regulator *laeA* in *A. versicolor* 0312 led to isolation of a new acetaminobutyric acid substance versicolor A (**1**) and four known compounds (**2**-**5**). The known compounds were identified as acetylapoaranotin (**2**) [14], acetylaranotin (**3**) [14], diisobutyl phthalate (**4**) [15], and ergosterol (**5**) [16] by comparison with the reported data (Figure 2). All the isolated compounds were tested for their cytotoxic activity by CCK-8 assay in vitro. Herein, we report the isolation, structure elucidation, as well as cytotoxic activities of these compounds.

2. Materials and Methods

2.1. General Experimental Procedures

The 1D and 2D NMR spectra were recorded on a Bruker DRX-600 MHz spectrometer with TMS as an internal standard. The HRESIMS data were recorded on an API QSTAR time-of-flight spectrometer. Optical rotations were measured with a JASCO P-1020 polarimeter. The IR spectra were obtained by a Bruker FT-IR Tensor 27 with KBr pellets. UV spectra were obtained with a Shimadzu UV-2401A spectrophotometer. Column chromatography was performed with silica gel (200-300 mesh, Qingdao marine), Spherical C18 Monomeric, 120A (50 μm, SILICYCLE), and Sephadex LH-20 gel (40-70 μm, GE Healthcare). Semipreparative HPLC was performed on a liquid chromatograph with a 250 × 10 mm column (Reprosil-Pur Basic C18). Fractions were monitored by thin layer chromatography, spots were visualized by UV light (254 nm and 365 nm) and by heating silica gel plates sprayed with 10% H_2SO_4 in EtOH. All solvents used in column chromatography were distilled.

2.2. Strains and Media

Aspergillus versicolor 0312 was isolated from the stems of *Paris polyphylla var. yunnanensis* collected in Kunming, Yunnan Province, People's Republic of China in 2017 and identified by ITS analysis (GenBank Accession No. MK311351). The strain cells were cultivated at 30 ℃, 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% $MgSO_4$•$7H_2O$) for three days, and used as a source for genomic DNA extracting [Quick Plant Genomic DNA Kit (TIANGEN)] and cloning of *laeA* gene. Standard DNA engineering experiments were carried out using *Escherichia coli* DH5α (Tsingke, Beijing,

China). *E. coli* cells harboring each plasmid were cultivated in Luria-Bertani (LB) medium and were selected with ampicillin.

2.3. Cloning of the LaeA Gene and Construction of the LaeA Overexpression Vector

pSilent-I [17] is a fungal expression vector containing the *trpC* promoter and *trpC* terminator. *LaeA* gene was amplified from *A. versicolor* 0312 genomic DNA with the primers 0312-*laeA*-F (aggtacgtacaagctATGTTTGGAAAGGGTCCGG) and 0312-*laeA*-R (taccacaggccttagTTATCTCGCG-ACGGTTTTCGCG) using the high-fidelity enzyme Phanta Max Super-Fidelity DNA Polymerase (Vazyme Biotech Co., Ltd). The full-length *laeA* genes was purified, and introduced into the *Hind*III and *Sph*I double digested *pSilent-I* vector to afford *pSilent-I-laeA* overexpression vector using an In-Fusion® HD Cloning Kit (Clontech Laboratories, Inc.) according to the manufacturer's protocol.

2.4. Transformation of Aspergillus versicolor 0312

Transformation of *A. versicolor* 0312 protoplasts was done using polyethylene glycol. The *pSilent-I-laeA* plasmid was used for the transformation to construct the *laeA* overexpressed *A. versicolor* 0312 strain. To construct the other strains without *laeA* gene, the corresponding empty vector of *pSilentI* harboring a single gene were used. The preparation of *A. versicolor* 0312 protoplasts and the PEG-mediated transformations were performed as previously described [18-19]. The *A. versicolor* 0312 cells transformed with the *pSilent-I* empty vector or *pSilent-I-laeA* plasmid for the overexpression of *laeA* were selected on plates containing hygromycin (ca. 200 μg/mL), prepared as described previously [20]; 20 μg of DNA were thus used for the transformation, and 20-30 transformants were obtained. Of these transformants were chemically examined to afford the versicolor A (**1**) production strain.

2.5. Fermentation, Isolation, and Purification

The over-expressing strain was used to inoculate three Erlenmeyer flasks (500 mL), each containing 200 mL of media (potato glucose medium). Three flasks of the inoculated media were incubated at 28 ℃ on a rotary shaker at 200 rpm for 2 days to prepare the seed culture. Fermentation was carried out in a fermentation tank (10 L), incubated at 30 ℃, 200 rpm for 3 day. The fermented whole broth was filtered through multilayer gauze to remove the mycelia, and the broth was then extracted 4 times with EtOAc. The mycelia were extracted 3 times with acetone under ultrasonic-assisted extraction method. The solvent was evaporated under reduced pressure to afford a crude residue. The crude residue was extracted 4 times with EtOAc. Finally, the filtrates were combined and evaporated in vacuum to yield a crude extract (6 g).

The crude extract was subjected to column chromatography on silica gel (chloroform: acetone 1:0, 10:1, 8:2, 5:5, 0:1) to yield five fractions (Fr. A-Fr. E). Compounds **1** and **4** were detected mainly in Fr. B. Fr. B (300mg) was purified by RP-18 medium pressure column chromatography (acetonitrile: water 30:70 ~ 90:10), and then semi-preparative HPLC to obtain compound **1** (2 mg), **4** (5.3mg). Fr. C (700 mg) was purified on silica gel column (petroleum ether: ethyl acetate 10:1 ~ 1:1) followed by semi-preparative HPLC, to afford compound **2** (7 mg), **3** (9 mg) and **5** (6 mg).

*2.6. Spectral Data of Versicolor A (**1**)*

White amorphous powder; $[\alpha]_D^{23.6}$ -150.0 (*c* 0.02, CH_3OH); UV (CH_3OH) λ_{max} (log ε): 202 (4.06), 254 (2.74) nm; IR (KBr) ν_{max}: 3442, 2976, 2925, 2855, 1738, 1670, 1647, 1636, 1385, 1046 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 481.0864 (calcd for $C_{22}H_{22}N_2O_5S_2$ $[M + Na]^+$, 481.0868).

2.7. Determination of Cytotoxic Activity

The cytotoxicity of all compounds against 3 human cancer cell lines, MCF-7 (breast cancer), MOLT-4 (leukemia), and CaCo-2 (colon cancer), was performed by the CCK-8 method in 96-well microplates [21].

The human cancer cell lines was used in the cytotoxic activity assay and the cell were cultured in DMEM or RPMI-1640 medium (Hyclone, USA) supplemented with 10% fetal bovine serum (Hyclone, USA) and 1% antibiotic mixture of penicillin (100 U/mL) and streptomycin (100 mg/mL) in a humidified environment with 5% CO_2 at 37 ℃. Cells viability was assessed using the CCK-8 method in 96-well microplates. Cells in logarithmic growth phase were selected, were seeded just before sample addition with an initial density of 1×10^6 cells/mL in 100 μL medium. Each tumour cell line was exposed to each test compound at various concentrations in triplicate for 48 h, with cisplatin as a positive control. In this study we set the blank group, negative control group and 5 samples (30, 20, 10, 5, 2.5 μg/mL) to be tested at different concentrations, each concentration has three parallel. The blank group consisted of 110 μL of medium. The negative control group consisted of 100 μL of cells suspension and 10 μL of medium. The sample group consisted of 100 μL of cells suspension and 10 μL of different compounds. After incubation for 44 h under growth conditions, then 10 μL of the CCK-8 solution was added to each well, and the incubation of cells was continued for 4 h. Absorbance was then determined on a Spectra Max Plus plate reader at 450 nm. The IC_{50} value of each sample was calculated following Reed and Muench's method [22].

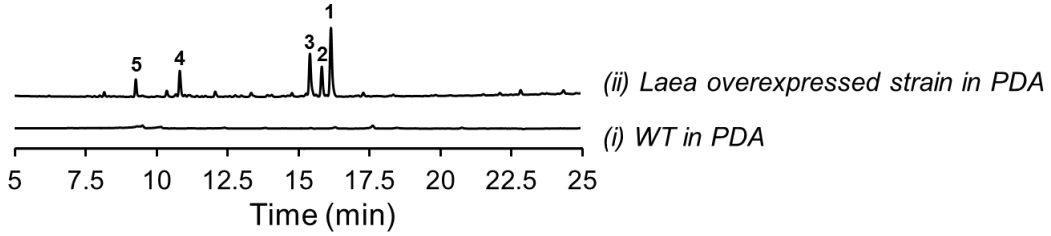


Figure 1. HPLC profiles of culture supernatant extracts from *Aspergillus versicolor* 0312: (i) 0312 WT (wide type) wild strain in PDB (potato dextrose medium); (ii) 0312 overexpressed strain in PDB; The chromatograms were monitored at 254 nm

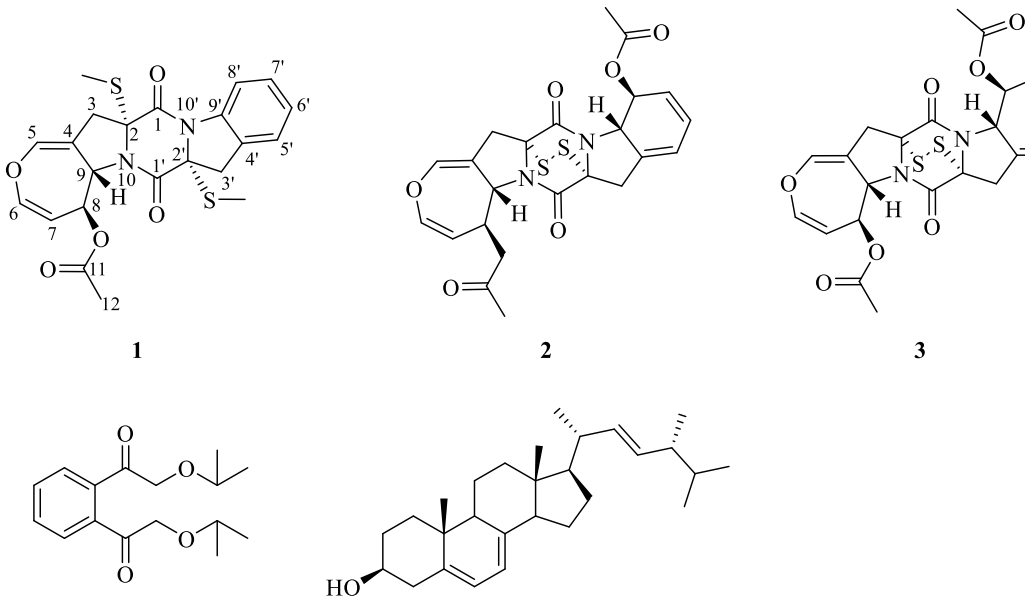
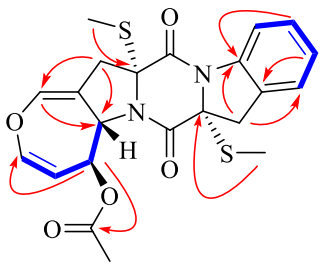


Figure 2. Chemical structures of compounds **1**-**5**

Table 1. The ^{1}H and ^{13}C NMR spectroscopic data of **1** (at 600 MHz and 150 MHz in methanol-d_4) and the ^{13}C NMR spectroscopic data of acetylapoaranotin[23] (at 150 MHz in $CDCl_3$) (δ in ppm, J in Hz)

Position	**δ_C (ppm)**	**δ_H (ppm)**	**HMBC**	**^{1}H-^{1}H COSY**	**δ_C (ppm)** (acetylapoaranotin)
1	163.1 (C)				164.5 (C)
2	70.2 (C)				70.2 (C)
2-SCH_3	13.4 (CH_3)	2.37 (3H, *s*)	2		14.7 (CH_3)
3a	38.9 (CH_2)	3.26 (1H, *d*, *J* = 15.4)	2, 4, 5, 9	H-3a	40.5 (CH_2)
3b		3.22 (1H, *d*, *J* = 15.4)	2, 4, 5	H-3b	
4	110.4 (C)				109.7 (C)
5	137.4 (CH)	6.74 (1H, *s*)	3, 4, 6, 9		137.7 (CH)
6	139.6 (CH)	6.41 (1H, *dd*, *J* = 8.2, 1.8)	5, 7, 8	H-7	139.6 (CH)
7	105.2 (CH)	4.72 (1H, *dd*, *J* = 8.2, 1.8)	6, 8, 9	H-6, H-8	105.7 (CH)
8	72.0 (CH)	5.83 (1H, *ddd*, *J* = 8.1, 2.1, 2.1)	6, 7, 9, 11	H-9	71.8 (CH)
9	60.3 (CH)	5.16 (1H, *d*, *J* = 8.1)	4, 5, 8	H-8	60.4 (CH)
11	170.3 (C)				170.0 (C)
12	19.9 (CH_3)	2.09 (3H, *s*)	11		21.0 (CH_3)
1'	164.9 (C)				164.7 (C)
2'	73.0 (C)				74.4 (C)
2'-SCH_3	12.7 (CH_3)	2.22 (3H, s)	2'		14.3 (CH_3)
3'a	38.8 (CH_2)	3.80 (1H, *d*, *J* = 16.8)	1', 2', 4', 9'	H-3'b	40.1 (CH_2)
3'b		3.51 (1H, *d*, *J* = 16.8)	2', 4', 5', 9'	H-3'a	
4'	129.7 (C)				133.8 (C)
5'	124.7 (CH)	7.39 (1H, *d*, *J* = 7.6)	3', 7', 9'	H-6'	119.8 (CH)
6'	125.5 (CH)	7.21 (1H, *t*, *J* = 7.6)	4', 5', 8', 9'	H-5', H-7'	125.2 (CH)
7'	127.2 (CH)	7.31 (1H, *t*, *J* = 7.6)	5', 8', 9'	H-8'	128.0 (CH)
8'	117.3 (CH)	7.94 (1H, *d*, *J* = 7.6)	4', 6'	H-7'	75.2 (CH)
9'	140.3 (C)				64.4 (CH)
11'					170.5 (C)
12'					21.3 (CH_3)



1

Figure 3. Key HMBC ( ) and ^{1}H-^{1}H COSY ( ) correlations of **1**

3. Results and Discussion

3.1. Structure Elucidation

Compound **1** was isolated as a white amorphous powder. The molecular formula of **1** was established as $C_{22}H_{22}N_2O_5S_2$ on the basis of sodium adduction at *m/z* 481.0864 $[M + Na]^+$ (calcd for 481.0868) in the positive HRESIMS spectrum, in association with ^1H and ^{13}C NMR spectra, indicating thirteen indices of hydrogen deficiency. The IR spectrum showed characteristic absorption peak signals at 1738 cm^{-1} and 1670 cm^{-1} for carbonyl and amide carbonyl functional groups. Its ^{13}C NMR and DEPT spectra exhibited 22 carbon signals assignable for three methyls, two methylenes, nine methines including two peroxymethine groups (δ_C 137.4 and 139.6), five olefinic (δ_C 105.2, 117.3, 124.7, 125.5 and 127.2), one dioxymethine (δ_C 72.0), and one diazomethine (δ_C 60.3), and eight quaternary carbons (two amide carbonyls at δ_C 163.1 and 164.9, one ester carbonyl at δ_C 170.3). The full assignment of the ^1H and ^{13}C NMR spectra was confirmed by the analysis of HSQC and HMBC spectroscopic data. The ^1H and ^{13}C NMR data (Table 1) of compound **1** were similar to acetylapoaranotin [23]. The significant differences were the absence of the ester carbonyl at the C-8' position and the presence of a double bond between C-8' (δ_C 117.3, d) and C-9' (δ_C 140.3, s). The ^1H NMR spectrum displayed four aromatic protons at δ_H 7.39 (1H, d, J = 7.6 Hz, H-5'), 7.21 (1H, t, J = 7.6 Hz, H-6'), 7.31 (1H, t, J = 7.6 Hz, H-7'), and 7.94 (1H, d, J = 7.6 Hz, H-8'), indicating the presence of an aromatic benzene ring moiety in **1** [24]. This assumption was also verified by the loss of 60 daltons in the HRESIMS spectrum. In the ^1H NMR spectrum compound **1** showed a doublet proton at δ_H 7.94 (1H, d, J = 7.6 Hz, H-8') which showed HMBC correlations to δ_C 129.7 (s, C-4') and 125.5 (d, C-6'). In addition, the HMBC correlations from δ_H 7.21 (1H, t, J = 7.6 Hz, H-6'), to δ_C 129.7 (s, C-4') and 117.3 (d, C-8'), and from δ_H 7.31 (1H, t, J = 7.6 Hz, H-7'), to δ_C 124.7 (d, C-5'), 117.3 (d, C-8') and 140.3 (s, C-9') confirmed our speculation. The large coupling constant (J = 8.1 Hz) H-8 and H-9 in compound **1** is similar to that of acetylapoaranotin (C-8 (δ_C 71.8), C-9 (δ_C 60.4) and $J_{8,9}$ = 8.2 Hz) [23], indicating that the configurations of C-8 and C-9 are consistent with acetylapoaranotin, of which the absolute configuration was confirmed by a crystal analysis [25]. Thus the configurations of the C-8 and C-9 positions are 8*S* and 9*S*, respectively (Figure 3). Thus, the structure of compound **1** was established and named as versicolor A.

3.2. Cytotoxic Activity

All the isolated compounds were tested for their cytotoxicity against MCF-7, MOLT-4, and CaCo-2 human cancer cell lines by the CCK-8 method in vitro. Compound **1** showed cytotoxicity against MOLT-4 cell lines with IC_{50} values of 29.6 μM, while **2** showed significant cytotoxicities against MOLT-4, MCF-7, and CaCo-2 cell lines with IC_{50} values of 7.8, 19.9, and 15.9 μM, respectively. The IC_{50} values of cisplatin for MOLT-4, MCF-7, and Caco-2 cell lines are 3.5, 16.6, and 17.2 μM, respectively.

Acknowledgments

Project supported by the NSFC (Nos. 31760096 and 31960095), Yunnan Ten-thousand Talents Program (W.-G., Wang), the National Natural Science Foundation of Yunnan provinces (No. 2019FB027), Yunnan Innovative Research Team for Discovery and Comprehensive Utilization of Functional Small Molecules in Medicinal Plants, and the Science Foundation of Yunnan.

Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

ORCID
Ting He: 0000-0002-4018-6239
Yu-Dan Wang: 0000-0001-8072-7944
Lian-Qiong Du: 0000-0001-9349-1459
Fang-Ru Li: 0000-0002-7788-5754
Qiu-Fen Hu: 0000-0003-4721-9248
Gui-Guang Cheng: 0000-0002-5553-8399
Wei-Guang Wang: 0000-0003-3395-767X

References

[1] R. Geris and T. J. Simpson (2009). Meroterpenoids produced by fungi, *Nat. Prod. Res.* **26**, 1063-1094.
[2] D. J . Newman and G. M. Cragg (2016). Natural products as sources of new drugs from 1981 to 2014, *J. Nat. Prod.* **79**, 629-661.
[3] S. Shi, Y. Li, Y. Ming, C. Li, Z. Li, J. Chen and M. Luo (2018). Biological activity and chemical composition of the endophytic fungus Fusarium sp. TP-G1 obtained from the root of dendrobium officinale Kimura et Migo, *Rec.Nat.Prod.* **12**, 549-556.
[4] R. N. Kharwar, A. Mishra, S. K. Gond, A. Stierle and D. Stierle (2011). Anticancer compounds derived from fungal endophytes: their importance and future challenges, *Nat. Prod. Res.* **28**, 1208-1228.
[5] P. Wiemann and N. P. Keller (2014). Strategies for mining fungal natural products, *J. Ind. Microbiol. Biotechnol.* **41**, 301-313.
[6] E. J. Hong, N. K. Kim, D. Lee, W. G. Kim and I. Lee (2015). Overexpression of the laeA gene leads to increased production of cyclopiazonic acid in *Aspergillus fumisynnematus*, *Fungal. Biol.* **119**, 973-983.
[7] P. Cimermancic, M. H. Medema, J. Claesen, K. Kurita, L. C. W. Brown, K. Mavrommatis, A. Pati, P. A. Godfrey, M. Koehrsen and J. Clardy (2014). Insights into secondary metabolism from a global analysis of prokaryotic biosynthetic gene clusters, *Cell* **158**, 412-421.
[8] Y. Z. Luo, B. Enghiad and H. M. Zhao (2016). New tools for reconstruction and heterologous expression of natural product biosynthetic gene clusters, *Nat. Prod. Res.* **33**, 174-182.
[9] P. J. Rutledge and G. L. Challis (2015). Discovery of microbial natural products by activation of silent biosynthetic gene clusters, *Nat. Rev. Microbiol.* **13**, 509-523.
[10] A. A. Brakhage (2013). Regulation of fungal secondary metabolism, *Nat. Rev. Microbiol.* **11**, 21-32.
[11] S. Bouhired, M. Weber, A. Kempf-Sontag, N. P. Keller and D. Hoffmeister (2007). Accurate prediction of the *Aspergillus nidulans* terrequinone gene cluster boundaries using the transcriptional regulator LaeA, *Fungal. Genet. Biol.* **44**, 1134-1145.
[12] S. Amaike and N. P. Keller (2009). Distinct roles for VeA and LaeA in development and pathogenesis of *Aspergillus flavus*, *Eukaryot. Cell* **8**, 1051-1060.
[13] W. G. Wang, L. Q. Du, S. L. Sheng, A. Li, Y. P. Li, G. G. Cheng, G. P. Li, G. Sun, Q. F. Hu and Y. Matsuda (2019). Genome mining for fungal polyketide-diterpenoid hybrids: discovery of key terpene cyclases and multifunctional P450s for structural diversification, *Org. Chem. Front.* **6**, 571-578.
[14] C. J. Guo, H. H. Yeh, Y. M. Chiang, J. F. Sanchez, S. L. Chang, K. S. Bruno and C. C. Wang (2013). Biosynthetic pathway for the epipolythiodioxopiperazine acetylaranotin in *Aspergillus terreus* revealed by genome-based deletion analysis, *J. Am. Chem. Soc.* **135**, 7205-7213.
[15] W. Zhang, H. X. Lou, G. Y. Li and H. M. Wu (2003). A new triterpenoid from *Entodon okamurae* Broth, *J. Asian. Nat. Prod. Res.* **5**, 189-195.
[16] V. Chobot, L. Opletal, L. Jáhodář, A. V. Patel, C. G. Dacke and G. Blunden (1997). Ergosta-4, 6, 8, 22-tetraen-3-one from the edible fungus, *Pleurotus ostreatus* (oyster fungus), *Phytochemistry* **45**, 1669-1671.
[17] H. Nakayashiki, S. Hanada, N. B. Quoc, N. Kadotani, Y. Tosa and S. Mayama (2005). RNA silencing as a tool for exploring gene function in ascomycete fungi, *Fungal. Genet. Biol.* **42**, 275-283.
[18] K. Gomi, Y. Iimura and S. Hara (1987). Integrative transformation of *Aspergillus oryzae* with a plasmid containing the *Aspergillus nidulans* argB gene, *Agr. Biol. Chem.* **51**, 2549-2555.
[19] C. de Bekker, A. Wiebenga, G. Aguilar and HA. Wösten (2009). An enzyme cocktail for efficient protoplast formation in *Aspergillus niger*, *J. Microbiol. Meth.* **76**, 305-306.
[20] K. F. Nielsen, J. M. Mogensen, M. Johansen, T. O. Larsen and J. C. Frisvad (2009). Review of secondary metabolites and mycotoxins from the *Aspergillus niger* group, *Anal. Bioanal. Chem.* **395**, 1225-1242.

[21] H. Tominaga, M. Ishiyama, F. Ohseto, K. Sasamoto, T. Hamamoto, K. Suzuki and M. Watanabe (1999). A water-soluble tetrazolium salt useful for colorimetric cell viability assay, *Anal. Commun.* **36**, 47-50.

[22] L. J. Reed and H. Muench (1938). A simple method of estimating fifty percent endpoints, *Am. J. Hyg.* **27**, 493-497.

[23] Z. J. Lin, T. J. Zhu, Y. C. Fang and Q. Q. Gu (2008). ^{1}H and ^{13}C NMR assignments of two new indolic enamide diastereomers from a mangrove endophytic fungus *Aspergillus* sp., *Magn. Reson. Chem*, **46**, 1212-1216.

[24] Q. Wu, N. Jiang, W. B. Han, Y. N. Mei, H. M. Ge, Z. K. Guo, N. S. Weng and R. X. Tan (2014). Antibacterial epipolythiodioxopiperazine and unprecedented sesquiterpene from *Pseudallescheria boydii*, a beetle (coleoptera)-associated fungus, *Org. Biomol. Chem.* **12**, 9405-9412.

[25] J. W. Moncrief (1968). Molecular structure of didethiobis (methylthio) acetylaranotin including absolute configuration, *J. Am. Chem. Soc.* **90**, 6517-6518.