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Design, synthesis and antifungal activity evaluation of isocryptolepine derivatives

Jun-cai Li[a,1], Ren-xuan Wang[a,1], Yu Sun[a], Jia-kai Zhu[a], Guan-fang Hu[b], Yu-ling Wang[b], Rui Zhou[a], Zhong-min Zhao[a], Ying-qian Liu[a,*], Jing-wen Peng[a], Yin-fang Yan[a], Xiao-fei Shang[a]

[a] School of Pharmacy, Lanzhou University, Lanzhou 730000, People's Republic of China
[b] Institute of Plant Protection, Gansu Academic of Agricultural Sciences, Lanzhou 730070, People's Republic of China

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ABSTRACT

In this paper, the nitrogen atom was inserted into the anthracycline system of the isocryptolepine nucleus to obtain the "Aza"-type structure benzo[4,5]imidazo[1,2-c] quinazoline. A series of "Aza"-type derivatives were designed, synthesized and evaluated for their antifungal activity against six plant fungi *in vitro*. Among all derivatives, compounds **A-0**, **B-1** and **B-2** showed significant antifungal activity against *B. cinerea* with the EC_{50} values of 2.72 μg/mL, 5.90 μg/mL and 4.00 μg/mL, respectively. Compound **A-2** had the highest activity against *M. oryzae* with the EC_{50} values of 8.81 μg/mL, and compound **A-1** demonstrated the most control efficacy against *R. solani* (EC_{50}, 6.27 μg/mL). Moreover, compound **A-0** was selected to investigate the *in vivo* tests against *B. cinerea* and the results indicated that the preventative efficacy of it up to 72.80% at 100 μg/mL. Preliminary mechanism studies revealed that after treatment with **A-0** at 5 μg/mL, the *B. cinerea* mycelia appeared curved, collapsed and the cell membrane integrity may be damaged. The reactive oxygen species production, mitochondrial membrane potential and nuclear morphometry of mycelia have been changed, and the membrane function and cell proliferation of mycelia were destroyed. Compounds **A-0**, **A-1**, **B-1** and **B-2** presented weaker toxicities against two cells lines than isocryptolepine. This study lays the foundation for the future development of isocryptolepine derivatives as environmentally friendly and safe agricultural fungicides.

1. Introduction

Fungi have been known to be an extensive threat to plant species for a long time which account for more than 70% of plant diseases, and pathogenic fungi cause about 10% of the world's major crops to be reduced each year [1–3]. Moreover, the fungal are easy to spread with air and water flow which increases the difficulty of their prevention [4]. Currently, people are mainly using chemical agents for prevention and control of fungal diseases of crops in agricultural production. But the long-term and increasing application of chemical fungicides caused ever-increasing resistance [5]. Therefore, it is necessary to develop novel scaffolds or action mode fungicides for controlling these crop diseases.

Natural products have been a rich source and are the leading inspiration for the development of new drugs [6]. Compared to traditional chemical synthetic pesticides, these natural products based pesticides showed lower toxicity and selectivity or specificity to the target organisms, which also have better environmental compatibility, which meets the requirements of new pesticides [7–10]. According to statistics published by Agranova last year, natural medicines and biomimetic drugs derived from natural products of fungicides are accounted more than 50% in the global pesticide market composition in 2016. It indicates that active natural products play a pivotal role in the discovery of new pesticides by serving as novel scaffolds, and it is efficiency to modify natural active structures in the development of new drugs [11], such as the discovery of the strobilurin fungicides [12]. Based on these results, we selected natural product as the scaffold to develop new candidate fungicides.

Isocryptolepine, cryptolepine and neocryptolepine are naturally occurring isomeric indoloquinoline alkaloids isolated from the *Cryptolepis sanguinolenta*, a plant that is used in Central and West Africa. These alkaloids are exhibited a wide range of biological activities such as antimalarial, antihyperglycemic, antibacterial, antifungal, antitumoral, anti-inflammatory, hypotensive, antithrombotic and vasodilation [13–19]. Recently, the significant antifungal activities of some this kind of alkaloids have been reported. For example, cryptolepine

* Corresponding author.
E-mail address: yqliu@lzu.edu.cn (Y.-q. Liu).
[1] These authors contributed equally to this work.

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presented the antimicrobial activity against *Campylobacter* strains with the MIC_{50} values of 6.25 μg/mL, and cryptolepine hydrochloride demonstrated the bactericidal and antimicrobial activity against *S. aureus* [20,21]. Nevertheless, because of the scarcity and high toxicity, the applications of cyptolepine are restrained [22,23]. During our previous works, we discovered that the antifungal activity of neocryptolepine derivatives against plant pathogens [24]. In this study, our research team selected isocryptolepine as the lead compound, which has a skeleton analogous to cryptolepine. The replacement of a CH group with an N atom in aromatic and heteroaromatic ring systems to get the "Aza"-type structures can produce many important effects on molecular and physicochemical properties and intra- and intermolecular interactions that can translate to improved pharmacological profiles. The "N" atom is shown to be a gifted significant impact factor for multiparameter optimization [25–27].

In this paper, we were going to find more efficiency and ecofriendly compounds against plant pathogenic fungi. To lay the foundation for subsequent researches, the benzimidazole was used as intermediate, the "Intermediate Derivatization Strategy" was adopted to design and synthesize the "Aza"-type isocryptolepine analogous by introducing different substituents into the ring A, B and D to explore the effects of different substitutions on the antifungal activity and discuss the structure-activity relationships for guiding the development of novel isocryptolepine-based candidate fungicides. Finally, we investigated the mode of action of this type against *B. cinerea* by scanning electron microscope observations (SEM), testing the production of reactive oxygen species (ROS), mitochondrial membrane potential (MMP) and nuclear morphology.

2. Chemistry

As shown in Scheme 1, the benzimidazole was used as a synthetic intermediate in the synthesis of the target compounds. The modified isocryptolepine derivatives with small substituent groups such as halogen at ring A and D were synthesized and evaluated their antifungal activity according to the previous described methods [28]. Intermediate **Z-1** to **Z-6** were synthesized by condensation reaction of *O*-phenylenediamine and benzoic acids under the condition of PPA [29]. Under this reaction condition, a cyclization reaction occurs when C-2 of benzoic acid is an amino group, thus the target compound **A-38** was obtained [30]. And compound **A-0** was obtained by cyclization reaction with NaN_3, *p*-toluenesulfonic acid and *tert*-butyl hydroperoxide in DMA under the catalysis of CuI [28].

Subsequently, we modified ring B of compound **A-0** for investigating their antifungal activity. Scheme 1 and Table 1 presented the synthesized compounds **A-4** to **A-37** with different ring B substituents. As illustrated in Table 1, the hydrogen atom at ring B (C-8 position of the ring system) was replaced by introducing cyclopropyl, phenyl, substituted phenyl groups, thiazolyl, tetrahydrofuranyl, and thienyl groups to give compounds **A-4** to **A-37**, respectively [31,32]. Compounds **B-1** to **B-5** were synthesized as the same manner of compound **A-0**. The structures of these compounds were confirmed by ESI-MS, ^1H NMR and ^{13}C NMR, and the purity of the target compounds were checked by HPLC analysis (Supplemental Material).

3. Results and discussion

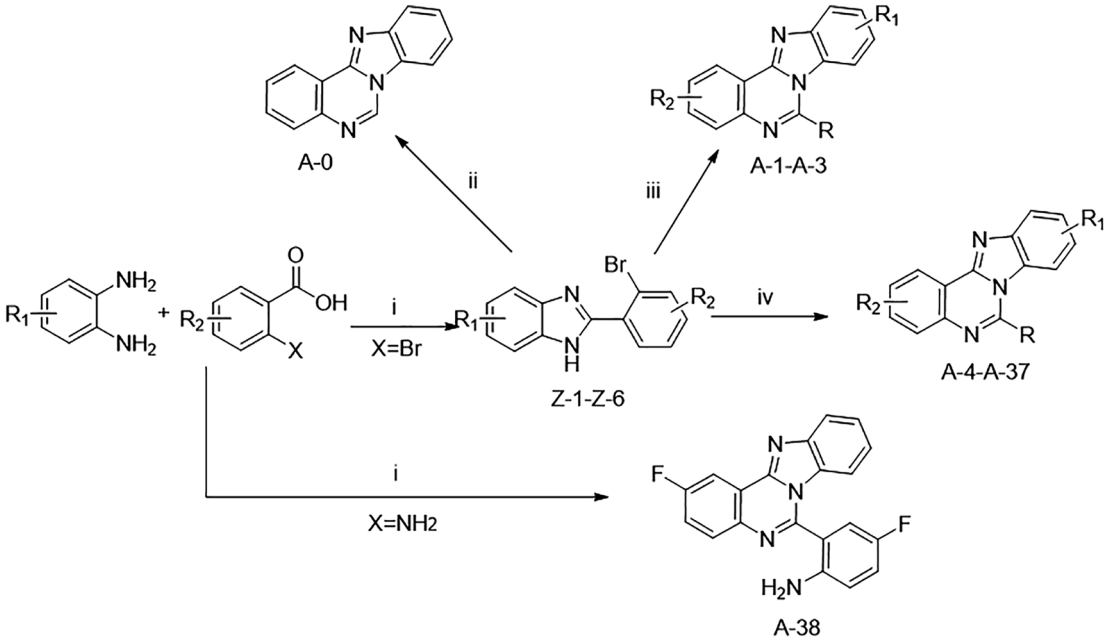
3.1. Effects of compounds on mycelial growth in vitro

3.1.1. In vitro antifungal activities of compounds A-0 to A-38

According to the results of biological activity evaluation (Fig. 1), demethylated isocryptolepine exhibited better antifungal activities than isocryptolepine, and its antifungal activities were further enhanced after the introducing of N atom into its skeleton. Which means that it's feasible to introducing the N atom to this heteroaromatic ring systems, and this study carries out further structural derivation based on the "Aza" type skeleton. A series of isocryptolepine "Aza"-type compounds derivatives were firstly designed, synthesized and evaluated the antifungal bioactivity of the compounds **A-1** to **A-38** for investigation.

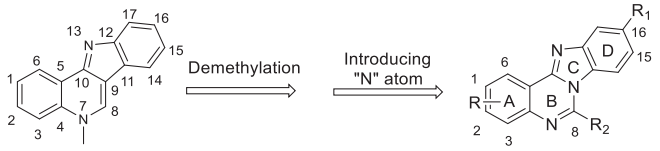
Through the mycelium growth rate tests, the antifungal activity of compounds **A-0** to **A-38** against six pathogenic fungi at the concentrations of 100 μg/mL and 50 μg/mL and the results were shown in Table 2.

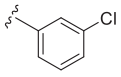
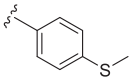
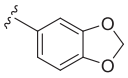
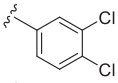
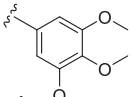
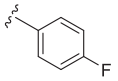
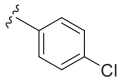
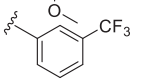
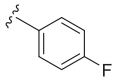
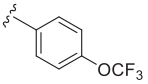
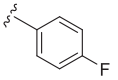
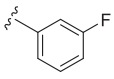
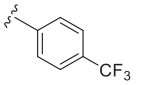
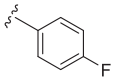
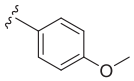
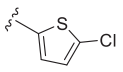
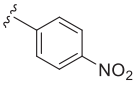
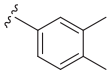
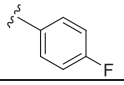
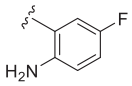
For *P. zeae*, compounds **A-0** and **A-2** presented the significant antifungal activity, the inhibition rates were 100.00%, 100.00%, and 99.31%, 99.15% at the concentrations of 100 μg/mL and 50 μg/mL,



Scheme 1. General synthesis of isocryptolepine "Aza" type derivatives. Reagents and conditions: (i) PPA, 140°C, 6 h; (ii) NaN_3, CuI, *p*-toluenesulfonic acid, *tert*-butyl hydroperoxide, DMA, 130°C, 10 h; (iii) Guanidine hydrochloride/Amidine hydrochloride, CuI, K_3PO_4, DMF, 100°C, 16 h; (iv) Substituted aldehyde, NaN_3, Cu powder, L-proline, Cs_2CO_3, DMF, 80°C, 16 h.

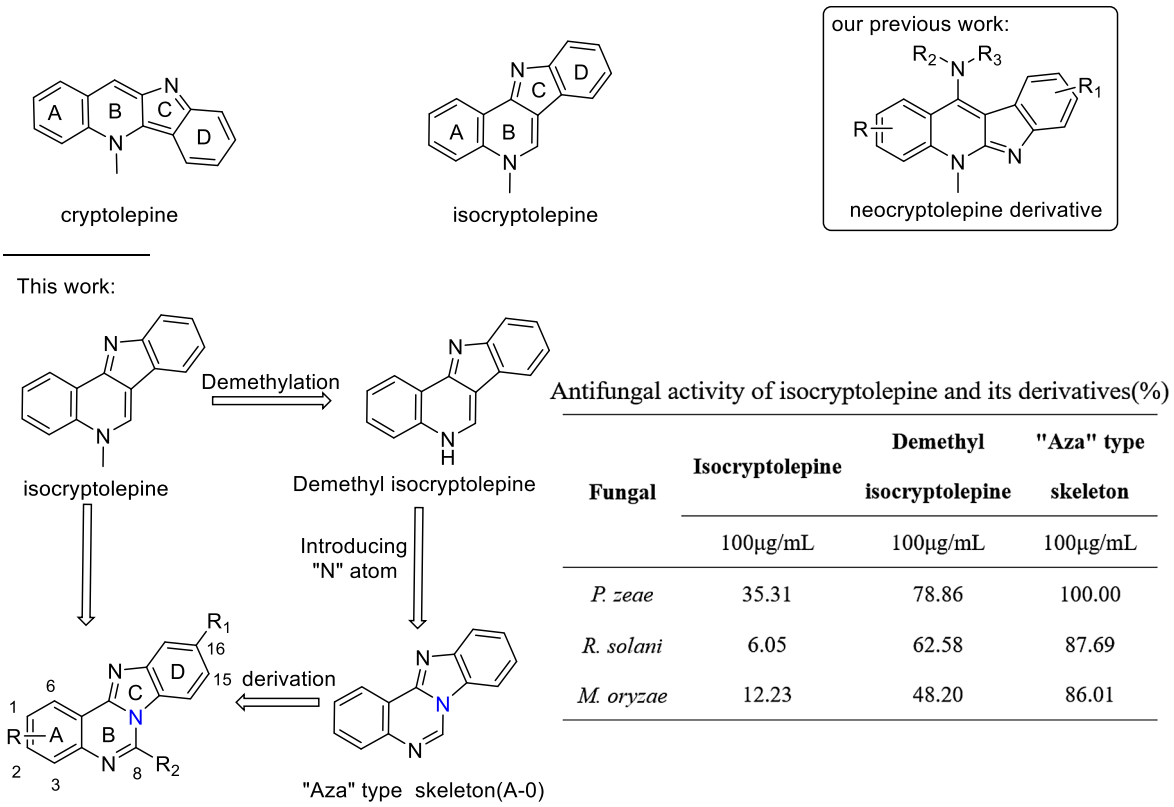
Table 1
Structures of the synthesized compounds.



No.	R	R_1	R_2	No.	R	R_1	R_2	No.	R	R_1	R_2
A-0	H	H	H	A-14	1-F	H		A-27	1-F	F	
A-1	H	H	-NH_2	A-15	1-F	H		A-28	1-F	F	
A-2	H	H	-Me								
A-3	1-F	H	-Me	A-16	1-F	H		A-29	1-F	H	
A-4	H	H		A-17	1-F	H		A-30	1-F	H	
A-5	1-F	H		A-18	1-F	H		A-31	1-F	F	
A-6	1-F	H		A-19	1-F	H		A-32	3-F	H	
A-7	1-F	H		A-20	1-F	H		A-33	3-F	H	
A-8	1-F	H		A-21	1-F	H		A-34	3-F	H	
A-9	1-F	H		A-22	1-F	H		A-35	1,2-di-F	H	
A-10	1-F	H		A-23	1-F	H		A-36	H	H	
A-11	1-F	H		A-24	1-F	H					
A-12	1-F	H		A-25	1-F	H		A-37	H	H	
A-13	1-F	H		A-26	1-F	F		A-38	1-F	H	

respectively. However, the inhibition rates of the positive control azoxystrobin were 68.17% and 83.33%. Similarly, these two compounds also demonstrated better antifungal activity against *B. cinerea*, and the inhibition rates were 87.13%, 85.46%, and 98.48%, 88.00%, respectively. As the positive control, the control efficiency of azoxystrobin against *B. cinerea* were lower than 50% at two concentrations. At the same time, compound **A-0** showed the remarkable antifungal activity against *M. oryzae* of 90.85% and 87.69%, and compound **A-2** with a methyl at C-8 position were 83.98% and 86.54%, which are more potent than azoxystrobin (the inhibition rates are 43.36% and 28.75%). However, compound **A-1** with an amino at R_2 group showed the best antifungal bioactivity against *R. solani* and *S. sclerotiorum* with the inhibition rate of 86.60% and 85.51%, respectively at 100 μg/mL, and the control efficiency were not decreased at 50 μg/mL, while azoxystrobin were 59.09%, 48.33%, and 55.70%, 74.58%. Meanwhile, compound **A-0** demonstrated the most notable antifungal activity against *Fusarium oxysporum f. sp. vasinfectum* of 89.51% and 90.51% at the same concentrations, while azoxystrobin were 55.95% and 52.50%. Base on this investigation, some interesting discoveries of the structure–activity relationships were summarized.

The isocryptolepine "Aza" type derivatives **A-0** to **A-3** which R_2 groups were substituted by amino, H atom and methyl demonstrated moderate to high antifungal activity against all the tested fungi strains. In detail, the results of the antifungal assay showed that the substituent of R_2 at Ring B which is C-8 position of the ring system has a significant effect. When there is H atom or methyl at R_2, the antifungal activities were enhanced. The amino substitution also has better inhibitory activity. But when the C-8 position (R_2) was substituted by an aromatic



Antifungal activity of isocryptolepine and its derivatives(%)

Fungal	Isocryptolepine	Demethyl isocryptolepine	"Aza" type skeleton
	100μg/mL	100μg/mL	100μg/mL
P. zeae	35.31	78.86	100.00
R. solani	6.05	62.58	87.69
M. oryzae	12.23	48.20	86.01

Fig. 1. Design of isocryptolepine "Aza" type skeleton.

substituent group, the biological activities of the derivatives against six pathogenic fungi become weak. Compared with phenyl group (compound **A-5**), when the C-8 position substituted by aromatic heterocyclic groups (compounds **A-6**, **A-13**, **A-14** and **A-15**) or alicyclic group (compound **A-16**) was introduced, the antifungal activities couldn't be enhanced.

When it comes to the ring A, as can be seen in Table 3, the antifungal activity was affected by both the electron properties and the substituted positions. The number of the electron-withdrawing groups and its substituted position on the ring A generated negative effects on its antifungal activity while the R_2 was substituted by 4-fluorophenyl (Table 2, compounds **A-8**, **A-32**, **A-35**, **A-36**). And the inhibitory rate of them against *M. oryzae* increased following H > 3-F > 1,2-di-F > 1-F at the concentration of 100 μg/mL. However, the effects were different when the R_2 group was changed (Table 2, **A-6**, **A-34**, **A-38**). Moreover, the substitution of fluorine at the C-15/C-16 position of the ring D don't exhibit a significant effect on its antifungal activities (Table 2, **A-6**, **A-8**, **A-14**, **A-15**, **A-26**, **A-27**, **A-28** and **A-31**).

In view of these results, as shown in Scheme 2, we designed and synthesized compounds **B-1** to **B-5** with the R_2 group substituted by H atom based on the structure of compounds **A-0** to **A-3** for further investigation Table 3.

3.1.2. *In vitro antifungal activities of compounds B-1 to B-5*

According to the antifungal activity results in Tables 2 and 3, compounds **B-1** to **B-5** exhibited better antifungal activity than the compounds **A-4**-**A-38** at the concentrations of 100 μg/mL and 50 μg/mL. The inhibition rates of them against six pathogenic fungi are mostly higher than 50% at the concentration of 100 μg/mL. The number and substituted positions of fluorine atoms on Ring A have significant effect on its antifungal activity and the antifungal activity of the compound with monofluoro substitution of ring A is superior to that of multiple fluorine substitutions (Tables 3 and 5, **B-1** to **B-4**). Meanwhile, as shown in Tables 3 and 5, the fluorine atom on ring D was not essential to the activity. Hence, compared with **B-2**, the antifungal efficacy of **B-5** against six pathogenic fungi were declined.

Among all the derivatives of **A-0** to **A-38** and **B-1** to **B-5**, the most potent activities against *P. zeae*, *R. solani*, *B. cinerea*, *M. oryzae*, *S. sclerotiorum*, *F. oxysporum f. sp. vasinfectum* were compounds **B-3** (EC_{50}, 4.77 μg/mL), **A-1** (EC_{50}, 6.27 μg/mL), **A-0** (EC_{50}, 2.72 μg/mL), **A-2** (EC_{50}, 8.81 μg/mL), **B-1** (EC_{50}, 15.88 μg/mL) and **B-2** (EC_{50}, 8.96 μg/mL) respectively, while the EC_{50} values of the positive control azoxystrobin are > 50 μg/mL, 18.36 μg/mL, > 100 μg/mL, 34.43 μg/mL, 16.96 μg/mL and > 50 μg/mL (Table 4 and Table 5). These results suggested that the small groups like H atom and methyl are very important for the antifungal activity. And introducing the F atom in ring A at different positions would make it selective to different fungi.

3.2. *In vivo antifungal activity of compound A-0 against B. Cinerea*

Compound **A-0** which demonstrated the best antifungal activity during the *in vitro* tests was evaluated against *B. cinerea* for the *in vivo* antifungal efficacies tests, and the efficacy was compared with the commercial fungicide Boscalid. Table 6 presents the results of the *in vivo* tests. The preventative efficacy of compound **A-0** were 72.07%, 58.82% and 52.40% at the concentrations of 100 μg/mL, 50 μg/mL and 25 μg/mL respectively, whereas Boscalid were 82.03%, 72.86% and 64.80%. These results indicated that compound **A-0** presents preventative efficacy to tomato fruits, and its effects exhibited concentration-dependent properties. It is noteworthy that there is no obvious phytotoxicity on the fruits after treated with compound **A-0** at 100 μg/mL from Fig. 2. Therefore, it was suggested that the tested compounds showed a certain application prospect.

3.3. *Effect of compound A-0 on the morphology of B. Cinerea*

The morphological changes of *B. cinerea* hyphae treated by compound **A-0** were observed under scanning electron microscope (SEM),

Table 2
Antifungal activity of isocryptolepine analogues against six plant pathogenic fungi (%)(μg/mL).

No.	Con.	*P. zeae*	*B. cinerea*	*S. sclerotiorum*	*M. oryzae*	*R. solani*	*F. oxysporum f. sp. vasinfectum*
A-0	100	**100.00**	**87.13 ± 0.87**	**77.64 ± 0.17**	**90.85 ± 0.31**	**86.01 ± 0.32**	**89.51 ± 0.19**
	50	**100.00**	**85.46 ± 0.37**	**77.35 ± 0.69**	**87.69 ± 0.29**	**78.58 ± 0.11**	**90.51 ± 0.84**
A-1	100	**94.60 ± 0.67**	38.13 ± 0.79	**85.01 ± 1.98**	**76.50 ± 0.62**	**86.60 ± 0.11**	**74.81 ± 1.34**
	50	**92.63 ± 0.73**	39.38 ± 1.47	**85.56 ± 2.06**	**77.83 ± 0.84**	**86.66 ± 1.19**	**74.57 ± 1.78**
A-2	100	**99.31 ± 0.39**	**98.48 ± 0.37**	**98.81 ± 1.58**	**83.98 ± 0.16**	**78.47 ± 0.35**	**67.94 ± 0.51**
	50	**99.15 ± 0.46**	**88.00 ± 0.77**	**61.36 ± 4.18**	**86.54 ± 0.29**	**73.01 ± 1.31**	**67.02 ± 0.68**
A-3	100	**98.16 ± 0.14**	**74.65 ± 0.28**	61.68 ± 0.50	**73.80 ± 0.35**	**65.75 ± 1.06**	**64.91 ± 0.22**
	50	**86.00 ± 3.64**	**58.94 ± 0.40**	41.90 ± 0.72	**78.84 ± 0.42**	**61.13 ± 0.29**	**63.52 ± 0.76**
A-4	100	0.00	12.78 ± 1.28	0.00	32.14 ± 0.89	27.03 ± 0.69	13.92 ± 0.62
	50	0.00	13.65 ± 1.47	0.00	30.61 ± 1.35	0.00	9.87 ± 0.30
A-5	100	15.11 ± 2.75	16.16 ± 0.34	0.00	36.00 ± 1.03	34.10 ± 0.45	10.75 ± 0.18
	50	0.00	13.93 ± 0.42	0.00	31.88 ± 1.17	25.80 ± 0.95	11.06 ± 0.51
A-6	100	58.49 ± 1.07	56.59 ± 0.16	0.00	35.09 ± 0.32	41.05 ± 0.47	24.74 ± 0.61
	50	42.91 ± 1.11	29.14 ± 0.87	0.00	33.65 ± 0.27	33.47 ± 1.34	19.08 ± 0.64
A-7	100	0.00	0.00	0.00	22.40 ± 0.65	10.81 ± 0.89	0.00
	50	0.00	0.00	0.00	19.69 ± 0.67	18.74 ± 3.15	0.00
A-8	100	0.00	0.00	0.00	24.66 ± 0.51	0.00	14.70 ± 2.13
	50	0.00	0.00	0.00	21.85 ± 0.56	0.00	0.00
A-9	100	0.00	0.00	0.00	29.21 ± 0.22	26.31 ± 2.73	12.48 ± 0.98
	50	0.00	0.00	0.00	28.65 ± 0.45	17.64 ± 0.94	15.09 ± 1.92
A-10	100	0.00	7.61 ± 1.24	0.00	24.40 ± 0.77	23.09 ± 0.83	0.00
	50	0.00	10.55 ± 1.27	0.00	31.72 ± 0.84	11.51 ± 0.41	0.00
A-11	100	35.49 ± 1.13	15.52 ± 2.05	0.00	33.35 ± 0.38	18.00 ± 0.64	18.54 ± 0.53
	50	0.00	17.28 ± 1.75	0.00	30.31 ± 0.41	15.25 ± 0.47	8.08 ± 0.46
A-12	100	19.48 ± 1.71	19.25 ± 1.57	0.00	32.48 ± 1.02	22.95 ± 0.10	16.25 ± 0.27
	50	31.08 ± 0.21	0.00	0.00	20.40 ± 1.18	28.35 ± 1.02	26.38 ± 0.61
A-13	100	36.78 ± 1.66	6.91 ± 0.57	0.00	16.49 ± 2.55	28.38 ± 0.98	30.40 ± 0.52
	50	25.61 ± 0.27	0.00	0.00	16.86 ± 1.94	27.02 ± 1.00	27.29 ± 0.72
A-14	100	24.05 ± 0.39	0.00	0.00	20.27 ± 2.16	28.49 ± 0.93	28.20 ± 1.07
	50	0.00	0.00	0.00	11.34 ± 0.29	38.28 ± 1.28	31.14 ± 0.61
A-15	100	33.11 ± 0.40	5.59 ± 0.38	0.00	20.43 ± 1.08	47.99 ± 0.35	29.73 ± 0.22
	50	44.71 ± 0.74	7.35 ± 0.68	0.00	17.68 ± 1.82	45.65 ± 0.36	30.19 ± 2.25
A-16	100	0.00	2.26 ± 1.40	0.00	20.03 ± 1.53	33.49 ± 1.75	25.13 ± 0.69
	50	0.00	12.40 ± 2.57	0.00	18.95 ± 0.94	34.75 ± 1.91	28.34 ± 0.17
A-17	100	25.92 ± 0.28	16.33 ± 0.52	19.98 ± 0.26	16.55 ± 0.35	56.48 ± 0.82	44.60 ± 0.47
	50	14.20 ± 0.60	6.06 ± 1.20	0.00	13.37 ± 0.31	46.40 ± 1.51	39.18 ± 1.67
A-18	100	0.00	6.50 ± 0.28	0.00	11.61 ± 0.83	21.45 ± 0.37	36.38 ± 0.42
	50	0.00	0.00	0.00	19.60 ± 2.71	10.85 ± 1.23	0.00
A-19	100	0.00	0.00	0.00	22.87 ± 2.06	15.72 ± 0.94	0.00
	50	0.00	0.00	0.00	20.81 ± 3.21	12.39 ± 0.69	0.00
A-20	100	37.38 ± 0.14	18.33 ± 0.97	22.73 ± 1.26	17.69 ± 0.66	21.57 ± 0.38	0.00
	50	24.42 ± 0.93	10.68 ± 0.59	0.00	17.44 ± 0.48	20.83 ± 0.52	0.00
A-21	100	0.00	0.00	0.00	10.21 ± 0.33	7.45 ± 1.76	26.99 ± 0.44
	50	0.00	0.00	0.00	9.06 ± 2.53	4.77 ± 0.43	23.36 ± 1.50
A-22	100	44.34 ± 0.45	11.12 ± 0.69	19.23 ± 0.28	20.96 ± 1.09	28.04 ± 0.99	0.00
	50	29.86 ± 0.96	26.04 ± 0.77	13.07 ± 4.63	15.25 ± 1.88	22.25 ± 0.75	0.00
A-23	100	0.00	0.00	0.00	9.30 ± 2.95	6.63 ± 0.25	32.36 ± 1.35
	50	0.00	0.00	0.00	5.78 ± 2.56	6.86 ± 0.64	36.12 ± 1.69
A-24	100	5.83 ± 0.30	13.07 ± 0.85	0.00	25.20 ± 0.71	12.34 ± 0.42	36.05 ± 0.99
	50	0.00	10.18 ± 0.32	0.00	19.53 ± 1.83	9.19 ± 0.36	35.19 ± 0.56
A-25	100	18.08 ± 0.69	16.48 ± 0.87	10.29 ± 0.26	24.98 ± 1.01	19.17 ± 0.72	39.15 ± 0.40
	50	12.64 ± 1.25	10.36 ± 1.91	6.97 ± 0.36	21.83 ± 1.51	4.77 ± 0.15	32.11 ± 1.36
A-26	100	13.68 ± 0.26	10.96 ± 1.08	18.83 ± 0.36	17.97 ± 0.61	12.68 ± 0.61	34.14 ± 0.91
	50	12.84 ± 1.80	6.72 ± 1.41	0.00	23.66 ± 0.77	14.09 ± 0.38	31.67 ± 1.19
A-27	100	59.78 ± 1.24	32.01 ± 1.04	12.39 ± 0.79	29.43 ± 1.26	41.18 ± 0.54	45.68 ± 0.33
	50	30.72 ± 1.51	18.53 ± 0.78	0.00	27.65 ± 0.15	14.93 ± 0.39	25.59 ± 0.50
A-28	100	19.62 ± 0.47	17.86 ± 0.40	0.00	31.34 ± 0.72	20.96 ± 0.65	19.88 ± 0.74
	50	19.13 ± 0.33	13.54 ± 0.89	0.00	32.54 ± 0.23	26.82 ± 1.05	21.69 ± 0.87
A-29	100	8.87 ± 0.23	10.56 ± 0.57	0.00	27.35 ± 1.15	5.87 ± 0.23	13.74 ± 1.03
	50	0.00	9.89 ± 1.48	0.00	24.48 ± 2.04	0.00	4.12 ± 1.06
A-30	100	15.57 ± 1.32	10.62 ± 0.49	0.00	26.12 ± 0.66	0.00	10.79 ± 0.76
	50	0.00	7.83 ± 1.00	0.00	19.28 ± 3.08	0.00	4.96 ± 1.28
A-31	100	29.32 ± 1.06	14.20 ± 0.66	0.00	24.61 ± 0.92	29.98 ± 1.18	18.68 ± 1.09
	50	17.10 ± 1.12	9.03 ± 0.37	0.00	17.46 ± 0.72	17.59 ± 2.09	20.27 ± 1.54
A-32	100	11.95 ± 0.53	7.86 ± 0.29	0.00	28.68 ± 1.33	9.55 ± 0.33	13.64 ± 0.48
	50	0.00	7.32 ± 0.59	0.00	21.42 ± 2.07	0.00	9.73 ± 1.64
A-33	100	13.69 ± 0.35	7.15 ± 0.23	0.00	22.21 ± 1.39	16.62 ± 0.45	20.80 ± 0.59
	50	0.00	10.51 ± 0.48	0.00	24.14 ± 1.45	9.93 ± 1.92	14.52 ± 1.08
A-34	100	37.25 ± 0.35	21.68 ± 1.41	0.00	27.01 ± 0.34	6.78 ± 0.80	27.70 ± 0.58
	50	22.20 ± 0.24	12.49 ± 2.01	0.00	25.57 ± 0.68	33.85 ± 1.33	25.32 ± 1.13
A-35	100	14.05 ± 1.11	14.93 ± 0.19	0.00	26.25 ± 0.73	32.09 ± 0.00	14.87 ± 0.38
	50	7.56 ± 0.32	12.27 ± 0.72	0.00	26.69 ± 1.13	31.10 ± 0.49	10.98 ± 1.90
A-36	100	10.95 ± 0.58	15.17 ± 1.43	0.00	32.01 ± 0.85	9.04 ± 0.66	10.77 ± 0.89
	50	8.12 ± 0.39	13.80 ± 0.40	0.00	28.45 ± 2.87	10.56 ± 0.87	4.54 ± 0.08

(*continued on next page*)

Table 2 (*continued*)

No.	Con.	*P. zeae*	*B. cinerea*	*S. sclerotiorum*	*M. oryzae*	*R. solani*	*F. oxysporum f. sp. vasinfectum*
A-37	100	0.00	11.32 ± 1.48	0.00	23.43 ± 0.50	11.69 ± 0.62	5.07 ± 0.70
	50	0.00	7.20 ± 1.09	0.00	27.85 ± 0.71	8.30 ± 0.74	8.14 ± 0.59
A-38	100	0.00	0.00	0.00	19.69 ± 2.06	0.00	16.56 ± 0.84
	50	0.00	9.50 ± 1.71	0.00	12.54 ± 0.57	0.00	18.00 ± 1.37
Azoxystrobin	100	68.17 ± 0.76	29.64 ± 0.81	55.70 ± 0.99	43.36 ± 0.34	59.09 ± 0.71	55.95 ± 0.47
	50	83.33 ± 0.47	42.08 ± 0.84	74.58 ± 0.72	28.75 ± 0.33	48.33 ± 1.44	52.50 ± 3.31

and the results were shown in Fig. 3. After treated by **A-0** at concentration of 5 μg/mL in DMSO, the micrograph of *B. cinerea* mycelia had been curving, collapsed, and the outer walls became shriveled and rough (Fig. 3, C and D). While the edge of the hyphae of the blank control only treated by DMSO was uniform, and the surface was relatively smooth (Fig. 3A and B). These results conjectured that compound **A-0** may destroyed the mycelium morphology and the structure, which may resulted the outflowing of cytoplasmic.

3.4. Effect of compound A-0 on reactive oxygen species (ROS) production of B. Cinerea

The production of reactive oxygen species (ROS) consisting mainly of the superoxide anion and its dismutation product hydrogen peroxide, on pathogen attack is generally be known as a defense mechanism for microbial killing in plants and animals [33]. In this test, DCFH-DA was selected as the indicator of ROS to reveal the changes in endogenous ROS production. As the result demonstrated in Fig. 4 A and B, fluorescence intensity of mycelia was stronger than the blank control, after treating with compound **A-0** at 5 μg/mL. These results leaked out that this compound could increase cellular ROS generation and accumulation of *B. cinerea* mycelia, and then accelerate the damage process of the membrane system.

3.5. Effect of compound A-0 on the mitochondrial membrane potential (MMP) of B. Cinerea

Mitochondria is the powerhouses of fungal mycelia and plays a pivotal roles in energy production and apoptosis. The changes of MMP is an important signal of mitochondria functions [34]. Fig. 5 was indicated the changes in MMP of *B. cinerea* after treating with compound **A-0** at 5 μg/mL. Compared with the blank control, the fluorescence intensity of the dye in mycelia obviously declined after treated by **A-0**. These results indicated that compound **A-0** might have damaged the mitochondrial membrane and dilapidated the normal physiological function of mitochondria of *B. cinerea* mycelia.

3.6. Effect of compound A-0 on nuclear morphology of B. Cinerea

Nucleus is the most important structure in eukaryotic cells, and it is the control center of vital activity [35]. In this part, we tested the effect of compound **A-0** on nuclear morphology of *B. cinerea*. After treating with compound **A-0** at 5 μg/mL, the nucleus of hyphal cells became smaller, and the fluorescence intensity also fell off. Meanwhile, compared to the blank control treating with 0.5% DMSO, the nuclear number of mycelia significantly decreased (Fig. 6). All these educts demonstrated that compound **A-0** might have affected the cell proliferation of *B. cinerea* mycelia, leading to apoptosis of mycelial cells.

3.7. Cytotoxicity

Compounds **A-0**, **A-1**, **B-1**, **B-2**, isocryptolepine and demethylated isocryptolepine were employed to evaluate the cytotoxicity against HL7702 and PC12 cell lines *in vitro* in this test, azoxystrobin was used as the positive control. The results showed that compounds **B-1** and **B-2** did not exhibit toxicities against these two cell lines, as well as the positive control azoxystrobin (IC_{50} > 100 μg/mL). Compounds **A-0** and **A-1** exhibited moderate to weak cytotoxicity against both cell lines with IC_{50} values ranging from 34.45 to 69.54 μg/mL (Table 7). Moreover, isocryptolepine and demethylated isocryptolepine demonstrated stronger cytotoxicity activities against both cell lines (IC_{50} values from 1 < to 13.93 μg/mL). Interestingly, it is also beneficial to remove methyl groups to reduce their toxicity. These results indicated that the introducing of N atom to the skeleton of isocryptolepine can reduce the cytotoxicity significantly.

4. Conclusion

In summary, a series of isocryptolepine “Aza”-type derivatives were designed, synthesized, and their antifungal activities against six pathogenic fungi were evaluated. The results indicated that some of the tested compounds displayed superior antifungal activities and broader antifungal spectrum compared to the positive control Azoxystrobin. Especially, compound **A-0** exhibited the strongest antifungal activity against *B. cinerea*. Moreover, it exhibited protective effects against *B. cinerea in vivo* tests. Furthermore, compound **A-0** inhibited the growth of *B. cinerea* by destroying mycelial morphology, devastating the cell membrane integrity, restraining the function of the mitochondria. The results of the cytotoxicity assay indicated that the toxicity of the “Aza”-type compounds were reduced significantly compared with the

Table 3
Antifungal activity of isocryptolepine analogues **B-1** to **B-5** against six plant pathogenic fungi (%) (μg/mL).

No.	Con.	*P. zeae*	*B. cinerea*	*S. sclerotiorum*	*M. oryzae*	*R. solani*	*F. oxysporum f. sp. vasinfectum*
B-1	100	100.00	96.97 ± 0.81	75.78 ± 0.64	67.70 ± 0.02	68.66 ± 0.11	86.95 ± 0.06
	50	98.85 ± 0.55	88.91 ± 0.98	67.46 ± 0.33	61.65 ± 1.69	65.35 ± 0.45	84.19 ± 0.16
B-2	100	100.00	79.58 ± 1.38	73.21 ± 1.10	74.63 ± 0.29	66.46 ± 0.46	83.77 ± 0.19
	50	94.11 ± 0.94	78.28 ± 1.54	68.25 ± 0.95	65.74 ± 0.25	65.73 ± 1.50	82.09 ± 0.04
B-3	100	99.31 ± 0.39	86.54 ± 0.60	100.00	90.40 ± 0.78	89.28 ± 0.67	92.92 ± 0.79
	50	99.15 ± 0.46	75.00 ± 0.75	74.38 ± 0.23	84.14 ± 1.25	72.43 ± 1.05	85.44 ± 0.29
B-4	100	100.00	73.56 ± 0.62	51.68 ± 3.07	16.07 ± 0.06	51.91 ± 1.14	49.96 ± 1.60
	50	100.00	70.83 ± 1.81	47.06 ± 1.11	7.00 ± 0.13	40.26 ± 2.06	42.46 ± 0.70
B-5	100	85.25 ± 1.15	53.96 ± 1.27	48.02 ± 1.31	35.79 ± 2.10	65.27 ± 2.17	58.80 ± 0.32
	50	57.81 ± 0.56	38.68 ± 0.59	25.62 ± 1.85	12.04 ± 0.86	50.82 ± 0.34	36.03 ± 1.01
Azoxystrobin	100	68.17 ± 0.76	29.64 ± 0.81	55.70 ± 0.99	43.36 ± 0.34	59.09 ± 0.71	55.95 ± 0.47
	50	83.33 ± 0.47	42.08 ± 0.84	74.58 ± 0.72	28.75 ± 0.33	48.33 ± 1.44	52.50 ± 3.31

Table 4
The EC_{50} values of compounds **A-0** to **A-3** against six plant pathogenic fungi (μg/mL).

No.	Fungal species	y = ax + b	EC_{50}	95%CI	R^2
A-0	*P. zeae*	y = 0.230x − 0.201	**8.45**	7.05–10.12	0.946
	R. solani	y = 0.518x − 1.223	23.30	17.54–30.97	0.989
	B. cinerea	y = 1.175x − 5.366	**2.72**	0.52–14.22	0.918
	M. oryzae	y = 0.483x − 1.215	15.13	10.81–21.16	0.891
	S. sclerotiorum	y = 0.293x + 0.126	40.87	31.39–53.22	0.927
	F. oxysporum f. sp. vasinfectum	y = 0.493x − 1.273	15.15	10.99–20.87	0.942
A-1	*P. zeae*	y = 0.260x − 0.301	**9.91**	8.48–11.58	0.984
	R. solani	y = 0.706x − 2.726	**6.27**	4.23–9.30	0.942
	B. cinerea	–	> 100	–	–
	M. oryzae	y = 0.739x − 2.547	14.40	9.38–22.12	0.960
	S. sclerotiorum	y = 0.157x + 0.884	48.59	41.43–56.99	0.868
	F. oxysporum f. sp. vasinfectum	y = 0.387x − 0.736	15.92	12.93–19.60	0.966
A-2	*P. zeae*	y = 0.139x + 0.434	13.59	12.10–15.27	0.909
	R. solani	y = 0.675x − 2.076	19.95	14.54–27.37	0.987
	B. cinerea	y = 0.401x − 0.748	17.62	13.89–22.35	0.845
	M. oryzae	y = 0.457x − 1.343	**8.81**	6.96–11.14	0.981
	S. sclerotiorum	y = 0.139x + 0.892	38.85	34.30–44.00	0.817
	F. oxysporum f. sp. vasinfectum	y = 0.440x − 1.146	11.34	9.08–14.15	0.965
A-3	*P. zeae*	y = 0.322x − 0.574	10.82	9.15–12.79	0.985
	R. solani	y = 0.183x + 0.360	19.40	16.66–22.58	0.932
	B. cinerea	y = 0.885x − 3.594	**6.42**	3.19–12.93	0.917
	M. oryzae	y = 0.502x − 1.474	11.49	8.48–15.56	0.878
	F. oxysporum f. sp. vasinfectum	y = 0.627x − 2.071	11.59	8.66–15.53	0.990
Azoxystrobin	*P. zeae*	–	> 50	–	–
	R. solani	y = 1.572x + 3.014	18.36	13.26–27.82	0.954
	B. cinerea	–	> 100	–	–
	M. oryzae	y = 0.571x + 4.122	34.43	13.09–90.56	0.995
	S. sclerotiorum	y = 0.812x-2.831	16.96	11.49–25.01	0.954
	F. oxysporum f. sp. vasinfectum	–	> 50	–	–

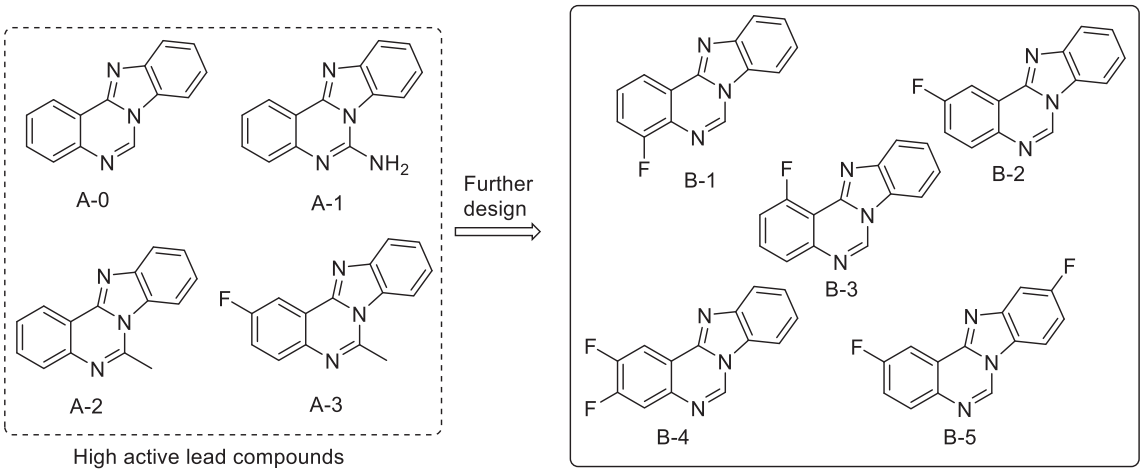
isocryptole-pine skeleton. Hence, this research work has provided valuable insights into the potential antifungal activity of isocryptolepine and its derivatives to develop novel, more effective and environmentally friendly fungicides.

5. Experimental

5.1. General information

Melting points were determined in an open capillary using WRS-2U melting point apparatus (Shanghai Precision Instrument Co., Ltd.) and are uncorrected. ESI-MS spectra were obtained on a Bruker Daltonics APEXII49e spectrometer (Bruker Company, USA). Nuclear magnetic resonance spectra (NMR) were recorded on a Bruker AM-400 spectrometer (Bruker Company, USA) in $CDCl_3$ and DMSO (^1H at 400 MHz, and ^{13}C at 100 MHz) with tetramethylsilane (TMS) as an internal standard and chemical shifts were recorded in δ values. Column chromatography was done with Select Scientific silica gel, and TLC was performed on silica gel plates using silica gel 60 F254 (Qingdao Haiyang Chemical Co., Ltd.). All reactions were performed with commercially available reagents without further purification. And the purity of the target compounds were tested by HPLC (Waters). The commercial fungicide azoxystrobin (98% purity) (Jiangsu Bailing Agrochemical Co., Ltd.) and boscalid (98% purity) (Shaanxi Sunger Road Bio-science Co., Ltd.) were used as positive control.

Plant Pathogenic Fungi. *Phyllosticta zeae*, *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Rhizoctonia solani* and *Fusarium oxysporum f. sp. vasinfectum* were obtained from Institute of Plant Protection, Gansu Academy of Agricultural Science. *Magnaporthe oryzae* was obtained from Pesticides Application Laboratory, Environment and Plant Protection Institute of Chinese Academy of Tropical Agricultural Science. The strains were retrieved from the storage tube and incubated in PDA at 23 ± 2 °C for a week to get new mycelia for the antifungal assay.



Scheme 2. Further design of active compounds A-0 to A-3.

Table 5
The EC_{50} values of compounds **B-1** to **B-5** against six plant pathogenic fungi (μg/mL).

No.	Fungal species	y = ax + b	EC_{50}	95%CI	R^2
B-1	*P. zeae*	y = 0.172x + 0.338	15.66	13.35–18.37	0.892
	R. solani	y = 0.888x − 3.097	22.41	13.90–36.15	0.867
	B. cinerea	y = 0.705x − 2.749	**5.90**	3.13–11.16	0.989
	M. oryzae	y = 0.729x − 2.265	24.04	16.44–35.15	0.949
	S. sclerotiorum	y = 1.032x − 3.937	15.88	7.50–33.65	0.928
	F. oxysporum f. sp. vasinfectum	y = 0.719x − 2.522	11.46	7.08–18.52	0.948
B-2	*P. zeae*	y = 0.296x − 0.417	11.46	9.26–14.19	0.958
	R. solani	y = 0.917x − 3.168	26.96	16.66–43.64	0.909
	B. cinerea	y = 1.059x − 4.687	**4.00**	1.22–13.14	0.990
	M. oryzae	y = 0.758x − 2.403	24.61	16.49–36.71	0.929
	S. sclerotiorum	y = 1.237x − 4.930	17.92	9.76–32.92	0.983
	F. oxysporum f. sp. vasinfectum	y = 0.855x − 3.287	**8.96**	4.68–17.16	0.917
B-3	*P. zeae*	y = 0.259x − 0.541	**4.77**	3.15–7.22	0.856
	R. solani	y = 0.673x − 2.103	18.05	12.23–26.65	0.984
	B. cinerea	y = 0.715x − 2.367	14.82	8.05–27.30	0.898
	M. oryzae	y = 0.472x − 1.081	18.59	13.10–26.39	0.954
	S. sclerotiorum	y = 0.179x + 0.480	22.09	19.40–25.15	0.829
	F. oxysporum f. sp. vasinfectum	y = 0.455x − 1.028	17.24	12.07–24.63	0.961
B-4	*P. zeae*	y = 0.758x − 2.580	14.95	9.21–24.27	0.806
	R. solani	–	> 100	–	–
	B. cinerea	y = 1.416x − 6.085	**9.63**	3.89–23.84	0.966
	M. oryzae	–	> 100	–	–
	S. sclerotiorum	y = 1.704x − 6.627	85.29	26.47–274.88	0.913
	F. oxysporum f. sp. vasinfectum	–	> 100	–	–
B-5	*P. zeae*	y = 0.459x − 0.756	34.59	27.24–43.94	0.907
	R. solani	y = 1.254x − 4.666	41.43	21.96–78.19	0.921
	B. cinerea	y = 0.732x − 1.742	82.63	51.55–132.45	0.999
	M. oryzae	–	> 100	–	–
	S. sclerotiorum	–	> 100	–	–
	F. oxysporum f. sp. vasinfectum	y = 0.821x − 2.218	82.51	47.25–144.07	0.925

Table 6
The results of *in vivo* antifungal activities of compound **A-0** against *B. cinerea* on tomato fruit.

Compounds	*Concentration (μg/mL)*	Protective effect	
		Lesion length (mm ± SD)	Control efficacy (%)
A-0	*100*	8.63 ± 0.22	72.07
	50	10.35 ± 0.20	58.82
	25	11.18 ± 0.07	52.40
Boscalid	*100*	7.33 ± 0.13	82.03
	50	8.52 ± 0.24	72.86
	25	9.57 ± 0.17	64.80
Control	–	17.98 ± 0.12	–

5.1.1. Chemistry

5.1.1.1. General synthesis of intermediate Z-1 to Z-6. The appropriate amount of polyphosphoric acid (PPA) was placed in a 100 mL round bottom flask and heated to 140 °C. The substituted *O*-phenylenediamine (6 mmol) and 2-bromobenzoic acid with substituent (6 mmol) were added and refluxed for 8 h with stirring. TLC was monitored the reaction till completed, and then the reaction mixture was cooled to 80–100 °C, and an appropriate amount of ice-water mixture was poured into the flask, neutralized with a saturated aqueous sodium hydroxide solution, filtered, and the cake was taken and dried to give a solid powder [29].

5.1.1.2. General synthetic procedure for target compound A-0. 2-(2-bromophenyl)benzimidazole (1 mmol), sodium azide (2 mmol), cuprous iodide (0.2 mmol), *p*-toluenesulfonic acid (2 mmol), *tert*-butyl hydroperoxide (1 mmol) were added to DMA (50 mL), refluxed under 130 °C for 10 h under nitrogen atmosphere, cooling to room temperature after completion the reaction. The mixture was filtered, 50 mL of dichloromethane was added to the residue, and then washed with brine. The organic layer was concentrated with evaporator, and the residue was purified by column chromatography on silica gel to give the target compound **A-0** [28].

5.1.1.3. General synthetic procedure for target compound A-1. 2-(2-Bromophenyl)benzimidazole (2 mmol), guanidine hydrochloride (3 mmol), CuI (5 mol%) and potassium phosphate (6 mmol) were added to DMF in a 50 mL round bottom flask, and refluxed at 100 °C for 16 h under nitrogen atmosphere. The reaction was detected by TLC. After the reaction was completed, cooled to room temperature. Then 50 mL of dichloromethane was added and washed by brine. The organic layer was concentrated with evaporator, and the residue was purified by column chromatography on silica gel to give the target compound **A-1** [31].

Compound **A-2** and **A-3** were synthesized in the same manner as Compound **A-1**.

5.1.1.4. General synthetic procedure for target compounds A-4 to A-37. 2-(2-Bromophenyl)benzimidazole (1 mmol), substituted aldehyde (1.2 mmol), NaN_3 (1.8 mmol), Cu (10 mol%), L-proline (20 mol%), and Cs_2CO_3 (1 mmol) were added to DMF in a 50 mL round bottom flask, and then the mixture was refluxed at 80 °C for 16 h under nitrogen atmosphere. The reaction was detected by TLC. After the reaction was completed, cooled to room temperature. The mixture was poured into the dichloromethane, washed with brine and water. The organic layer was concentrated with evaporator, and the residue was purified by column chromatography on silica gel to give the target compound **A-4** [32].

Compounds **A-5** to **A-37** were synthesized in the same manner as Compound **A-4**.

5.1.1.5. General synthetic procedure for target compound A-38. Compound **A-38** was synthesized as reported [29].

5.1.1.6. General synthetic procedure for target compound B-1 to B-5. Compound **B-1** to **B-5** were synthesized in the same manner as Compound **A-0.**

5.2. Biological activity testing

5.2.1. In vitro bioassays.

The effects of the compounds on the mycelial growth against *P. zeae, B. cinerea, S. sclerotiorum, R. solani* and *Fusarium oxysporum f. sp. vasinfectum* and *M. oryzae* were assessed using Poison Food Technique in solid media [36–38]. Firstly, the compounds were dissolved in 200 μL of DMSO and a drop of water containing Tween-80, then mixed with sterile molten potato dextrose agar (PDA) to obtain the compounds with final concentrations from 1 to 100 μg/mL. The mixture was poured into petri plates (20 mL) that were then inoculated with 5 mm plugs of the tested fungi, respectively. This experiments were repeated 3 times, and PDA containing a corresponding concentration of DMSO as a blank control. Mycelial growth diameters were measured while the fungal growth of the blank control had completely covered the petri dishes. And the inhibition rates were calculated by the previous described method [36]. I(%) = ([(C − d) − (T − d)])/((C − d)) × 100, where d is diameter of the cut fungus (5 mm), I is the inhibition rate (%), C and T are the arithmetic mean of mycelium diameters of the blank control and treatment, respectively.

5.2.2. In vivo antifungal bioassay

The *in vivo* fungicidal activity of the compound **A-0** was carried out on tomato. The *in vivo* assay was carried out according to the method described previously with minor modifications [39].

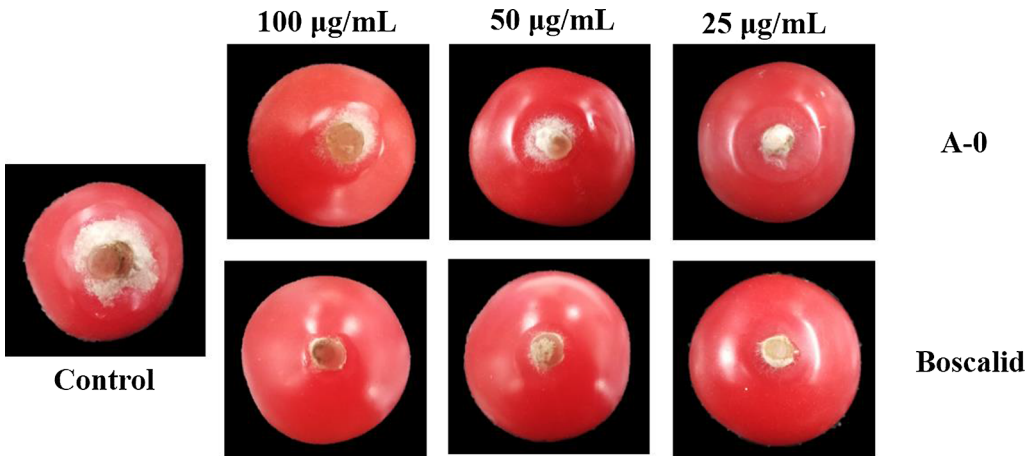


Fig. 2. Efficacy of compound A-0 at various concentrations on disease severity of gray mold caused by *B. cinerea* on tomato fruits stored at 23 ± 2 °C. Appearance of representative samples of tomato fruits on 4 days of storage.

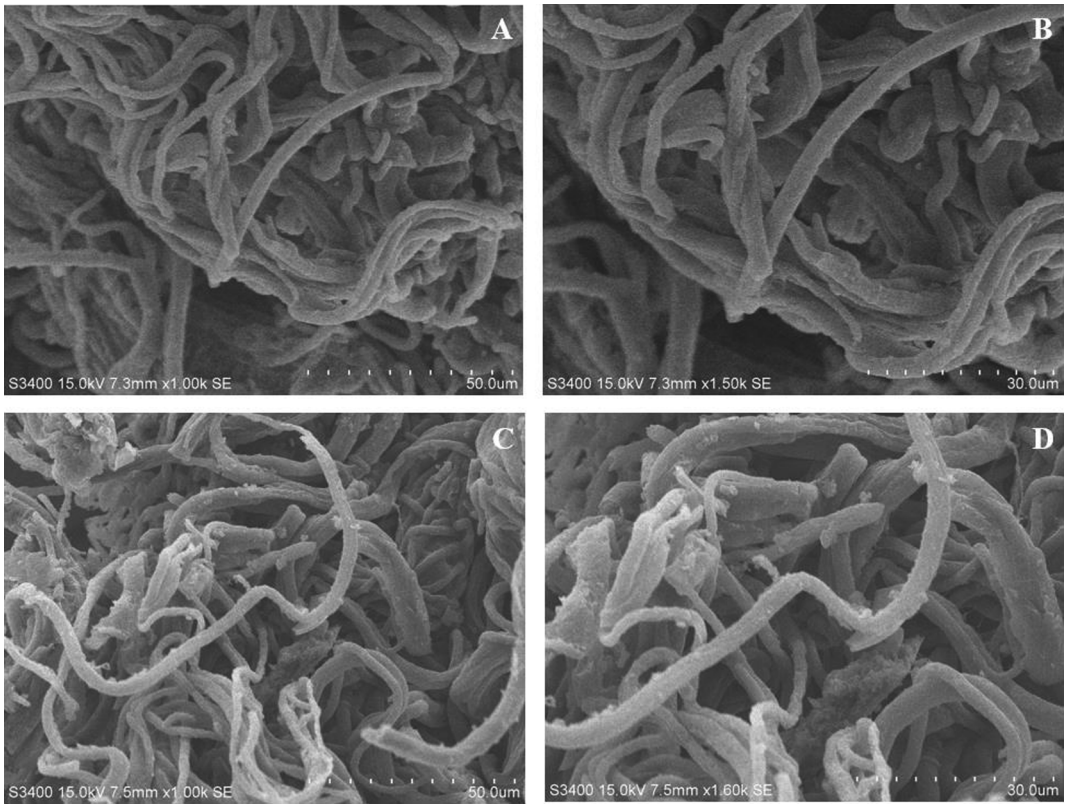


Fig. 3. Scanning electron micrographs of *B. cinerea* mycelia. (A) Control, 0.5% DMSO, ×600; (B) Control, 0.5% DMSO, ×1500; (C) Treated by Compound A-0 at 5 μg/mL, ×600; (D) Treated by Compound A-0 at 5 μg/mL, ×1500.

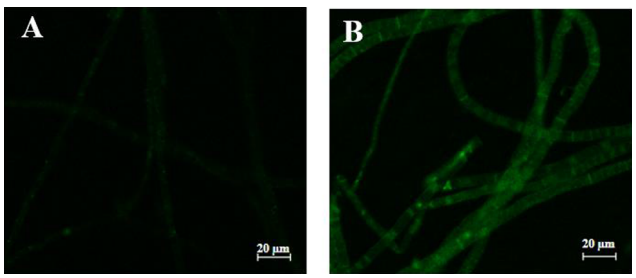


Fig. 4. Effects of Compound A-0 on the reactive oxygen species of *B. cinerea* mycelia. (A) Control, Treated by 0.5% DMSO; (B) Treated by Compound A-0 at 5 μg/mL.

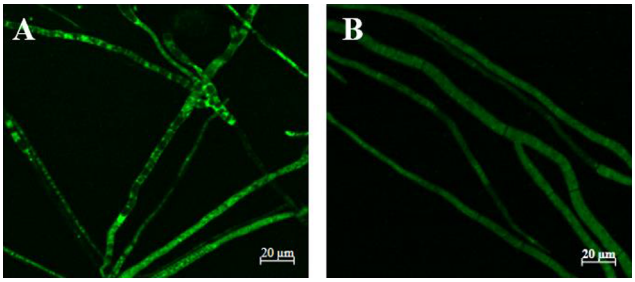


Fig. 5. Effects of Compound A-0 on the mitochondrial membrane potential of *B. cinerea* mycelia. (A) Control, 0.5% DMSO; (B) Treated with 0.5% DMSO plus Compound A-0 at 5 μg/mL.

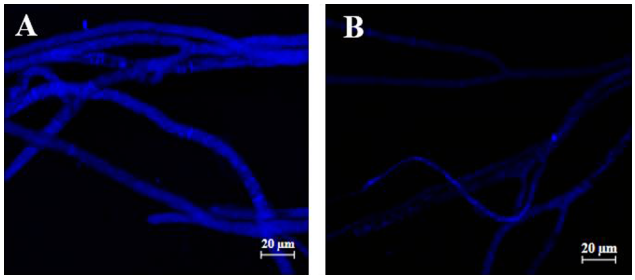


Fig. 6. Effects of Compound A-0 on the nuclear morphology of *B. cinerea* mycelia. (A) Control, 0.5% DMSO; (B) Treated with 0.5% DMSO plus Compound A-0 at 5 μg/mL.

The fruit of tomato (*Lycopersicum esculentum* Mill), which was washed and treated with water and 75% aqueous ethyl alcohol in advance, rinsed with water, and evaporation under room temperature. The fruits were wounded (d = 5 mm) using an inoculating needle and then each pathogen was inoculated. The test sample dissolved in 200 μL of DMSO, then 50 μL of Tween-80 was added and distilled water at concentrations of 25, 50 and 100 μg/mL respectively was added to form 10 mL suspension. The fruits treated by the aqueous of DMSO (2%) containing Tween-80 (0.5%) were used as the control. Commercial fungicide boscalid was used as the positive control. All of the treated fruits were then placed into an illumination incubator (23 ± 2 °C and 85% relative humidity) for 4 days. This experiments were repeated 3 times. The statistical analyses were performed by SPSS Statistic 19.0.

5.2.3. Scanning electron microscopy (SEM) observations

To examine the effects of **A-0** on *B. cinerea* microstructure, scanning electron microscopy (SEM) observations were performed according to described methods [40]. The mycelia blocks with the specification of 5.0 mm × 4.0 mm square was cut from the growth boundary of the fungi on PDA mediums treating with compound **A-0** at 5 μg/mL. After fixed with glutaraldehyde for 1 day and washed with PBS for three times, the blocks were then fixed with osmium tetraoxide for 2 h. And then the samples were washed with PBS again and dehydrated by aqueous ethanol from 20% to 100%. After drying at critical point and gold coating, SEM observations were carried out with a scanning electron microscope (Hitachi, S-3400 N, Japan).

5.2.4. Effect on the mitochondrial membrane potential (MMP)

Effect on the mitochondrial membrane potential (MMP) of *B. cinerea* mycelia was performed according to the previously described method [41]. The hyphae treated with **A-0** at 5 μg/mL were stained with 0.5 mL of Rhodamine 123 (Beyotime, China) and incubated in darkness (30 min at 37 °C). Then the samples were washed using pre-cooled PBS for two times in 10 min. Finally, the samples were observed and photographed under LSM 800 laser confocal microscope.

5.2.5. Production of reactive oxygen species (ROS)

The accumulation of reactive oxygen species (ROS) was measured by the method previously described [42]. The mycelia tips of *B. cinerea* (d = 5 mm) treated with **A-0** at 5 μg/mL for three days were placed on a sterile slide to incubate. Then 0.5 mL of 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) solution (Beyotime, China) was added and incubated for half an hour at 37 °C in coverture. After incubation, the mycelia were washed by pre-cooled PBS for three times in 15 min. Then, the samples were observed and photographed immediately (Carl Zeiss LSM 800, Germany).

5.2.6. Nuclear staining assay [41]

B. cinerea mycelia tips were treated with **A-0** (5 μg/mL) for three days and then fixed with stain fixative for half an hour under refrigerated temperature. Following, the hyphae were washed with PBS for two times and incubated at 25 °C for 20 min after treated with Hoechst 33,258 (Beyotime, China). Then a coverslip was placed on, and the samples were observed and photographed under LSM 800 laser confocal microscope immediately (Carl Zeiss LSM 800, German).

5.2.7. Cytotoxicity assay

The cytotoxicity against HL7702 and PC12 cell lines were performed using CCK-8 method according to previously described methods [43]. Cells were treated with different concentrations of tested samples in the growth medium for 24 h, and the absorbance was measured at 450 nm (Thermo Scientific Multiskan MK3 microplate reader, USA). Five replicates were performed.

5.3. Statistical analysis

Data were analyzed using SPSS software version 18.0 and expressed as the means ± SD. The concentration for 50% of maximal effect (EC_{50}) were obtained from the parameters in the regression curves for the different concentrations. The half maximal inhibitory concentration (IC_{50}) was calculated using the complementary log-log (CLL) model.

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HYPERLINK "SPS:role::contributing" Author contributions statement

Jun-cai Li and Ren-xuan Wang performed the chemical synthesis, biological tests; Jun-cai Li wrote the paper; Ying-qian Liu conceived this work; Yu Sun, Jia-kai Zhu, Zhong-min Zhao, Guan-fang Hu, Yu-ling Wang, Rui Zhou and Yin-fang Yan carried out the bioassay experiments and prepared the data; Jing-wen Peng conducted structure detection. Xiao-fei Shang assisted in writing the paper and checked the manuscript.

Declaration of competing interest

All authors declare that they have no competing interests.

Table 7
Cytotoxic activities of isolated compounds against HL7702 and PC12 cell lines (IC_{50} in μg/mL)*

	Isocryptolepine	Demethylated Isocryptolepine	A-0	A-1	B-1	B-2	Azoxystrobin
HL7702	6.89 ± 0.43	13.93 ± 0.91	59.75 ± 3.04	69.54 ± 4.37	> 100	> 100	> 100
PC12	1 <	13.28 ± 1.21	34.45 ± 2.60	42.21 ± 3.14	> 100	> 100	> 100

* The results are means ± SD deviation from five independent experiments.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.103266.

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