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Gene mining and efficient biosynthesis of a fungal peptidyl alkaloid

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ABSTRACT

Objective: Peptidyl alkaloids, a series of important natural products can be assembled by fungal non-ribosomal peptide synthetases (NRPSs). However, many of the NRPSs associated gene clusters are silent under laboratory conditions, and the traditional chemical separation yields are low. In this study, we aim to discovery and efficiently prepare fungal peptidyl alkaloids assembled by fungal NRPSs.
Methods: Bioinformatics analysis of gene cluster containing NRPSs from the genome of *Penicillium thymicola*, and heterologous expression of the putative gene cluster in *Aspergillus nidulans* were performed. Isolation, structural identification, and biological evaluation of the product from heterologous expression were carried out.
Results: The putative tri-modular NRPS AncA was heterologous-expressed in *A. nidulans* to give anacine (**1**) with high yield, which showed moderate and selective cytotoxic activity against A549 cell line.
Conclusion: Heterologous expression in *A. nidulans* is an efficient strategy for mining fungal peptidyl alkaloids.

1. Introduction

Natural products play a pivotal role in drug discovery and fungi have been proved to be important resource of bioactive natural products. Peptidyl alkaloids are a significant class of bioactive secondary metabolites with characteristic multicyclic, constrained rings in their chemical structures, and mainly produced in filamentous fungi (Walsh, Haynes, Ames, Gao, & Tang, 2013). Typical examples of peptidyl alkaloids included asperlicins as cholycystokinin antagonist (Liesch, Hensens, Zink, & Goetz, 1988), ardeemins with function of obstruction the multiple drug resistance in tumor cells (Boyes-Korkis et al., 1993; Hochlowski et al., 1993), as well as fumiquinazolines with diverse bioactivities (Takahashi et al., 1995).

Biosynthetically, the core scaffold of peptidyl alkaloids is assembled by short non-ribosomal peptide synthetases (NRPSs), and then further modified via a series of tailoring enzymes to increase structural complexity and diversity, such as oxidation, acylation and pentenylation (Haynes, Ames, Gao, Tang, & Walsh, 2011; Yin, Grundmann, Cheng, & Li, 2009). It was reported that NRPSs have ability to select, activate, and incorporate the nonproteinogenic amino acid anthranilate (Ant) as building block in peptidyl alkaloids biosynthesis, exampled by fumiquinazolines (Gao et al., 2012; Haynes, Gao, Tang, & Walsh, 2013). Therein, fumiquinazolines F (FQF), containing a 6–6–6 tricyclic core, has the simplest modification in this family. Biochemical characterization has confirmed that FQF is a tripeptidyl skeleton assembled by anthranilic acid (Ant), *L*-tryptophan (*L*-Trp), and *L*-alanine (*L*-Ala) (Ames, Liu, & Walsh, 2010). As one of the most structurally complex quinazoline alkaloids, alanditrypinone was characterized to be biosynthesized by only three enzymes in the *Aldp* cluster, where a tri-modular NRPS, AldpA, catalyzed the generation of a tripeptide 14-*epi*-FQF, then an α-KG-dependent oxygenase AldpC led to the formation of an iminium containing intermediate, and a mono-modular NRPS, AldpB, loaded an additional *L*-Trp moiety to construct the final structure (Yan et al., 2019).

In order to discover more peptidyl alkaloids from fungi, we commenced to mining gene clusters with tri-modular NRPS homologous to AldpA. When analyzing the gene clusters for natural product biosynthesis from the sequenced *Penicillium thymicola* genome, we found a new gene cluster (named *Anc*) containing a tri-modular NRPS which was supposed to be responsible for a fungal quinazoline tripeptidyl alkaloid biosynthesis with unknown structure. As *Aspergillus nidulans* has been established as a convenient expression host, which could increase the level of gene

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expression by strong/inducible promoters, amenable to large-scale fermentation, and easily manipulated (Nielsen, Nielsen, Anyaogu, Holm, Nielsen, & Larsen, 2013; Ryan, Moore, & Panaccione, 2013; Yin et al., 2013), it is a perfect host to express fungal natural products biosynthetic gene clusters.

Herein, we reported the genome mining, heterologous expression, and bioactivity of a fungal peptidyl alkaloid anacine, which presented the heterologous expression in *A. nidulans* as an efficient strategy for mining fungal natural products.

2. Materials and methods

2.1. Strains, and culture conditions

The strains used in this study are listed in Table 1. The fungal strain *Penicillium thymicola* was grown at 25 °C on potato dextrose agar (PDA, BD). The *Aspergillus nidulans* strain was grown at 37 °C on glucose minimum medium (GMM) and were supplemented with uracil 0.56 g/L, uridine 1.26 g/L, 0.5 μmol/L pyridoxine HCl when appropriate. The heterologous expression strains were fermented at 25 °C in liquid starch minimum medium (LSMM), with the carbon source in GMM to 2% soluble starch, and 0.5% tryptone addition. All strains were maintained as glycerol stocks at −80 °C. *Escherichia coli* strain XL1-Blue was used for plasmid multiplication, grown on Luria-Bertani (LB) agar plates, supplemented when necessary with ampicillin (100 μg/mL). *Saccharomyces cerevisiae* strain was used for yeast homologous recombination.

2.2. Sequencing and bioinformatic analysis

The genomic DNA (gDNA) of *P. thymicola* was prepared from mycelium stationary grown on PDA at 25 °C for 7 d. The mycelia (100 mg) was grinded in liquid nitrogen, and then gDNA was extracted from the resulting cell powder using cetyltrimethylammonium bromide (CTAB) method. Shotgun sequencing was performed at Novogene Bioinformatics Technology Co., Ltd. (Tianjin, China) using Illumina Hiseq2000 platform. Gene clusters were predicted by antiSMASH (https://fungismash.secondarymetabolites.org) and 2ndFind (http://biosyn.nih.go.jp/2ndfind) analysis. The amino acid sequences of AncA (the target NRPS encoded by *AncA* in *Anc* cluster) were submitted to the web-based DOMAIN SEARCH PROGRAM for NRPS (http://www.nii.ac.in/~zeeshan/search_only_nrps.html) to analyze the functional domain. The 10-residue specificity sequence (10AA code) of A domain in NRPS was exacted with NRPSpredictor2 server (http://abi-services.informatik.uni-tuebingen.de/nrps2/Controller?cmd=SubmitJob) (Rottig, Medema, Blin, Weber, Rausch, & Kohlbacher, 2011), and aligned with other NRPSs. Sequence alignment analysis of the active sites of C_T domains was performed using DNAMAN.

Table 1
Fungal strains and plasmids used in this study.

Strains/Plasmids	Description/Aim
A.n-*AncA*	Overexpression *AncA* in *A. nidulans*
Penicillium thymicola	Genomic origin
Aspergillus nidulans	*pyrG89, pyroA4, riboB2*, heterologous host
Saccharomyces cerevisiae	Yeast homologous recombination host
Escherichia coli XL1-Blue	Plasmids multiplication
pYTU	URA3, AMA1, *glaA::AfpyrG*, Amp
pYTP	URA3, AMA1, *amyB::AfpyroA*, Amp
pYTR	URA3, AMA1, *gpdA::AfriboB*, Amp
pYTU-*AncA*	*AncA* DNA in pANU under the control of *glaA* promoter

2.3. Gene amplification and plasmid construction

The plasmids utilized in this work were shown in Table 1. The PCR primers used were listed in Table 2. PCR reactions were performed with Q5® High-Fidelity DNA Polymerase (New England Biolabs). The gene sequence of *AncA* was divided into three parts and amplified from the genomic DNA of *P. thymicola*, respectively. The plasmid pYTU was digested with PacI and NotI. All of the fragments and linearized vector were gel purified using AxyPrep DNA Gel Extraction Kit (Axygen). The vector pYTU-*AncA* was constructed by *in vivo* yeast recombination. Three NRPS fragments and linearized vector pYTU were transformed into *S. cerevisiae* using yeast homologous recombination method as described by R Daniel Gietz subsequently (Gietz & Schiestl, 2007). All three DNA fragments for *in vivo* yeast recombination contained a minimum of 30 bp overlapping bases with the flanking fragments. The obtained yeast colonies were characterized by PCR. Yeast plasmids were isolated using Zymoprep™ (D2001) Kit (Zymo Research) and transformed into *E. coli* XL1-Blue cells. All plasmids were isolated using Axyprep Plasmid Miniprep Kit (Axygen) and confirmed by restriction enzyme digestion and sequencing.

2.4. Transformation of A. nidulans

A. nidulans strain was used as the recipient host. Fungal protoplast preparation and transformation were performed as the description from our laboratory (Yan et al., 2019). The plasmids pYTU-*AncA*, pYTR and pYTP were co-transformation into *A. nidulans* to own the *AncA* overexpression strain A.n-*AncA*. Co-Transformation with plasmids pYTU, pYTR and pYTP were used as control. Transformants were verified using diagnostic PCR with appropriate primers (Table 2).

2.5. Fermentation and LC-MS analysis

A. nidulans strain A.n-*AncA* was cultivated at 25 °C, 180 rpm in 5 mL liquid CD-ST medium (GMM liquid medium containing 20 g/L starch without glucose, 2% tryptone is added) for 5 d. The broth (2 mL) was extracted three times with ethyl acetate (EtOAc), the supernatant organic phase gave an oily residue (Labconco Corporation, Dry Evaporators, Concentrators & Cold Traps, MO, USA). The dried material was dissolved in 150 μL of acetonitrile (MeCN) and subjected to LC-MS analysis.

LC-MS were performed on a Waters ACQUITY H-Class UPLC-MS with QDA mass detector (ACQUITY UPLC® BEH, 1.7 μm, 50 mm × 2.1 mm, C_{18} column) using positive and negative mode electrospray ionization. LCMS grade MeCN and H_2O (both with 0.02% formic acid, volume percentage) were used as the mobile phases. The sol-

Table 2
PCR primer sets utilized in this study.

Primers		Oligonucleotide sequences (5′−3′)
For *AncA* amplification	AncA-P1	CTTCATCCCCAGCATC ATTACACCTCAGCATTAATTA AATGGCGGACTCTTGTTTATTT
	AncA-P2	GCCCACTTGCTCGTTAGG
	AncA-P3	CTTGCTGGAATTGAGGAGAT
	AncA-P4	GAAATAAAGTGGCACGAAAGT
	AncA-P5	GATTGTGAAGAAATGCCTCG
	AncA-P6	CTGCAGCCCGGGGGA TCCACTAGTTCTAGAGCGGCCG CGCGTCGTCGTAGATTGGAT
For transformant screening	AncA-test1-F	TTCCTGGAGACGAACTGGT
	AncA-test1-R	TAGGAGACCGCTTGATGTAG

vent gradient for LC-MS analyses changed as follows: 0–8 min 5%–99% MeCN, 8–12 min 99% MeCN, with a flow rate of 0.4 mL/min.

2.6. Extraction, purification and identification of product from heterologous expression

The desired product was purified from A.n-*AncA* 7 d cultures in 3 L liquid CD-ST medium. The aqueous phase was filtered from the fermentation broth and then extracted with analytical grade EtOAc (1:1, volume percentage) for three times to offer the EtOAc extract. After evaporation of the organic phase, the EtOAc extract was re-dissolved with HPLC grade MeOH and concentrated by centrifugation to give the MeOH-soluble extract (452.5 mg). The MeOH-soluble extract was fractionated by semi-preparative HPLC with a DAD detector (SSI series 1500, CoMetro Technology Ltd., NJ, USA) and using a Shiseido Capcell Pak 250 × 10 mm 5 μm C_{18} column. The target compound (**1**) was isolated by semi-prepared HPLC (MeCN: H_2O = 30: 70, flow: 3 mL/min, t_R = 14 min) with final yield of 35.23 mg/L. The obtained pure compound was performed NMR analysis at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR, on an Inova 500 instrument (Varian Associates Inc., Palo Alto, CA, USA) in $CDCl_3$ with solvent peaks used as references.

2.7. Biological assays

Cytotoxic activities against PC3 (human prostate cancer cell line), MDA-MB-231 (human breast cancer cell line), MCF-7 (human breast cancer cell line), A549 (human lung adenocarcinomic cell line), HL-60 (human leukemia cell line) and THP-1 (human leukemia cell line) cancer cell lines were assayed using the MTT method. All the cells were maintained in Hyclone 1640 or DMEM (low glucose) supplemented with 10% FBS and incubated at 37 °C, 5% CO_2. The cells were harvested using trypsin and diluted to 1.5–3.0 × 10^4/mL, then seeded 100 μL of cells per well in 96-well plates and incubated 24 h. Different concentrations (final concentrations were 0.625, 1.25, 2.5, 5 and 10 μmol/L) of compound **1** were added into the wells in triplicate and then incubated for 96 h. After added 20 μL of MTT (5 mg/mL) into each well, the plates were continue incubated for an additional 4 h. The cells were gently replaced by 200 μL of DMSO and treated on shaker for 10 min, the absorbance was measured at OD = 570 nm with reference at OD = 630 nm on a microplate reader (Synergy-HT, Bio-Tek) subsequently. A total of 200 μL of DMSO was used as control.

3. Results

3.1. Identification of Anc gene cluster containing a tri-modular NRPS by bioinformatic analysis

To identify more peptityl alkoilds from fungi, we analyzed gene clusters containing NRPSs from the sequenced *P. thymicola* genome, and found a gene cluster with a single tri-modular NRPS (named *Anc* cluster). The *Anc* cluster encodes a NRPS (AncA) with a domain sequence of A_1-T_1-C_1-A_2-T_2-C_2-A_3-T_3-C_T, and there are no genes encoding redox enzymes around *AncA* (Fig. 1), which suggested that *AncA* has potential to activate three amino acids and yield a tripeptide alkaloid without any modification.

Prediction of the 10AA code in AncA is useful for identification of the putative ant-activating modules. The amino acid sequence of AncA was submitted to the web-based NRPSpredictor2, and the 10AA code of the first A domain was extracted as GIILlAAGIK. The 10AA codes of AncA and other selected A domains were compared in Table 3. The 10AA code of AncA (A1) was similar to NFIA_057960 (A1) (90%), AldpA (A1) (90%), CtqA (A1) (90%) and AFUA_6g12080 (A1) (80%). Compared with 10AA codes of other nine fungal A domains (A1), the residues positions (Pos) 1 and 8 of AncA were strict conservation glycines, and the distinct residues at positions 2, 4, 5 and 9 are variable hydrophobic residues, which were in consistent with the conclusion (Ames & Walsh, 2010). Thus, module 1 (A_1-T_1) of AncA was likely to be responsible for selection, activation, and loading of anthranilic acid. AncA was proposed to activate anthranilate (Ant), and two other amino acids, to yield a tripeptide started with Ant (Table 3).

Sequence alignment analysis of the active sites of C_T domains in AncA with other fungal NRPSs C domains (using the C_T domains from AFUA_6g12080, TqaA, AldpA and CtqA, the fungal NRPSs which can produce macrocyclic peptidyl products) were shown in Fig. 2. The C_T domain in AncA contained highly conserved HXXXDXXS motif, and was responsible for the macrocyclization reaction (Gao et al., 2012).

3.2. Heterologous expression of AncA in A. nidulans led to production of anacine

To investigate the product encoded by this tri-modular NRPS, we expressed the putative gene cluster in *A. nidulans*, an established heterologous expression system for production of cryptic natural products. The gene *AncA* was amplified from gDNA and then cloned directly into pYTU which contains an inducible strong *glaA* promoter using yeast recombination. Following PCR verification of the integration of pYTU-*AncA*, the strain A.n-*AncA* was cultivated in CD-ST media, and the metabolites were extracted and analyzed by LC-MS (Fig. 3A), where a new metabolite (t_R = 3 min) with m/z = 343 $[M + H]^+$ was observed compared to the control

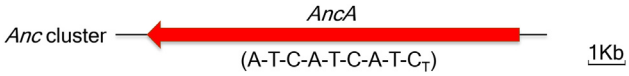

Fig. 1. Gene cluster of *Anc*.

Table 3
Comparison of 10-residue specificity sequences for selecting NRPS adenylation domains.

Names (module)	Pos1 (235)	Pos2 (236)	Pos3 (239)	Pos4 (278)	Pos5 (299)	Pos6 (301)	Pos7 (322)	Pos8 (330)	Pos9 (331)	Pos10 (517)
NFIA_057960(A1)	G	I	I	L	G	A	A	G	I	K
AnaPS/NFIA_055290(A1)	G	A	L	F	F	A	A	G	V	K
ACLA_017890(A1)	G	V	I	F	L	A	A	G	V	K
ACLA_076770(A1)	G	V	I	F	V	A	G	G	V	K
ACLA_095980(A1)	G	V	I	I	L	A	G	G	L	K
TqaA (A1)	G	V	I	F	M	A	A	G	V	K
AFUA_6g12080(A1)	G	V	I	I	L	A	A	G	I	K
AldpA(A1)	G	I	I	L	G	A	A	G	I	K
CtqA (A1)	G	I	I	L	G	A	A	G	I	K
AncA (A1)	G	I	I	L	L	A	A	G	I	K
Consensus[a]	G	X_h	I/L	X_h	X_h	A	A/G	G	X_h	K

[a] The abbreviation "X_h" stands for variable hydrophobic residues.

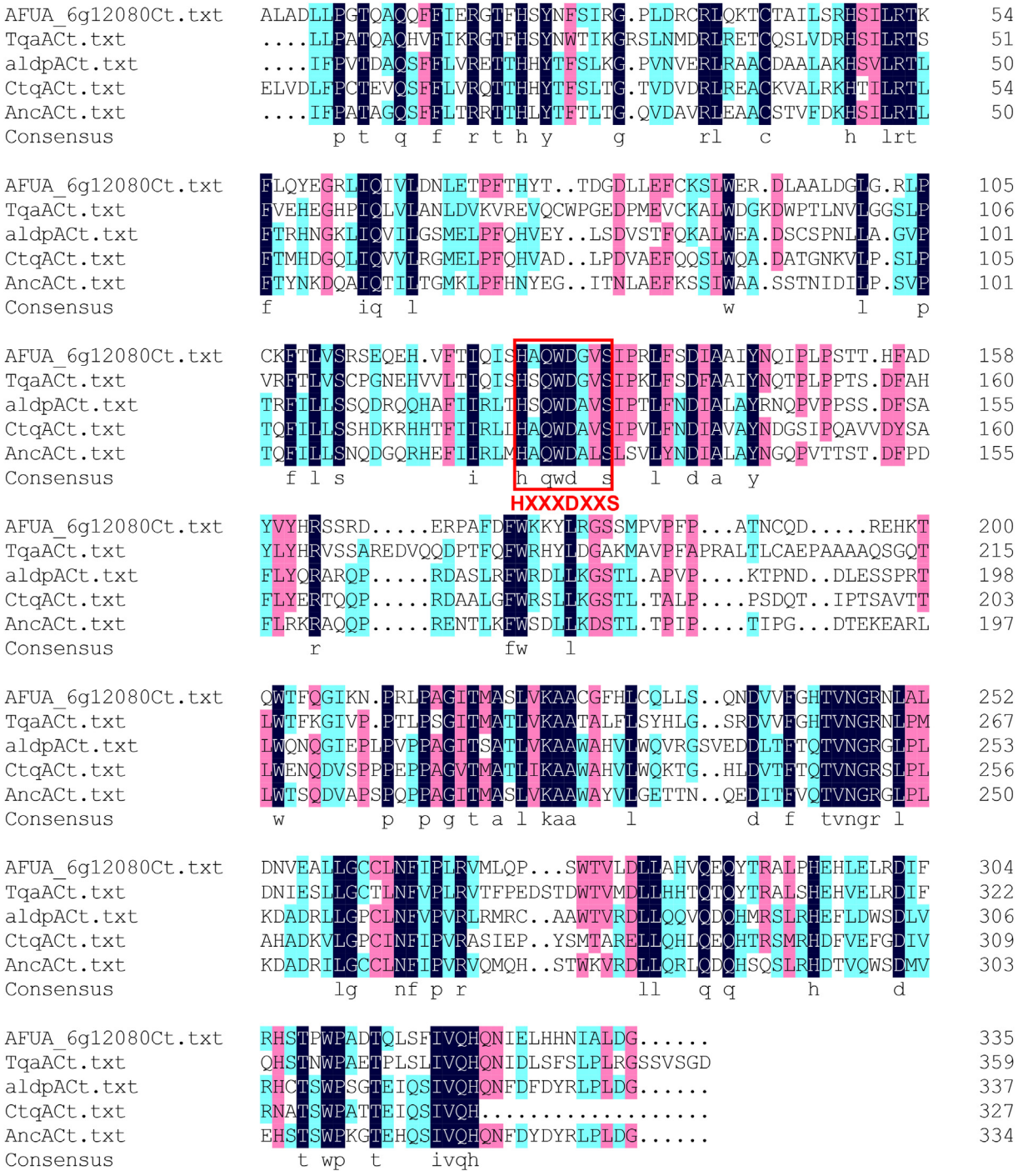

Fig. 2. Sequence alignment analysis of active sites of C_T domain with other fungal NRPSs C domains.

(Fig. 3C). The new metabolite (**1**) was purified as amorphous powder (452.5 mg) from the crude extract and fully characterized by NMR analyses (Fig. 4). The 1D NMR spectra data (Table 4) indicated the presence of one *ortho*-disubstituted benzene ring, three carbonyls, four aromatic protons, three methines, three methylenes, and two methyl groups. Also, the ^{13}C NMR data (Table 4) also revealed the presence of two olefinic groups at δ_C 126.6, 127.0, 127.3, and 135.1. Comparison of the observed and reported spectroscopic data, compound **1** was identified as anacine, a known natural compound previously found in *P. aurantiogriseum* (Wang & Sim, 2001). Based on the specific optical rotation ([α]24 D + 142.9, *c* 0.02 in MeOH), the absolute configuration of **1** could be confirmed. The identified configuration of **1** was also consistent with the feature that no E domain in the tri-modular NRPS AncA. Although **1** was identified as known compound, heterogeneous expression was able to obtain sufficient amount of material for biological assay.

3.3. Biological evaluation of anacine (1)

The cytotoxicities of anacine (**1**) were evaluated against a series of cancer cell lines by MTT method, including PC3, MDA-MB-231 and MCF-7, A549, as well as HL-60 and THP-1, with doxorubicin as positive control. Anacine showed moderate cytotoxic activity

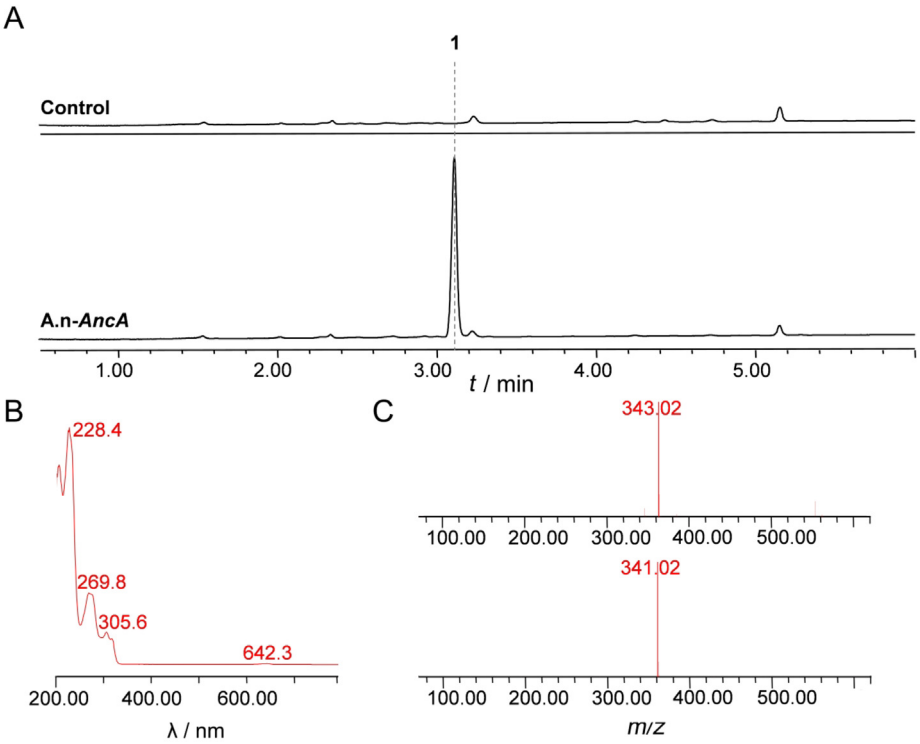

Fig. 3. LC-MS analysis of *A. nidulans* heterologous transformants. LC-MS analysis of *A. nidulans* strains (A). UV absorption of compound **1** (B). Positive and negative masses of compound **1** (C), *m/z* 343.02 $[M + H]^+$, 341.12 $[M - H]^-$.

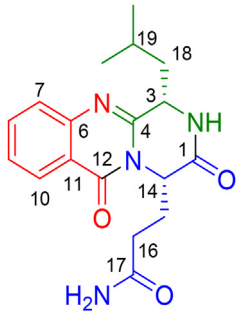

Fig. 4. Structure of compound **1**.

against A549 cell (IC_{50} = 17.3 μmol/L), which was weaker than that of positive control (IC_{50} = 0.1 μmol/L). Anacine was inactive against other tested cancer cell lines.

4. Discussion and conclusion

Anacine was firstly identified as benzodiazepine alkaloids from *P. aurantiogriseum*. Later investigation indicated that anacine had a quinazoline instead of a benzodiazepine core, which was further confirmed by total synthesis (Wang & Sim, 2001). Biosynthetically, it can be synthesized by a tri-modular NRPS with three adenylation domains that incorporates anthranilate (Ant), *L*-glutamine (*L*-Gln) and *L*-leucine (*L*-Leu). The proposed biosynthetic mechanisms of anacine (**1**) were shown in Fig. 5. Non-ribosomal peptide synthetase AncA with three modules are responsible for the assembly of the tricyclic scaffold via a process from recognition of Ant to upload the $_L$-Gln and $_L$-Leu in turn. Nucleophilic attack from Ant-free amine to the thioester carbonyl contributes to achieve cyclization and release of the product. As the final step, there are two possible routs of cyclization catalyzed by C_T domain in the formation of anacine (**1**). In route A, the Ant amine attacks on the thioester carbonyl to yield a macrocyclic intermediate, which can later give rise to the ancine (**1**) through a bridging amide bond cyclization and dehydration. In route B, the *L*-Gln amide act as an initial nucleophile attacks on the thioester carbonyl to yield a diketopiperazine (DKP) intermediate. Subsequently, The Ant amine and the *L*-Leu amide carbonyl undergo intramolecular attack and dehydration to form anacine (**1**).

Anacine has been reported to be produced by strain *P. aurantiogriseum* with a yield of 0.4 mg/L (Boyes-Korkis et al., 1993; Xin

Table 4
^1H and ^{13}C NMR data of compound 1 ($CDCl_3$)[b].

Positions	δ_C	δ_H (J/Hz)
1	168.3	/
2	/	/
3	54.9	4.62 dt (9.6, 4.6)
4	151.2	/
5	/	/
6	147.0	/
7	127.0	7.65 d (8.3)
8	135.1	7.75 t (8.3, 7.1)
9	127.3	7.47 t (7.8, 7.1)
10	126.9	8.23 d (7.8)
11	119.4	/
12	160.9	/
13	/	/
14	54.9	5.21 dd (10.0, 5.4)
15a	29.5	2.38 m
15b	/	2.22 m
16	32.4	2.68 t (7.1)
17	174.2	/
18	47.2	1.93 m
19	24.8	1.93 m
20	23.4	1.05 dt (6.2, 3.0)
21	21.3	1.05 dt (6.2, 3.0)

[b] ^1H and ^{13}C spectra were obtained at 500 and 125 MHz, respectively.

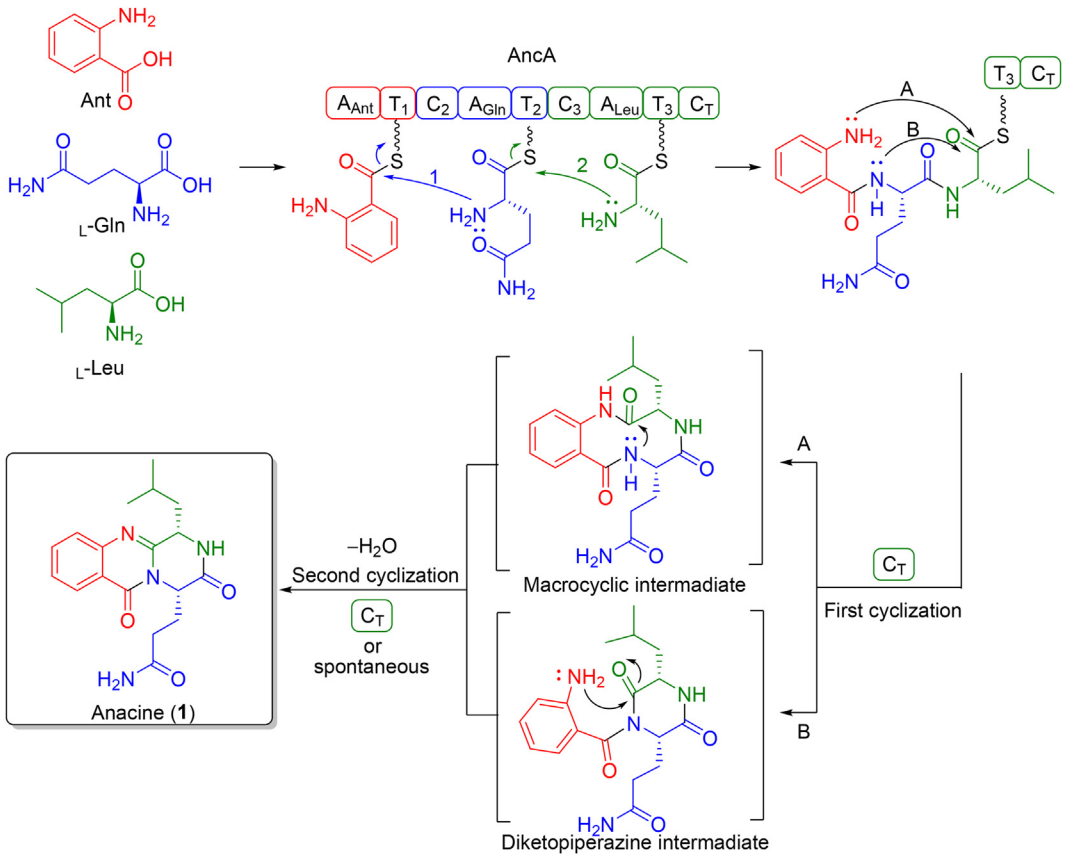

Fig. 5. Proposed biosynthetic pathway of anacine (**1**).

et al., 2007). Here, we obtained this compound with high yield (35.23 mg/L) through concise biosynthesis by only one single tri-modular NRPS. Our research also supported that heterologous biosynthesis is an efficient strategy for mining cryptic gene clusters from fungi and to obtain enough material of minor secondary metabolites.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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