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Genomics-driven discovery of a new cyclodepsipeptide from the guanophilic fungus *Amphichorda guana*†

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Two potential non-ribosomal peptide synthetases (NRPSs) were identified in the genome of a guanophilic fungus *Amphichorda guana* by bioinformatics analysis and gene knockout experiments. Liquid chromatography coupled with mass spectrometry (LC-MS) guided isolation led to the discovery of a new cyclodepsipeptide isaridin H (1) and seven known analogs, desmethylisaridin E (2), isaridin E (3), isariin A (4), iso-isariin B (5), iso-isariin D (6), isariin E (7), and nodupetide (8). The absolute configuration of isaridin H (1) was achieved by Marfey's method. Isaridin H (1) showed significant antifungal activity against *Botrytis cinerea* and *Alternaria solani*.

Introduction

Natural products such as peptides, polyketides, and terpenoids are important drug resources due to their highly diverse structures.[1] The skeletons of these compounds are constructed by core biosynthetic enzymes for each class of natural products, namely non-ribosomal peptide synthetases (NRPSs) for peptides,[2] polyketide synthases (PKSs)[3] for polyketides, and terpene cyclases[4] for cyclic terpenoids. Among these, non-ribosomal peptides (NRPs) represent a broad range of important medicinal agents, such as the immunosuppressant cyclosporine, the antifungal caspofungin, the antibacterial daptomycin and so on.[5]

Cyclodepsipeptides (CDPs) are a class of NRPs featuring molecular ring formation involving an ester group. Most CDPs (*i.e.*, PF1022A, enniatins, destruxins and beauvericins) have been widely applied as pharmaceuticals and agrochemicals,[6–9] and some are known as mycotoxins.[10–13] They are mainly distributed in the genera *Acremonium*, *Aspergillus*, *Beauveria*, *Fusarium*, *Isaria* and *Amphichorda*.[10,14–17] *Amphichorda*, a rare genus with long white synnemata, is isolated from humid habitats.[18–20] *Amphichorda* was first established by Fries in 1825, and currently comprises only two species, *A. felina* and *A. guana*, among which, *A. guana* is a novel bat guano-loving fungus recently found in a Karst cave in Guizhou province, China.[21] A number of bioactive secondary metabolites (SMs) have been isolated from *A. felina*, such as cyclosporin C, isariins and isaridins.[17,20,22–24] However, the secondary metabolic profile of *A. guana* was fully unknown. In this study, we sequenced the genomic DNA of *A. guana* and found that it contains two NRPSs which were predicted to assemble CDPs. Guided by LC-MS, a novel CDP isaridin H (**1**), along with seven known analogs, desmethylisaridin E (**2**), isaridin E (**3**), isariin A (**4**), iso-isariin B (**5**), iso-isariin D (**6**), isariin E (**7**) and nodupetide (**8**), were isolated. The antifungal and antibacterial activities of the isolates were also described.

Results and discussion

To mine novel CDPs, the genomic DNA of *A. guana* LC5815 was subjected to PacBio sequencing. *In silico* analysis of the draft genome of *A. guana* LC5815 using antiSMASH 4.0.1 revealed 54 secondary metabolite biosynthesis gene clusters (BGCs) including 21 PKSs, 8 NRPSs (Table S3), and 6 hybrid PKS-NRPSs. NRPs are biosynthesized by large, modular, multienzyme NRPS machinery. Each module of an NRPS is responsible for the selection, loading, and condensation of a single amino acid building block. The general NRPS module comprises three enzyme domains: adenylation (A), peptidyl carrier protein (PCP; also known as the thiolation domain), and condensation (C). Proteinogenic and non-proteinogenic amino acid building blocks are selected and activated by A domains, and then other domains incorporate these amino acid sub-

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strates into the final NRP products.[25] Importantly, modifying enzymes increases the diversity of products. To identify the BGCs of CDPs, we retrieved the sequenced genome data of *A. guana* LC5815 using a local Basic Local Alignment Search Tool Protein (BLASTP) program. According to the structures of CDPs which have been reported in *A. felina* and bioinformatics tool analysis (antiSMASH, NRPSsp, CDD, interPro), two candidate BGCs in our novel species *A. guana* (cluster 20 and cluster 31) were expected to be involved in the biosynthesis of CDPs. Cluster 20 includes a 22 906 bp core gene (*AG06652*) encoding a putative NRPS with five A-domains. Meanwhile, cluster 31 includes a 24 262 bp core gene (*AG03264*) encoding a putative NRPS with six A-domains. The candidate gene clusters are illustrated by using the native BLAST (Fig. S1, S2 and Tables S4, S5†) and the A domain specificities are shown in Table S6† by bioinformatics software analysis (NRPSsp and NRPSpredictor3). We used the amino acid sequences of AG06652 and AG03264 as a search query for the BLASTP program. The results indicated that AG06652 had 58% identity and 97% similarity to NRPS of *Thermothelomyces thermophilus* ATCC 42464 (GenBank accession number XP_003664570.1), and 33% identity and 92% similarity to destruxin synthetase in *Metarhizium robertsii* ARSEF 23 (GenBank accession number XP_007826232.2). Meanwhile, AG03264 had 62% identity and 99% similarity to destruxin synthetase of *M. robertsii* ARSEF 23 (GenBank accession number XP_007826232.2), and 39% identity and 96% similarity to cyclosporin C synthetase of *Beauveria felina* (GenBank accession number ATQ39428.1) respectively. Similarly, AG03265 had 74% identity and 93% similarity to aldo-keto reductase of *M. robertsii* ARSEF 23 (GenBank accession number XP_007826234.2) (Fig. S3†). The biosynthetic pathway for potential CDPs can also be proposed based on a mode for destruxin biosynthesis.[26]

To prove the two BGCs related to CDP production, we performed gene deletion experiments. The *AG06652* (NRPS) and *AG03264* (NRPS) genes were disrupted by homologous replacements in *A. guana*, respectively.[9] Correct transformants were verified by diagnostic PCR (Fig. S4†). Subsequently, mutants were cultured in rice medium for 7 days and subjected to metabolite analysis. HPLC analysis of fermentation extracts of wild-type *A. guana* and mutants (Δ*AG06652* and Δ*AG03264*) showed that most of those compounds are absent in the mutants compared to the wild-type (WT) (Fig. 1). Interestingly, the phenotypes of the mutants showed significant differences in comparison with the WT because mutants cannot produce synnemata (Fig. S5†). It is reported that synnemata play important roles in ecological significance and are related to CDP production (Fig. S5†).[27,28,29–32]

To identify these compounds missing from the mutant strains, we performed comparative metabolite analysis between deletion strains and the WT by LC-MS analysis of their extracts. The results showed that many high molecular weight compounds exist in the WT, but not in the mutants (Fig. 2A), implying the possible novel CDPs. The results were further confirmed by LC-ESI-MS/MS profiling which pinpointed the successive cleavage of amino acid moieties from

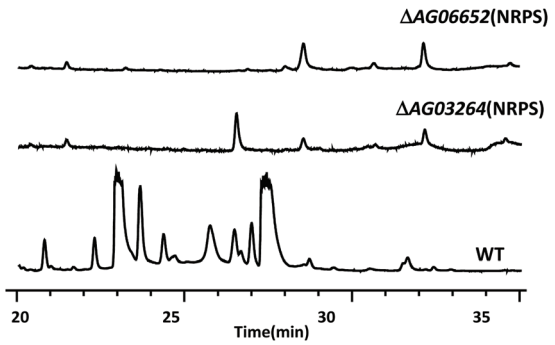

Fig. 1 HPLC analysis of fermentation products from WT *A. guana* and mutants (Δ*AG06652*, Δ*AG03264*). The control strain and mutants were grown in rice medium at 28 °C for 7 days; their fermentation products were extracted using ethyl acetate (for detailed information see the ESI†). Detection was carried out at 210 nm.

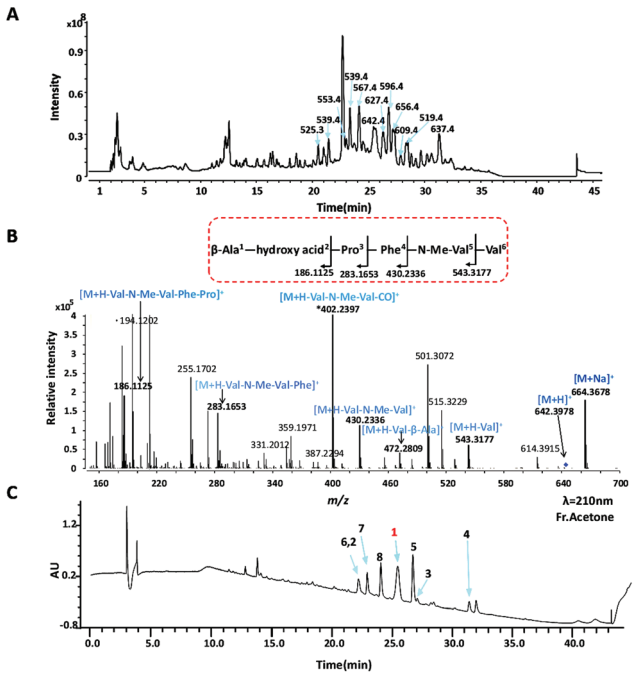

Fig. 2 (A) Total ion chromatography (TIC) from the positive ionization mode of LC-MS of the *A. guana* LC5815 chemical extract in the range of 500–800 Da. *A. guana* LC5815 was grown in rice medium at 28 °C for 7 days, and then extracted using ethyl acetate. (B) The putative novel CDP sequence indicated by the pronounced fragment ions in its LC-MS/MS spectrum. (C) HPLC analysis of the target fraction isolated from the crude extract of the wild type.

the protonated molecular ion at *m/z* 642.3978 $[M + H]^+$ (Fig. 2B). Meanwhile, two know CDPs, isariin A (*m/z* 638.4315) and iso-isariin B (*m/z* 596.3779), were deduced (Fig. S6†).

Combined with the results of knockout experiments and mass spectrometry, it was speculated that the missing compounds were CDPs. To mine more CDPs in the bat guano-loving fungus *A. guana*, scale-up fermentation was carried out. The ethyl acetate extracts were separated by silica gel column

chromatography using different organic solvents to give four fractions, and the targeted fraction was selected for further purification using Sephadex LH-20 and semipreparative HPLC (Fig. 2C) (for detailed information see the ESI†). Fortunately, a novel CDP isaridin H (**1**), along with seven known analogs, desmethylisaridin E (**2**), isaridin E (**3**), isariin A (**4**), iso-isariin B (**5**), iso-isariin D (**6**), isariin E (**7**), and nodupetide (**8**), were isolated from *A. guana* (Fig. 3). The known compounds were determined by the comparison to the reported data (Tables S9–S15†).[24,28,30,33–35] Even the known isariin E (**7**) was reported; however, no detailed NMR spectra were provided. In this study, we report the NMR spectra of isariin E (**7**) for the first time (for detailed information see the ESI†).

Isaridin H (**1**) was obtained as a white powder with $[\alpha]_D^{25}$ −27 (*c* 0.1, MeOH), which was further proved to have a molecular formula of $C_{34}H_{51}N_5O_7$ from the protonated molecular ion at *m*/*z* 642.3859 (calculated for $C_{34}H_{52}N_5O_7N$, 642.3890) in its high-resolution electrospray ionization mass spectrometry (HR-ESI-MS). The UV spectrum showed absorption at 207 nm (Fig. S16†). The IR spectrum indicated the presence of amine (3274 cm^{-1}), ester carbonyl (1740 cm^{-1}), amide carbonyl (1647 cm^{-1}), and aromatic (1545 cm^{-1}) functionalities (Fig. S17†). The ^1H NMR spectrum of **1** revealed the presence of three NH protons (δ_H 9.11, 8.28, and 7.33), five aromatic protons (δ_H 7.38–7.25, for H-19 to H-23), five CH protons [δ_H 4.95 (H-16), 4.91 (H-30), 4.62 (H-10), 4.50 (H-25), and 4.16 (H-5)], six methyl groups (δ_H 0.94–0.64, for H_3-7, H_3-8, H_3-12, H_3-13, H_3-33, and H_3-34), and one *N*-methyl group (δ_H 2.62, for H_3-14) (Fig. S10†). Analysis of the ^{13}C NMR spectrum of **1** indicated the presence of six carbonyls, one quaternary aromatic carbon, five tertiary aromatic carbons, one tertiary oxymethine, four tertiary nitrogenated methines, seven methylenes, three methines, six methyls, and an *N*-methyl, suggesting a CDP structure (Fig. S11† and Table 1).[33] With this information in mind, the assignment of proton and carbon signals to the amino acid residues was subsequently accomplished by interpreting their ^1H–^1H COSY, HSQC, and HMBC spectra (Fig. S12–S14†), indicating that **1** was composed of Ala, HMPA (2-hydroxy-4-methylpentanoic acid), Pro, Phe, *N*-Me-Val, and Val residues. The sequence of amino acids in **1** was also confirmed on the basis of MS analysis (Fig. 2B). The absolute configurations of the four alpha amino acids in **1** were determined through a combination of Marfey's method and the use of reversed-phase HPLC,[34,36] and thus, the four amino acid units (Val, *N*-Me-Val, Phe, and Pro) in **1** were assigned as L-configured (Fig. S7†). The absolute configuration of C-30 in HMPA was deduced as *S* from the ROESY correlation between H-25 and H-30 (Fig. 4 and Fig. S15†).[33] Hence, the structure of **1** was assigned as a new CDP and named isaridin H (Fig. 4). Based on the CDPs isolated, we further confirmed that two gene clusters were responsible for the biosynthesis of CDPs.

The new CDP isaridin H (**1**) and two analogs (**2** and **3**) were also subjected to antimicrobial bioassays. These compounds showed weak inhibitions against *Escherichia coli* and *Staphylococcus aureus*. However, the novel CDP isaridin H (**1**)

Table 1 ^1H and ^{13}C NMR spectroscopic data for isaridin H (**1**) (DMSO-d_6, 500 MHz for ^1H NMR, 125 MHz for ^{13}C NMR)

Position	δ_H (J Hz)	δ_C
1		171.3
2	2.65 m, 2.37 dd (15.2, 11.8)	33.8
3	3.71 m, 3.08 m	34.2
4		170.3
5	4.16 m	57.9
6	2.04 ddd (16.6, 11.6, 4.9)	30.4
7	0.64 d (6.7)	17.9
8	0.81 d (6.7)	19.6
9		168.3
10	4.62 d (10.4)	65.3
11	2.24 m	25.9
12	0.82 d (6.4)	19.0
13	0.94 d (6.4)	19.5
14	2.62 m	29.0
15		172.7
16	4.95 m	49.8
17	3.04 m, 2.84 m	37.2
18		137.4
19	7.38 d (7.5)	129.4
20	7.32 d (7.5)	128.3
21	7.25 m	126.7
22	7.32 m	128.3
23	7.38 m	129.4
24		173.7
25	4.50 t (7.8)	58.6
26	2.19 m, 1.43 m	27.5
27	1.78 m	25.1
28	3.78 m, 3.22 m	47.3
29		167.4
30	4.91 dd (10.1, 4.9)	75.0
31	1.78 m, 1.52 m	37.6
32	1.65 m	24.4
33	0.85 d (6.6)	22.6
34	0.91 d (6.6)	21.5

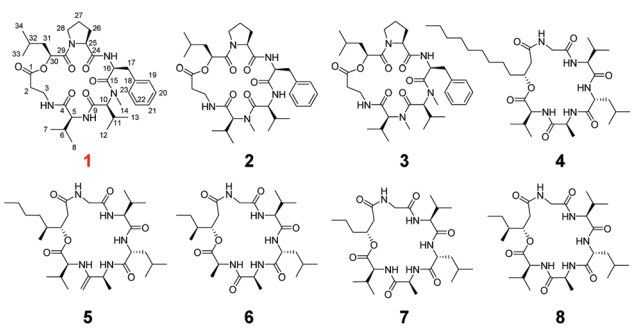

Fig. 3 Structures of the isolated compounds **1**–**8**.

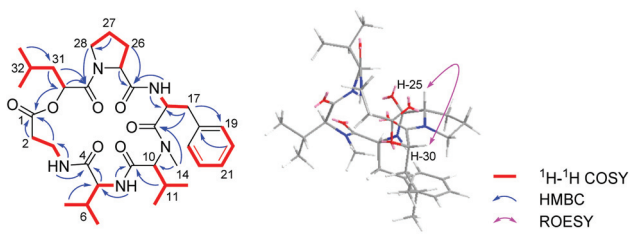

Fig. 4 ^1H–^1H COSY, key HMBC and ROESY correlations of **1**.

showed inhibition against *Bacillus subtilis* at 12.5 μM (Fig. S8†). Most notably, **1** exhibits significant antifungal activities against *Botrytis cinerea* and *Alternaria solani* (Fig. S8 and Table S8†). The IC_{50} value of antifungal activity was 10.0 μg mL^{-1} (15.6 μM) against *A. solani* (Fig. S8†).

Conclusions

To summarize, we identified two NRPS BGCs responsible for the biosynthesis of CDPs by bioinformatics analysis and gene deletion experiments in *A. guana*. LC-MS analysis showed that *A. guana* could produce many high molecular weight compounds which contain many CDPs. In this study, we have identified eight CDPs, including one new CDP isaridin H (**1**) and seven known analogs. The structure of new CDP was identified by NMR, Mafey's method and LC-ESI-MS/MS. In addition, the new CDP isaridin H (**1**) was found to have antifungal activity against several phytopathogens. Furthermore, we proposed a biosynthetic model for the new CDP isaridin H (**1**) according to the domain organisations of AG06652 and AG03264 (Fig. S9†). Since neither of the two non-ribosomal peptide synthases individually recognizes all assembled amino acids according to A domain specificities, it is not clear whether the two enzymes perform assembly functions together.[37] These isolated CDPs indicate that *A. guana* has great potential to mine novel SMs. Our data also provide insights into the biosynthetic origin of CDPs (isaridins and isariins) in *Amphichorda.* It is worth noting that the discovery of new active CDPs could accelerate the development of these important molecules for agricultural and pharmacological applications.

Conflicts of interest

There are no conflicts to declare.

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