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Discovery of the New Plant Growth-Regulating Compound LYXLF2 Based on Manipulating the Halogenase in *Amycolatopsis orientalis*

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Abstract Analysis of the *Amycolatopsis orientalis* HCCB10007 genome revealed new gene clusters involved in natural product biosynthesis that were not associated with the production of known compounds. Halogenases are a type of tailoring enzymes that are usually found within these secondary gene clusters. In this study, we identified an indole-type halometabolite 6-chrolo-1H-indole-3-carboxamide, named LYXLF2, by whole genome mining and metabolic profiling of a flavin-dependent halogenase mutant. LYXLF2 is a new plant growth-regulating compound that promotes root elongation. The results of this study demonstrated that the special gene knock-out/comparative metabolic profiling approach provides a powerful tool for the discovery of novel natural products by genome mining.

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Introduction

The term "genome mining" has been used to describe the exploitation of genomic information for the discovery of new processes, targets, and products [2]. It is possible to estimate the biosynthetic potential of a given organism by mining the whole genome sequence [16], especially the polyketide (PK) and non-ribosomal peptide (NRP) biosynthetic gene clusters. However, it is generally not possible to predict the exact structures of new products by analyzing the backbone synthetases [9]. Tailoring enzymes, such as glycosyltransferases, methyltransferases and halogenases, are important for forming the final products and can affect their activity.

Flavin-dependent halogenases play a vital role in the biosynthesis of organohalogens that display a wide range of biological activities, such as vancomycin (antibacterial), cryptophycin (antitumor), and salinosporamide A (proteasome inhibition). The genes that encode the flavin-dependent halogenases are usually discovered among secondary gene clusters, and the enzymes display strict substrate specificity. The flavin-dependent halogenases recognize tryptophan/indole, phenol/pyrrole and aliphatic moieties [18]. Additionally, these halogenases are found to require the cofactor $FADH_2$, which can be produced from FAD and NADH by nonspecific flavin reductases [17]. Genome mining or scanning strategies have led to the discovery of novel flavin-dependent halogenases distributed across diverse microbes in recent years [1, 8, 12, 20].

Amycolatopsis orientalis HCCB10007 is an important species of actinobacteria that produces vancomycin and a linear polyene antibiotic ECO-0501. Recently, the whole genome of *A. orientalis* HCCB10007 was sequenced by our group (GenBank Accession No. CP003410), and more than 20 secondary metabolites biosynthetic gene clusters

were found based on genome scanning strategy [19]. Among them, four halogenase genes (*AORI_5336*, *AORI_5364*, *AORI_6626* and *AORI_6627*) embedded in different gene clusters were predicted. In the present study, we characterized the halogenase gene *AORI_5336* and deposited it as a flavin-dependent halogenase. In an attempt to find new compounds, we compared the changes of metabolites before and after *AORI_5336* gene disruption and a new halogenated compound LYXLF2, with plant growth-regulating activity was found in the broth of *A. orientalis*. This work provides an alternative method for mining natural products.

Materials and Methods

Strains, Plasmids, Medium and Growth Conditions

The strains and plasmids used in this study are listed in Table S1. All of the *A. orientalis* strains were grown at 28 °C on Gause's synthetic agar medium. Ten types of production media were used in the metabolic profiling comparisons. Details regarding the media and culture conditions are shown in Table S2.

Phylogenetic Analysis of Halogenase Genes

The sequences of 15 known halogenases, including PrnA (U74493), RebH (AJ414559), PyrH (AY623051), Thal (EF095207), CmdE (AM179409), CrpH (EF159954), BhaA (Y16952), McnD (DQ075244), PltA (AF081920), HalB (AF450451), Pyr29 (EF140901), Clo-Hal (AF329398), CalO3 (AF497482), CmlS (AY026946) and NapH2 (EF397638), were located in the NCBI database. These halogenases are involved in the halogenation of different structures. Multiple sequence alignments were performed using the BioEdit sequence alignment editor. phylogenetic analysis of the halogenases was performed using the Mega 4.1 tool. The tree topography and evolutionary distances were determined using the neighbor-joining method with bootstrap analysis of 1000 replicates.

Disruption of AORI_5336 Gene

The primer pairs 5336UF/5336UR and 5336DF/5336DR were designed to amplify the upstream and downstream regions of the *AORI_5336* gene, respectively. The upstream and downstream fragments were digested by *Bgl*II/*Xba*I and *Xba*I/*Hin*dIII, and linked with the pLYZL102 fragment obtained by *Bg* II/*Hin*dIII digestion, respectively, to construct the deletion plasmid pLYXL5336. Plasmid pLYXL5336 was isolated from *E. coli* JM110 and subsequently introduced into *A. orientalis* DVE by electroporation performed in 2 mm electrode gap cuvettes at 7.5 kV/cm for approximately 13 ms. Single-crossover cells were selected with apramycin (50 μg/ml) on Bennet agar medium. The apramycin-resistant colonies were used to inoculate antibiotic-free liquid tryptic soy broth to allow for the formation of double-crossover clones. The construction of the mutant was confirmed by PCR using primers 5336VF and 5336VR with genomic DNA isolated from the parental *A. orientalis* DVE and potential mutants as templates (Fig. S2). The resulting mutant strain was designated as D5336. All of the primers used in this study are listed in Table S3.

Complementation of the AORI_5336 Disrupted Mutant

To complement the mutant strain D5336, *AORI_5336* and a 300 bp upstream region were amplified from genomic DNA of *A. orientalis* DVE using the primers 5336CF/5336CR and introducing *Xba*I sites at both ends. The PCR fragment was digested with *Xba*I and ligated with pULVK2A [4], which was digested by the same endonuclease, to generate pLYXL5336c. The expression vector pLYXL5336c was introduced into *A. orientalis* D5336 by electroporation to yield the strain C5336.

Compound Purification

Large-scale cultures of *A. orientalis* HCCB10007 were grown, and cells were harvested by centrifugation at 4000*g* for 30 min. A PS25-300 column was used to remove impurities, followed by a column (70 × 1000 mm) filled with XAD 1600 resin. Then, fractions containing LYXLF2 were applied to an Agilent SB C_{18} column (5 μm, 9.4 × 250 mm) for final purification with solutions A (0.05 % aqueous TFA solution) and B (0.05 % TFA-acetonitrile) as the mobile phase.

HPLC, MS and NMR Analysis

Samples were analyzed on an Agilent Poroshell 120 SB C_{18} column (2.7 μm, 4.6 × 150 mm) and eluted with 0.05 % aqueous TFA solution and 0.05 % TFA-acetonitrile solution at 1 ml/min. The metabolites were detected by monitoring the absorbance at 223 nm. ESI–MS spectra were taken with a Waters Q-TOF–MS Premier spectrometer in *m/z* (rel. %), and NMR spectra were measured with a Buker-Avance-400 spectrometer in DMSO-*d6* (δ in ppm, *J* in Hz).

Plant and Bioassay

Arabidopsis thaliana and wheat seeds were used in this test. The growth-promoting activity of LYXLF2 was

evaluated in regards to seed growth. The compounds were evaluated at concentrations of 0.005, 0.05, 0.5 and 5 mg/l, respectively. Seeds were placed on absorbent paper in a plate that had been sufficiently moistened with 3 ml of the test solution. After 7 days of cultivation in the dark, root lengths were measured. The growth-regulating rate was calculated as a percentage of the average length of a root from the treated plant relative to the control water. The compounds indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) were also assayed with the same methods. All experiments were performed in triplicate.

Results

Identification of a Halogenase Gene in the New NRPS/PKS Hybrid Gene Cluster Encoded by the *A. orientalis* Genome

An NRPS/PKS hybrid gene cluster named *n_p2* that encodes for the biosynthesis of an unknown compound in the *A. orientalis* HCCB10007 genome has been identified, and the transcription levels of *n_p2* were significantly higher than most other genes [19]. The *n_p2* cluster is approximately 58 kb and contains 24 genes, one of which was proposed to encode a halogenase (*AORI_5336*) (Fig. 1). Fifteen known halogenases that catalyze the halogenation of different substances were chosen for multiple alignment and phylogenetic analyses with the putative halogenases (*AORI_5336*) in the genome. The multiple alignment analysis revealed that AORI_5336 had two conserved regions, a G × G × G motif near the *N*-terminus exhibited the FAD-binding domain, and another domain W × W × IP in the middle of the sequence was responsible for specifically binding a substrate and catalyzing an enzymatic reaction. In addition, a gene downstream *AORI_5336* that encodes a flavin reductase (*AORI_5335*) was proposed to provide the cofactor $FADH_2$. Phylogenetic analysis showed that *AORI_5336* fell into the same clade as Thal, a tryptophan-6-halogenase (Fig. 2) [13]. These characteristics belong to the Flavin-dependent halogenases [17] (Fig. S1).

Deletion of AORI_5336 Revealed a Halogenated Natural Product

To avoid the known metabolites, the strain *A. orientalis* DVE, with the disruption of both vancomycin and ECO-0501 biosynthesis, was chosen as the parental strain. The deletion mutant *A. orientalis* D5336 was assessed for secondary metabolite production during growth in ten different fermentation media (Table S2). Using a strategy combining gene knockouts and metabolic profiling, a peak eluting with a retention time of approximately 19 min was observed by HPLC in the wild-type strain *A. orientalis* DVE (Fig. 3a) in F2 media, and was absent in the mutant. The substance responsible for this peak was designated LYXLF2. To confirm that the production of this substance relied on *AORI_5336*, *AORI_5336* was reintroduced into D5336 to generate the strain C5336. The product was restored in C5336. Accordingly, it was proposed that LYXLF2 was an organohalogen, and *AORI_5336* performed the halogenation of this compound.

Structure Identification of LYXLF2

LYXLF2 was obtained as a pale powder. The molecular formula of LYXLF2 was established as $C_9H_7ClN_2O$ on the basis of HRESI-MS at *m/z* 195.0293 $[M+H]^+$ (calcd. for 195.0325). In the HRESI-MS, a $[M+H]^+$ ion cluster at m/z 195.0293/197.0283 in the ratio of 3:1 was observed, characteristic of a mono-chlorinated structure (Fig. 3b).

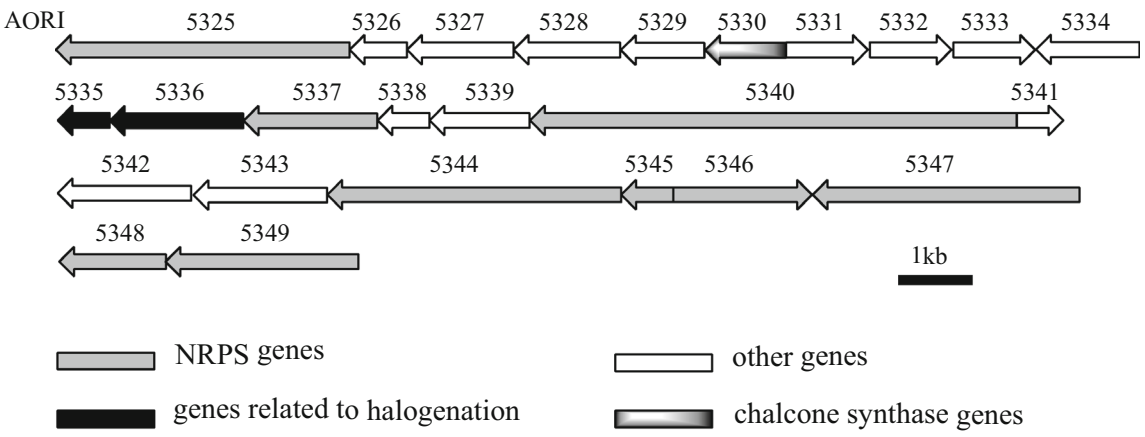

Fig. 1 The putative NRPS–PKS hybrid gene cluster (*n_p2*)

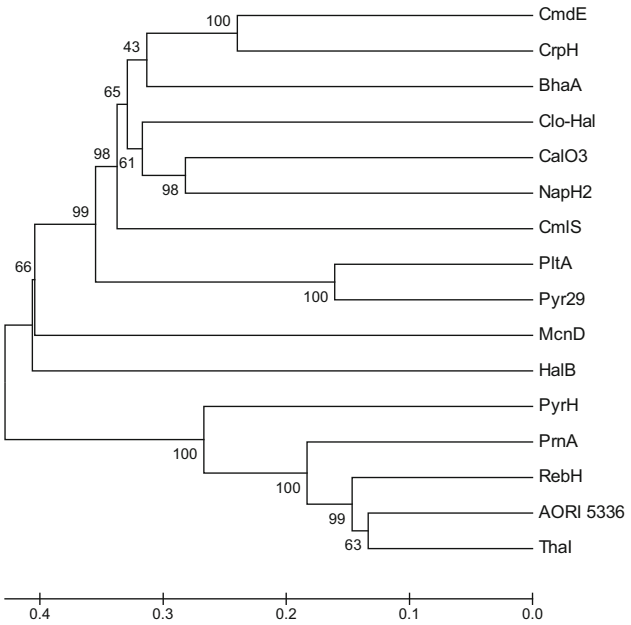

Fig. 2 Phylogenetic tree constructed by the neighbor-joining method based on amino acid sequences of known halogenases and AORI_5336 from *A. orientalis*. Bootstrap values, expressed as percentages of 1000 replications, are given at the branching points. *Scale bar* represents amino acids substitutions per 100 residues

Analysis of the ^{1}H NMR data[(11.71(brs, H-CONH$_2$), 8.16 (d, J = 8.5, H-4), 8.09 (s, H-2), 7.51 (d, J = 1.8 Hz, H-7), 7.13 (dd, J = 8.5, 1.8 Hz, H-5)] for LYXLF2 revealed the presence of nearly identical structural features to those found in compound **6**, except that the ^{1}H–^{1}H coupling constant observed between H-4 and H-5 or H-6 were different (J = 8.5 Hz in LYXLF2; J = 2.2 Hz in compound **6**) (Fig. S4) [3]. The data implied that LYXLF2 was an isomer of compound **6**; thus, the Cl atom should be located at C-6 in LYXLF2. The structure of LYXLF2 was therefore concluded to be 6-chrolo-1H-indole-3-carboxamide (Fig. 3c). Database (Sci Finder and Reaxy) search results revealed that LYXLF2 could be a novel natural product that has not been previously reported.

Flavin-dependent halogenases are involved in the halogenation of different structures, such as tryptophan, phenolic and indole moieties, pyrrole moieties, phenolic moieties and aliphatic moieties [18]. In this study, the structure of LYXLF2 was identified as an indole-like substance. Therefore, *AORI_5336* catalyzed the halogenations at C6 of 1H-indole-3-carboxamide.

Biological Activity of LYXLF2

Plant growth activities of LYXLF2, indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) were examined using bioassays with *Arabidopsis thaliana* and wheat seedling (Fig. 4). Compound LYXLF2 and IBA promoted *A. thaliana* root growth within the range of 0.005–0.5 mg/l. The root elongation of *A. thaliana* treated with LYXLF2 was maximally improved by 23 % of the control at a concentration of 0.05 mg/l, and when treated with IBA it was improved by 10 % at a concentration of 0.5 mg/l, however, IAA showed no improvement effects on root elongation at the same concentration. When the root growth of wheat was examined, both LYXLF2 and IAA had stimulation roles within the range of 0.005–5 mg/l. LYXLF2 accelerated root elongation maximally by 37 % of the control at a concentration of 0.5 mg/l. The compound IAA showed maximal acceleration by 17 % of the control at the same concentration. In contrast, IBA inhibited wheat root growth from 0.005 to 5 mg/l. The results indicated that LYXLF2 is a novel plant growth-regulating substance.

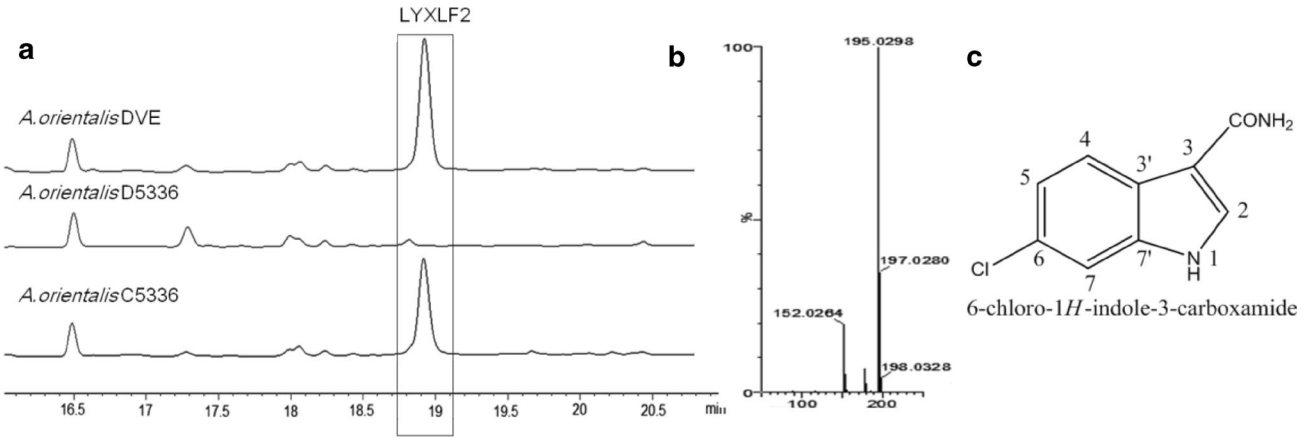

Fig. 3 **a** HPLC analysis of the relevant products from AORI_5336. *A. orientalis* DVE cannot produce vancomycin and ECO-0501. *A. orientalis* D5336 is a mutant with a disrupted AORI_5336 gene. *A. orientalis* C5336 is the complementation strain for the D5336 mutant. The peaks are designated LYXLF2. **b** The MS spectrum of LYXLF2. **c** The structure of the halogenated compound LYXLF2

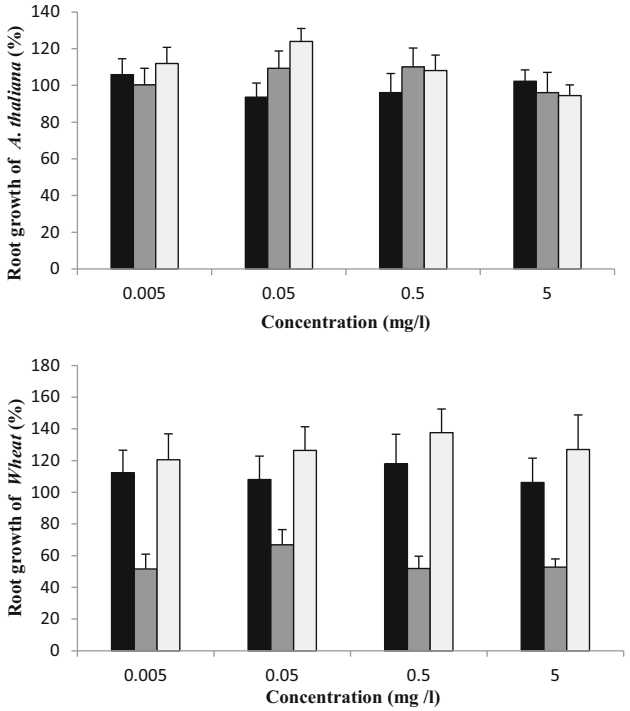

Fig. 4 Effects of IAA, IBA and LYXLF2 on seed rooting in *A. thaliana* and wheat. *Black* indicated IAA; *Gray* indicated IBA; *White* indicated LYXLF2

Discussion

Genome-sequencing projects on different filamentous actinomycetes have revealed numerous natural product biosynthetic gene clusters in each genome. In addition, sequence-guided predictions and subsequent identification of novel metabolites have been processed well in some strains, such as siderophores from *Streptomyces coelicolor* [11] and pentalenolactone or pentaene from *Streptomyces avermitilis* [15]. *A. orientalis*, a type of rare actinomycetes, has 26 gene clusters related to secondary metabolism, including the vancomycin and ECO-0501 gene clusters, which were predicted in the genome [19]. This indicates that there is an abundance of undiscovered secondary metabolites in this organism waiting for us to explore.

In this study, to exclude the effects of vancomycin and ECO-0501, the mutants had both biosynthetic pathways blocked. The halogenase *AORI_5336* was first predicted by phylogenetic analysis and defined as a flavin-dependent halogenase. Then, AORI_5336 was knocked out and a new compound was found by comparing the metabolic profiling of the mutants in up to ten different media. Hornung et al. identified 103 novel putative halogenase genes from 550 randomly selected actinomycetes strains by PCR screening and found two halometabolites by mass spectrometric analysis of cultured filtrates [8]. Gao et al. also used the same method to detect 54 potential gene clusters for organohalogen compounds from 228 actinomycetes and did not find new bioactive compounds [7]. In comparison, our results demonstrated that the gene knockout/comparative metabolic profiling approach was an efficient method to discover new compounds from well-known strains.

There are two classes of flavin-dependent halogenases according to the status of the substrates, including halogenase activity with a free substrate, such as PrnA [5], and halogenase activity requiring a peptidyl carrier protein-tethered substrate, such as PltA [6]. In this study, LYXLF2 disappeared when *AORI_5336* was knocked out, and the theoretical precursor, 1H-indole-3-carboxamide, was also not found, which suggested that *AORI_5336* did not catalyze a free substrate. Additionally, *AORI_5337*, a gene upstream of *AORI_5336*, encoded NRPS. The disruption of *AORI_5337* blocked the biosynthesis of the compound LYXLF2 (Fig. S5). Therefore, it was suggested that *AORI_5336* reacted with a peptidyl carrier protein-tethered substrate. Additionally, the structure of LYXLF2 was far different from the predicted product of *n_p2*, suggesting that only several of the genes in the cluster, including *AORI_5335*, *AORI_5336* and *AORI_5337*, were essential for the biosynthesis of this compound. The gene cluster encoding LYXLF2 might be independent of *n_p2*.

LYXLF2, 6-chrolo-1H-indole-3-carboxamide, an indole alkaloid with halogen substituent, has an active role on plant seeds rooting. It is well-known that the indole nucleus is one of the most important ring systems for pharmaceutical development [14]. In this study, LYXLF2 showed better effect on the root growth of *A. thaliana* and wheat compared to IBA and IAA. In addition to LYXLF2, brevicompanines were another plant growth regulator found in the microorganism *Penicillim brevicompactum* [10]. LYXLF2 was the first *Amycolatopsis* metabolite identified that promotes the growth of plant seedlings.

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References

1. Bayer K, Scheuermayer M, Fieseler L, Hentschel U (2013) Genomic mining for novel FADH2-dependent halogenasesin marine sponge-associated microbial consortia. Mar Biotechnol 2:63–72
2. Challis GL (2008) Genome mining for novel natural product discovery. J Med Chem 51:618–2628
3. Choy J, Figueroa SJ, Jiang L, Wagner P (2008) Novel practical deprotection of N-Boc compounds using fluorinated alcohols. Synth Commun 38:3840–3853
4. Ding XM, Tian YQ, Chiao JS, Zhao GP, Jiang WH (2003) Stability of plasmid pA387 derivatives in *Amylcolatopsis mediterranei* producing rifamycin. Biotechnol Lett 25:1647–1652

5. Dong CJ, Flecks S, Unversucht S et al (2005) Tryptophan 7-halogenase (PrnA) structure suggests a mechanism for regioselective chlorination. Science 309:2216–2219
6. Dorrestein PC, Yeh E, Garneau-Tsodikova S, Kelleher NL, Walsh CT (2005) Dichlorination of a pyrrolyl-S-carrier protein by FADH(2)-dependent halogenase PltA during pyoluteorin biosynthesis. Proc Nat Acad Sci 102:13843–13848
7. Gao P, Huang Y (2009) Detection, distribution, and organohalogen compound discovery implications of the reduced flavin adenine dinucleotide-dependent halogenase gene in major filamentous actinomycete taxonomic groups. Appl Environ Microbiolog 75:813–4820
8. Hornung A, Bertazzo M, Dziarnowski A et al (2007) A genomic screening approach to the structure-guided identification of drug candidates from natural sources. Chem Bio Chem 8:757–766
9. Katz L, Baltz RH (2016) Natural product discovery: past, present, and future. J Ind Microbiol Biotechnol 43:155–176
10. Kimura Y, Sawada A, Kuramata M et al (2005) Brevicompanine C, cyclo-(D-Ile-L-Trp), and cyclo-(D-Leu-L-Trp), plant growth regulators from *Penicillium brevi-compactum*. J Nat Prod 68:237–239
11. Lautru S, Deeth RJ, Bailey LM, Challis GL (2005) Discovery of a new peptide natural product by *Streptomyces coelicolor* genome mining. Nat Chem Biol 1:265–269
12. Li XG, Tang XM, Xiao J et al (2013) Harnessing the potential of halogenated natural product biosynthesis by mangrove-derived *Actinomycetes*. Mar Drugs 11:3875–3890
13. Milbredt D, Patallo EP, van Pée KH (2014) A tryptophan 6-halogenase and an amidotransferase are involved in thienodolin biosynthesis. Chem bio chem. 15:1011–1020
14. Netz N, Opatz T (2015) Marine indole alkaloids. Mar Drugs 13:4814–4914
15. Tetzlaff CN, You Z, Cane DE et al (2006) A gene cluster for biosynthesis of the sesquiterpenoid antibiotic pentalenolactone in *Streptomyces avermitilis*. Biochemistry 45:6179–6186
16. Van Lanen SG, Shen B (2006) Microbial genomics for the improvement of natural product discovery. Cur Opin Microbiol 9:252–260
17. Van Pée KH, Patallo EP (2006) Flavin-dependent halogenases involved in secondary metabolism in bacteria. Appl Microbiol Biotechnol 70:631–641
18. Wagner C, Omari ME, König GM (2009) Biohalogenation: nature's way to synthesize halogenated metabolites. J Nat Prod 72:540–553
19. Xu L, Huang H, Wei W et al (2014) Complete genome sequence and comparative genomic analyses of the vancomycin-producing *Amycolatopsis orientalis*. BMC Genom 15:363–373
20. Zeng J, Zhan J (2010) A novel fungal flavin-dependent halogenase for natural product biosynthesis. Chem Bio Chem 11:2119–2123