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Design, Synthesis, and Antifungal Evaluation of Cryptolepine Derivatives against Phytopathogenic Fungi

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ABSTRACT: Inspired by the widely antiphytopathogenic application of diversified derivatives from natural sources, cryptolepine and its derivatives were subsequently designed, synthesized, and evaluated for their antifungal activities against four agriculturally important fungi *Rhizoctonia solani*, *Botrytis cinerea*, *Fusarium graminearum*, and *Sclerotinia sclerotiorum*. The results obtained from *in vitro* assay indicated that compounds **a1**−**a24** showed great fungicidal property against *B. cinerea* (EC_{50} < 4 μg/mL); especially, **a3** presented significantly prominent inhibitory activity with an EC_{50} of 0.027 μg/mL. In the pursuit of further expanding the antifungal spectrum of cryptolepine, ring-opened compound **f1** produced better activity with an EC_{50} of 3.632 μg/mL against *R. solani* and an EC_{50} of 5.599 μg/mL against *F. graminearum*. Furthermore, **a3** was selected to be a candidate to investigate its preliminary antifungal mechanism to *B. cinerea*, revealing that not only spore germination was effectively inhibited and the normal physiological structure of mycelium was severely undermined but also detrimental reactive oxygen was obviously accumulated and the normal function of the nucleus was fairly disordered. Besides, *in vivo* curative experiment against *B. cinerea* found that the therapeutic action of **a3** was comparable to that of the positive control azoxystrobin. These results suggested that compound **a3** could be regarded as a novel and promising agent against *B. cinerea* for its valuable potency.

KEYWORDS: *cryptolepine, Botrytis cinerea, antifungal activity, structure activity relationship, antifungal mechanism*

INTRODUCTION

Epidemic diseases of crops caused by fungi deeply affected the course of human history and processed a major restriction on social and economic development. Research revealed that more than 19,000 different types of fungi could cause plant diseases and some of them could be dormant in dead plants until opportunities were conducive to their proliferation.[1] The loss of crops caused by fungi had become a severe issue that cannot be ignored. For example, rice blast could bring about 10−35% of harvest losses in 85 countries around the world because of the spread of *Magnaporthe oryzae*; more than 200 crop species including different fruits and vegetables could be severely damaged by *Botrytis cinerea* as it is a necrotrophic, haploid, heterothallic ascomycete with a unique infection approach;[2,3] *Phytophthora* could transmit from potatoes and tomatoes to other important corps, causing symptoms like fruit rot, trunk ulcers, branch wilting, and serious economic losses.[4] Ever since, the most effective way to control harvest deterioration in crops still remained the common use of chemical fungicides, although long-term and widespread utilization of them had led to a developed increase in fungal resistance. In fact, the disadvantages of using agricultural chemicals had already surfaced due to their influences on food, environment, and other side effects on humans like carcinogenicity and teratogenicity.[3,5,6]

Since penicillin was accidentally discovered and successfully used in clinical treatment, natural products provided a clear direction for the discovery of antimicrobial drugs.[7] Natural alkaloids were the metabolites produced by natural organisms interacting with multiple biological targets in the process of evolution, which were easily degraded and had low impact to the environment and nontarget organisms.[8] Searching for lead compounds from natural products by structural modification and simplification had proven its great potency and success. For instance, dimethomorph, created in 1988 as a novel systemic fungicide with curative, protectant, and antifungal activity, was inspired from the natural cinnamate; by modifying the structure of strobilurin A, the shortcomings of its poor light stability and bad volatility were well solved, leading to the creation of azoxystrobin, trifloxystrobin, fluoxastrobin, and so on;[9] hymexazol was obtained by modifying the ibotenic acid isolated from higher fungi *Amanita ibotengutake*.[10] Therefore, searching for active lead compounds from natural alkaloids was a valid and bright routine to deal with fungal infections of crops.

In our proceeding pursuit of antifungal natural compounds, a traditional African climbing herbal medicinal plant named *Cryptolepis sanguinolenta* caught our greatest interest.[11] As a shrub indigenous to West Africa, *C. sanguinolenta* had long been employed by Ghanaian traditional healers in the treatment of various fevers, including malaria.[12] The extraction

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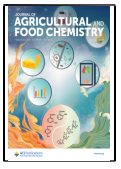

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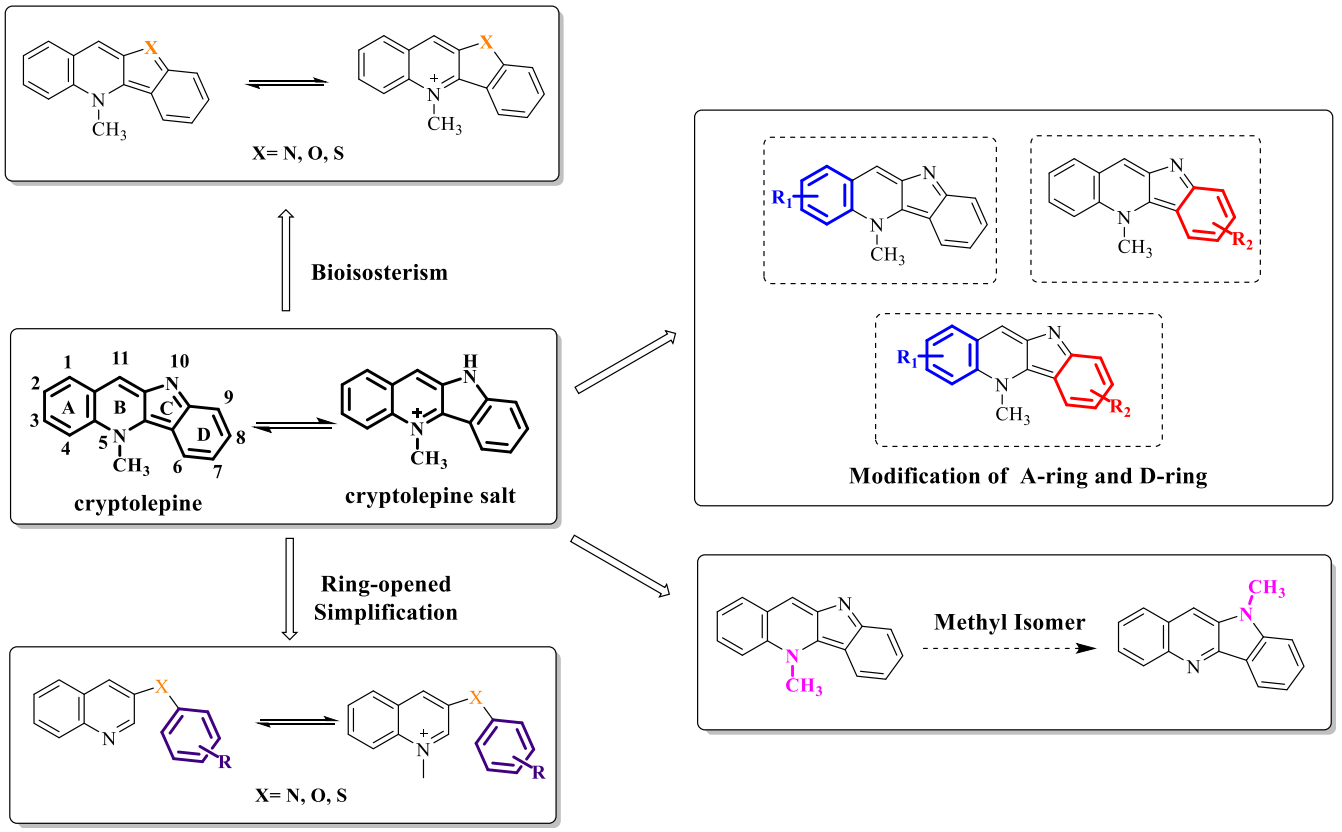
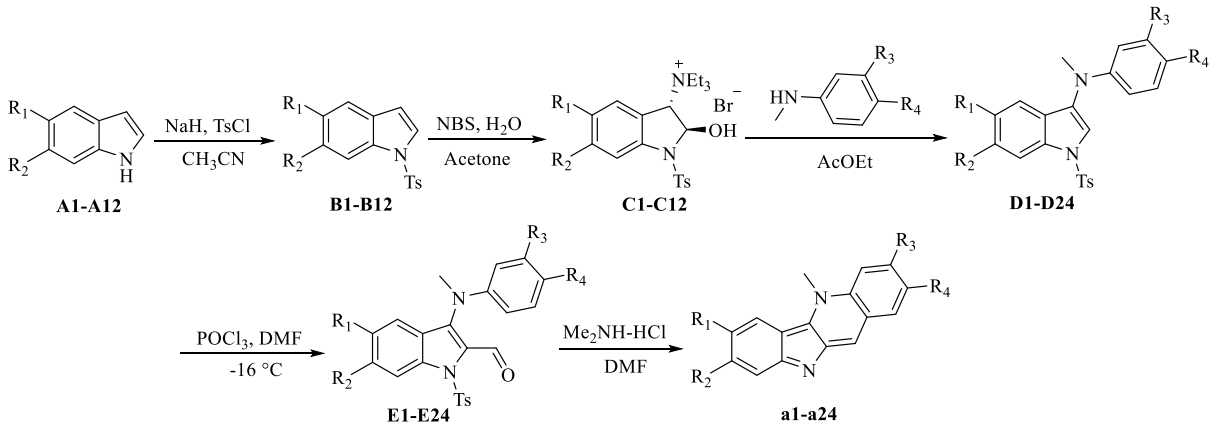


Figure 1. Strategies of structural modification and simplification of cryptolepine.



a1 R_1 = H, R_2 = H, R_3 = H, R_4 = H
a2 R_1 = H, R_2 = H, R_3 = H, R_4 = Cl
a3 R_1 = H, R_2 = H, R_3 = H, R_4 = OCH_3
a4 R_1 = H, R_2 = H, R_3 = H, R_4 = CF_3
a5 R_1 = H, R_2 = H, R_3 = H, R_4 = CH_3
a6 R_1 = H, R_2 = H, R_3 = H, R_4 = Br
a7 R_1 = H, R_2 = H, R_3 = H, R_4 = t-Bu
a8 R_1 = H, R_2 = H, R_3 = CH_3, R_4 = H
a9 R_1 = H, R_2 = H, R_3 = CF_3, R_4 = H
a10 R_1 = H, R_2 = H, R_3 = OCH_3, R_4 = H
a11 R_1 = H, R_2 = H, R_3 = t-Bu, R_4 = H
a12 R_1 = H, R_2 = H, R_3 = Cl, R_4 = H
a13 R_1 = Br, R_2 = H, R_3 = H, R_4 = H
a14 R_1 = F, R_2 = H, R_3 = H, R_4 = H
a15 R_1 = Cl, R_2 = H, R_3 = H, R_4 = H
a16 R_1 = CH_3, R_2 = H, R_3 = H, R_4 = H
a17 R_1 = OCH_3, R_2 = H, R_3 = H, R_4 = H
a18 R_1 = H, R_2 = F, R_3 = H, R_4 = H
a19 R_1 = H, R_2 = Cl, R_3 =H, R_4 = H
a20 R_1 = H, R_2 = Br, R_3 = H, R_4 = H
a21 R_1 = H, R_2 = CH_3, R_3 = H, R_4 = H
a22 R_1 = Br, R_2 = H, R_3 = H, R_4 = F
a23 R_1 = H, R_2 = Cl, R_3 = H, R_4 = F
a24 R_1 = H, R_2 = F, R_3 = H, R_4 = Cl

Figure 2. Synthesis of target compounds **a1**−**a24**.

and separation test revealed that it contains cryptolepine, neocryptolepine, quindoline, hydroxycryptolepine, and other ingredients. Further research revealed that the main component cryptolepine, whose total synthesis was prior to separation and determination, had exhibited unprecedented pharmacological effects, including antimalarial activity,[13−18] antibacterial activity,[19−22] anticancer activity,[23−26] antifungal activity,[27−29] anti-inflammatory activity,[30,31] antidiabetic activity,[32] antituberculosis activity,[33] and so on.[34−36] Although fruitful achievements had been widely reported, the exploration of cryptolepine and its derivatives in fungicide chemistry was barely conducted. This implied us that more emphasis should be laid on the study of cryptolepine and its derivatives in the control of crop fungi.

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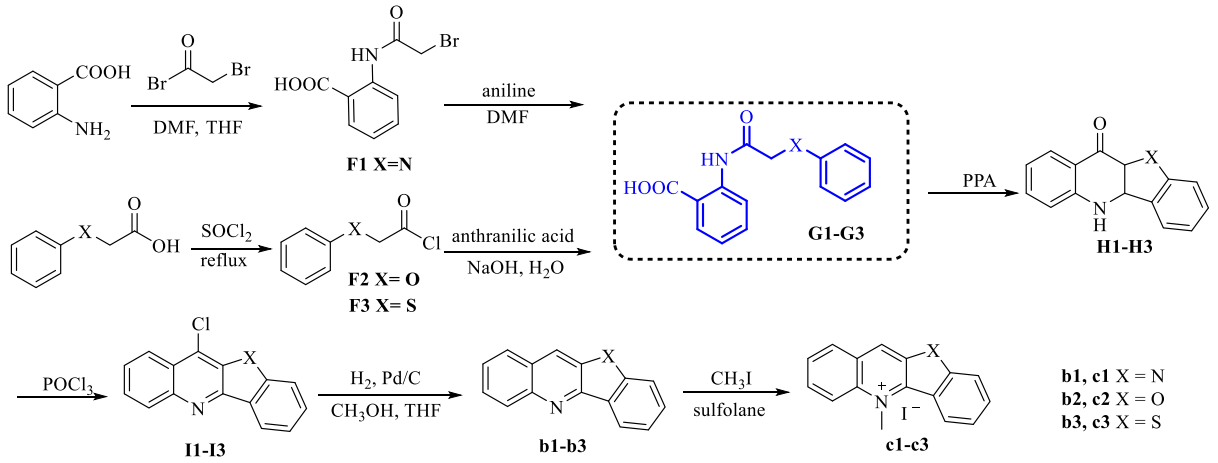

Figure 3. Synthesis of target compounds **b1**−**b3** and **c1**−**c3**.

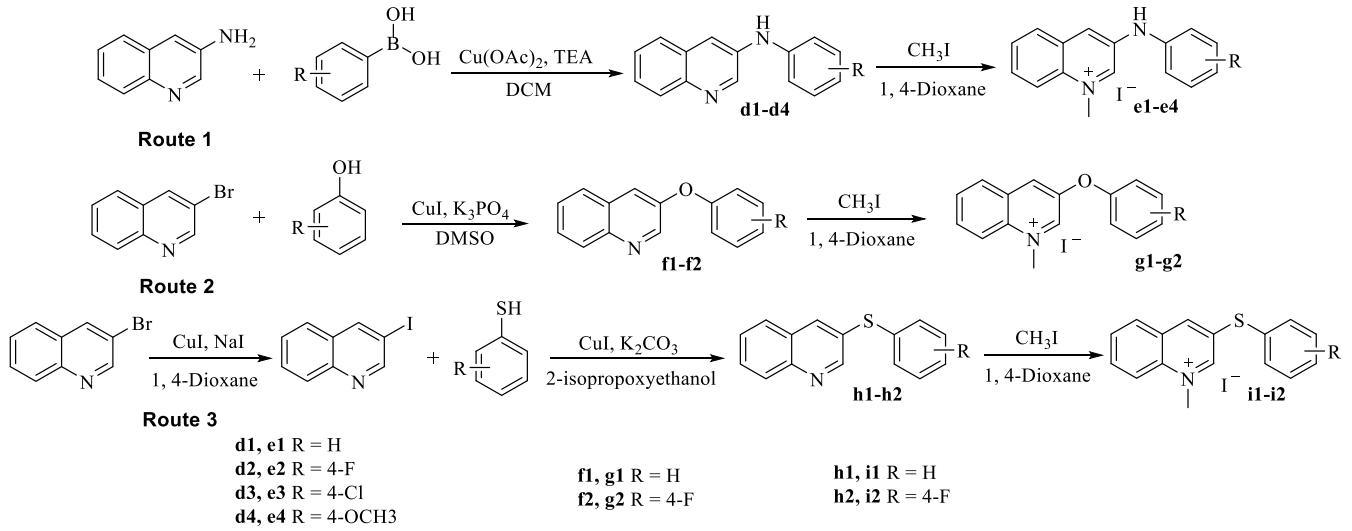

Figure 4. Synthesis of target compounds **d1**−**d4**, **e1**−**e4**, **f1**−**f2**, **g1**−**g2**, **h1**−**h2**, and **i1**−**i2**.

Enlightened from our previous study of neocryptolepine[37] and structural simplification of natural products,[38,39] a library of derivatives and simplifications of cryptolepine had been availably designed and synthesized in various ways to evaluate their antiphytopathogenic activities. As shown in Figure 1, a succession of substituted A-ring, D-ring, and A, D-ring derivatives **a1**−**a24** was synthesized to explore their influence on activity. Meanwhile, some different types of bioisosterisms **b1**−**b3** and **c1**−**c3** were created by replacing N at 10-position with O and S to study the results of different ring-forming atoms. Then, ring-opened simplifications **d1**−**d4**, **e1**−**e4**, **f1**−**f2**, **g1**−**g2**, **h1**−**h2**, and **i1**−**i2**, and methyl isomer **j1** had been synthesized to further hunt for active compounds. By virtue of its excellent activity to *B. cinerea* (EC_{50} = 0.027 μg/mL), compound **a3** was chosen to be a privileged candidate to evaluate its mechanisms of action. Preliminary results showed that spores and hyphae were inhibited and destroyed, prompting us to study the influence of **a3** on the changes of reaction oxygen species (ROS), cell nuclear morphology, and mitochondrial membrane potential (MMP) of *B. cinerea*. Based on the above research results, *in vivo* antifungal experiments on apples had been progressed.

MATERIALS AND METHODS

General. All of the compounds of quinolones and indoles were purchased from commercial companies (Energy Chemical Co., Ltd.) without further purification. All reactions were performed with commercially available reagents without further purification. ^{1}H and ^{13}C NMR spectra were recorded at 400 and 100 MHz on a Bruker AM-400 (Bruker Company) spectrometer using TMS as a reference. Mass spectra were recorded on a Bruker Daltonics APEXII49e spectrometer (Bruker Daltonics Inc.) with the ESI source as ionization. The relative conductivity of hyphae was performed by a conductivity meter (Leici DDS-307, China). The microscopic morphology of fungal hyphae was observed by a scanning electron microscope (Hitachi, S-3400 N, Japan). The commercial fungicide azoxystrobin (analytical grade, 98% purity) (Jiangsu Bailing Agrochemical Co., Ltd., China) was used as a positive control in antifungal activity assays.

Plant Pathogenic Fungi. Four plant pathogenic fungi species *Rhizoctonia solani*, *B. cinerea*, *Fusarium graminearum*, and *Sclerotinia sclerotiorum* were isolated from susceptible plants cultivated at the Gansu Academy of Agricultural Sciences, Gansu Province of China. Then, they were purified and identified by the Institute of Plant Protection, Gansu Academy of Agricultural Science, and maintained during the experiments on potato dextrose agar medium (PDA: potato 200 g, dextrose 20 g, agar 15 g, and distilled water 1000 mL) at 25 °C for the antifungal assay.

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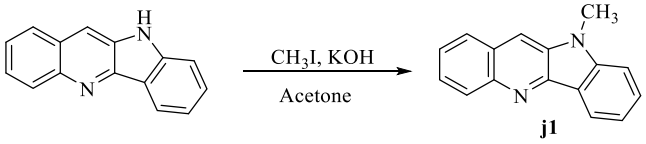
Synthetic Procedures. The synthetic routes of series of **a1−a24**, **b1−c3**, **c1−c3**, **d1−d4**, **e1−e4**, **f1−f1**, **g1−g2**, **h1−h2**, **i1−i2**, and **j1** are outlined in Figures 2−5.



j1

Figure 5. Synthesis of target compound **j1**.

*General Synthetic Procedure for Target Compounds **a1−a24** (Figure 2).*[40] To a solution of different indoles **A1−A12** (15 mmol) in CH_3CN was added 60% NaH (21 mmol) at 0 °C. The mixture was stirred for 10 min, and TsCl (16.5 mmol) was added. Then, the mixture was allowed to reach room temperature and stirred for 4 h. The reaction was quenched with a saturated aqueous solution of NH_4Cl. The resulting solution was extracted with ethyl acetate (3 × 100 mL). The combined organic phases were washed with brine (100 mL), dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo to provide the corresponding compounds **B1−B12**.

To a solution of **B1−B12** (11 mmol) and H_2O (110 mmol) in acetone (110 mL) was added NBS (12 mmol). The mixture was stirred at room temperature until the complete disappearance of the starting material as indicated by TLC. Et_3N (12 mmol) was added to the mixture and stirred further 1 h. The resulting precipitate was separated by filtration, washed with acetone, and dried in vacuo to give the corresponding compounds **C1−C12**.

A mixture of **C1−C12** (0.5 mmol), different *N*-methylanilines (0.55 mmol), and Et_3N (1.0 mmol) in AcOEt (5 mL) was heated for 0.5−6 h. After addition of H_2O, the whole was extracted with AcOEt (3 × 15 mL) and washed with brine (15 mL). The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. Then, the mixture was dissolved in AcOEt (5 mL). To this solution was added $BF_3{\cdot}Et_2O$ (2.5 mmol), and the mixture was heated at 50 °C with stirring for 3 h. After addition of saturated $NaHCO_3$ aq, the whole mixture was extracted with AcOEt (3 × 15 mL) and washed with brine (15 mL). The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the corresponding compounds **D1−D24**.

Phosphoryl chloride (2 mmol) was added to dimethylformamide (DMF) (1 mL) at −16 °C and stirred for 0.5 h. A solution of **D1−D24** (1 mmol) in DMF (1 mL) was added to the mixture and stirred at room temperature for 1 h. The resultant mixture was added to saturated $NaHCO_3$ aq (5 mL) at 0 °C, and the whole was extracted with AcOEt (3 × 10 mL) and washed with saturated $NaHCO_3$ aq (10 mL). The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the corresponding compounds **E1−E24**.

To a solution of $Me_2NH{\cdot}HCl$ (0.1 mmol) in DMF (1 mL) was added **E1−E24** (0.05 mmol), and the mixture was heated at 150 °C with stirring for 1.5 h. The mixture was cooled to room temperature and added with 5% Na_2CO_3 aq (5 mL). After stirring at room temperature for 10 min, the resultant mixture was extracted with AcOEt (3 × 10 mL) and washed with saturated Na_2CO_3 aq (5 mL). The organic layer was dried over Na_2CO_3 and concentrated under reduced pressure. The residue was purified by alumina column chromatography to give target compounds **a1−a24**.

*General Synthetic Procedure for Target Compounds **b1−b3** and **c1−c3** (Figure 3).*[41] To a solution of anthranilic acid (96 mmol) in DMF and tetrahydrofuran (THF) (vol/vol = 1:1) was added bromoacetyl bromide (96.6 mmol) at 0 °C. The mixture was allowed to reach room temperature and stirred for overnight. The resulting solution was added to water (150 mL) to form a light-yellow precipitate. The precipitate was filtered and washed with water until neutral pH, and the solid residue was dried in vacuo to give the corresponding compound **F1** (**X** = **N**).

To a round-bottomed flask was added phenoxyacetic acid or 2-phenylsulfanylacetic acid (2.4 mmol) and thionyl chloride (20 mL). Then, the mixture was heated at 80 °C for 2 h. When the reaction was over, excess thionyl chloride was removed under reduced pressure to obtain **F2** (**X** = **O**) and **F3** (**X** = **S**) and directly put into the next step without purification.

For **X** = **N**, a mixture of **F1** (90 mmol) and aniline (100 mmol) in DMF was heated under reflux for 18 h. When the reaction was completed, a solution of 10% sodium hydroxide was slowly added to adjust the pH of the mixture to 9. The whole mixture was extracted with DCM, and the aqueous phase was collected. Then, the pH of aqueous phase was adjusted to neutral with a solution of 10% hydrochloric acid to obtain a large amount of precipitate. The obtained solid was washed with water repeatedly to get **G1** without further purification.

For **X** = **O** or **S**, anthranilic acid (50 mmol) was added to aqueous solution of sodium hydroxide and stirred until completely dissolved. Then, a dropping funnel was used to slowly add the prepared **F2** or **F3** to the mixture at 0 °C. The reaction would be quickly completed, and a large amount of white solid formed. The precipitate was filtered and dried in vacuo to give **G2** and **G3**.

A mixture of **G1−G3** (21.5 mmol) and polyphosphoric acid (5 w/w) was heated at 130 °C for 2 h. Then, the mixture was cooled to room temperature and poured into ice water (250 mL) and neutralized with saturated solution of KOH. The precipitated solid was filtered and dried to give **H1−H3** and used for the next step without further purification.

A suspension of **H1−H3** (23.8 mmol) in $POCl_3$ (60 mL) was refluxed at 110 °C for 3 h. After evaporating excess $POCl_3$, the reaction mixture was poured into ice water and neutralized with solution of sodium hydroxide. The precipitated solid was purified by column chromatography using petroleum ether and ethyl acetate as an eluent to give **I1−I3**.

Pd/C (10%, 40 mg) and Et_3N (0.32 mmol) were orderly added into a CH_3OH/THF = 1:1 (vol/vol) solution of **I1−I3**. After removing the air in the flask and flushing hydrogen for 5 min, the system was closed and reacted at room temperature for overnight. When the reaction was completed, Pd/C was removed by filtration, and the filtrate was concentrated under reduced pressure. The residue was purified by column chromatography using petroleum ether and ethyl acetate as eluents to give target compounds **b1−b3**.

CH_3I (0.6 mL) was added to a solution of compounds **b1−b3** (0.09 mmol) in sulfolane (1 mL). The mixture was heated at 55 °C for 8 h until the solid formed. Target compounds **c1−c3** could be obtained by filtering and washing the solid with a small amount of ethyl acetate.

*General Synthetic Procedure for Target Compounds **d1−d4** and **e1−e4** (Figure 4, Route 1).*[42] To a solution of 3-aminoquinoline (10 mmol) in DCM (30 mL) was added substituted phenylboronic acid (13.36 mmol), $Cu(OAc)_2$ (10.41 mmol), TEA (10.41 mmol), and molecular sieves (2 g). The mixture was stirred at room temperature for 24 h. Then, small amounts of ammonium hydroxide was added into the mixture and continued to stir for 30 min. When the reaction was completed, the mixture was extracted with DCM. The combined organic phases were washed with saturated brine and dried with anhydrous sodium sulfate, and the solvent was removed in vacuo. Compounds **d1−d4** were gained by column chromatography using petroleum ether and ethyl acetate as eluents.

CH_3I (2.0 mmol) was added to a solution of **d1−d4** (1.0 mmol) in 1,4-dioxane (15 mL). After stirring at 55 °C for 8 h, the solid was precipitated and target compounds **e1−e4** could be obtained by washing the solid with a small amount of ethyl acetate without further purification.

*General Synthetic Procedure for Target Compounds **f1−f2** and **g1−g2** (Figure 4, Route 2).*[43] To a sealed tube was added 3-bromoquinoline (1.0 mmol), CuI (0.1 mmol), substituted phenol (1.2 mmol), and K_3PO_4 (2 mmol) and then was filled with nitrogen. Dimethyl sulfoxide (DMSO) (2 mL) was added to the mixture with a syringe and heated at 90 °C for 24 h. When the reaction was completed, ethyl acetate and water were added to the resulting

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mixture, the organic phase was separated, and the aqueous phase was extracted by ethyl acetate. The combined organic phases were washed with saturated brine and dried with anhydrous sodium sulfate, and the solvent was removed in vacuo. Target compounds **f1**−**f2** were gained by column chromatography using petroleum ether and ethyl acetate as eluents.

CH_3I (2.0 mmol) was added to a solution of **f1**−**f2** (1.0 mmol) in 1,4-dioxane (15 mL). After stirring at 55 °C for 8 h, the solid was precipitated and target compounds **g1**−**g2** could be obtained by washing the solid with a small amount of ethyl acetate without further purification.

*General Synthetic Procedure for Target Compounds **h1**−**h2** and **i1**−**i2** (Figure 4, Route 3).*[44] To a solution of 3-bromoquinoline (47.3 mmol) in 1,4-dioxane was added CuI (2.4 mmol), NaI (94.5 mmol), and *N*,*N*′-dimethyl-1,2-ethanediamine (0.5 mL). The mixture was heated at 110 °C for 48 h. When the reaction was completed, 30 mL of 30% ammonium hydroxide was added and stirred at room temperature for 10 min. Then, water was added to the resulting mixture, and the whole mixture was extracted with ethyl acetate. The combined organic phases were washed with saturated brine and dried with anhydrous sodium sulfate, and the solvent was removed in vacuo. Target intermediate 3-iodoquinoline was gained by column chromatography using petroleum ether and ethyl acetate as eluents.

To a round-bottomed flask was added 3-iodoquinoline (1.96 mmol), CuI (0.1 mmol), K_2CO_3 (3.92 mmol), substituted thiophenol (1.96 mmol), and 2- isopropoxyethanol (3.92 mmol). Then, the mixture was heated at 80 °C for 16 h. When the reaction was completed, a solution of 10% hydrogen peroxide was added and the whole mixture was stirred at room temperature for 10 min. Then, water and ethyl acetate were added to the resulting mixture, the organic phase was separated, and the aqueous phase was extracted by ethyl acetate. The combined organic phases were washed with saturated brine and dried with anhydrous sodium sulfate, and the solvent was removed in vacuo. Target compounds **h1**−**h2** were gained by column chromatography using petroleum ether and ethyl acetate as eluents.

CH_3I (2.0 mmol) was added to a solution of **h1**−**h2** (1.0 mmol) in 1,4-dioxane (15 mL). After stirring at 55 °C for 8 h, the solid was precipitated and target compounds **i1**−**i2** could be obtained by washing the solid with a small amount of ethyl acetate without further purification.

*General Synthetic Procedure for Target Compound **j1** (Figure 5).* To a solution of compound **b1** in acetone was added potassium hydroxide (1.5 mmol) and CH_3I (1.2 mmol). The mixture was reacted at room temperature for 15 min. When the reaction was over, the solvent was removed in vacuo, and the residue was purified by silica gel column chromatography to give target compound **j1**.

***In Vitro* Antifungal Assay.** Cryptolepine and its derivatives were tested by the mycelium growth rate method[45] for their antifungal activities against four fungi, including *R. solani*, *B. cinerea*, *F. graminearum*, and *S. sclerotiorum*. All the synthetic compounds were dissolved in DMSO and then were added to PDA medium that was prepared and sterilized to obtain a series of concentrations (50, 25, 10, 5, 2.5, 1, 0.5, 0.1, 0.05, and 0.01 μg/mL). The antifungal activities of target compounds **a1**−**a24**, **b1**−**c3**, **c1**−**c3**, **d1**−**d4**, **e1**−**e4**, **f1**−**f1**, **g1**−**g2**, **h1**−**h2**, **i1**−**i2**, and **j1** in detail were included in the Supporting Information. The blank control was maintained with 0.5% DMSO (vol/vol) mixed with PDA (The same amount of DMSO was added to the sterile medium as a blank control.), and azoxystrobin was used as a positive control. The mycelial disks (5 mm) of phytopathogenic fungi were inoculated on PDA plates and then were incubated at 25 °C in the dark. Each sample was measured in triplicates, and its diameters (mm) of inhibition zones were measured by the cross-bracketing method. The growth inhibition rates were calculated when the blank control hyphae grew to the edge of the Petri dish according to the following formula

$$\text{mycelial growth inhibition (\%)} = [(d_c - d_t)/(d_c - 5\ \text{mm})] \times 100$$

where d_c and d_t were average diameters of the fungal colony of black control and treatment, respectively.

Spore Germination Inhibition Assay. According to the described method,[46] the phytopathogenic fungi included in the assay was grown on PDA plates in darkness at 25 °C for 2 weeks. The spores were harvested from sporulating colonies and suspended in sterile distilled water containing 0.1% (vol/vol) Tween 80. The concentrations of spores in the suspension were determined using a hemocytometer and adjusted to 1×10^6 spores per mL.

The stock solutions of the tested compounds dissolved in DMSO were diluted 100-fold with sterile distilled water, which were further prepared as 2-fold dilutions for the assay, in which the final concentration of DMSO was 1% (vol/vol). Sterile distilled water with 1% DMSO was used as a negative control. A 30 μL spore suspension was placed on separate glass slide containing 30 μL of prepared test solutions for a final volume of 60 μL. The inoculated glass slides were incubated in a moisture chamber at 25 °C for 12 h. A spore was considered to be germinated if the germ tube length was longer than the short radius of the spore, and the number of spores germinated at each concentration was counted under the microscope. The percentage of spore germination was calculated, and the half maximal effective concentration (EC_{50}) values were derived from the data analysis of the concentration-inhibition rate. The experiment was independently performed three times under the same conditions. The experiment was repeated three times, and the inhibition rates were calculated according to the following formula

$$\text{inhibition germination of spores (\%)} = [(N_0 - N_1)/N_0] \times 100$$

where N_0 and N_1 were average values of the spore germination rates of the black control and treatment, respectively.

Scanning Electron Microscopy Observations. (After treating with **a3** at a concentration of 0.05 μg/mL for 72 h, mycelia blocks (5.0 mm × 4.0 mm) were cut from the fungi.). All the samples were treated by 4% glutaraldehyde for 4 h, washed three times with 0.01 M PBS (pH = 7.2), and then fixed with 1% osmium tetraoxide solution (wt/vol) for 2 h. After that, each sample was dehydrated with graded ethanol series (20, 50, 80, and 90%) for 10 min. Subsequently, the samples were dried at a critical point and gold-sprayed and observed by using a scanning electron microscope (Hitachi, S-3400N, Japan).

Determination of Cell Membrane Permeability. Effects of compound **a3** on the cell membrane permeability were determined by the conductivity method.[47] The mycelial disk of *B. cinerea* (5 mm) was placed in 60 mL of PD broth medium and shook at 140 rpm for 4 days at 27 °C. After that, the mycelia were filtered and added into the solution of **a3** with different concentrations (50, 25, 10, and 5 μg/mL). Eventually, the conductivity values were determined with a conductivity detector (0 h was marked as L_0, and 0.5, 1, 2, 4, 6, 8, 10, 12, and 24 h were marked as L_1). The conductivities of samples treated by boiling water for 30 min were remarked as L_2. The relative permeability rate of the cell membrane was calculated by the following formula

$$\text{relative electric conductivity (\%)} = [(L_1 - L_0)/(L_2 - L_0)] \times 100$$

ROS Production of *B. Cinerea*. The accumulation of ROS was measured by the method previously described.[48] Mycelia tips (0.5 cm) were treated with compound **a3** (0.05 and 0.1 μg/mL) for 48 h and then placed on a sterile slide in a culture dish. After the samples were cultured at 25 °C for 24 h, the PDA medium was removed carefully and the hyphae were stained with 10 μM DCFH-DA solution (Beyotime, Shanghai, China). The hyphae were incubated at 37 °C for 30 min in the darkness and washed twice with phosphate-buffered saline (PBS). A coverslip was placed on the hyphae, and the samples were observed and photographed using the microscopic morphology of fungal hyphae that was observed by a scanning electron microscope (Hitachi, S-3400 N, Japan).

MMP of *B. Cinerea*. Effects of compound **a3** on the MMP of *B. cinerea* mycelia were analyzed according to the previously described method.[49] After staining with rhodamine 123 solution (Beyotime,

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Table 1. *In Vitro* Antifungal Activities of Cryptolepine Derivatives against Four Phytopathogenic Fungi at 50 and 25 μg/mL[a]

		inhibition rate ± SD (%)			
compd	concn (μg/mL)	*S. s.*	*B. c.*	*F. g.*	*R. s.*
a1	50	79.91 ± 0.44	98.43 ± 0.21	72.15 ± 0.76	26.40 ± 0.71
	25	70.56 ± 0.23	96.26 ± 0.52	51.57 ± 0.33	23.67 ± 0.24
a2	50	89.82 ± 0.12	99.71 ± 0.11	76.84 ± 0.37	53.98 ± 0.49
	25	82.86 ± 0.71	99.19 ± 0.03	67.79 ± 0.71	47.66 ± 0.02
a3	50	84.34 ± 0.02	99.21 ± 0.02	71.29 ± 0.35	52.15 ± 0.47
	25	75.78 ± 0.04	99.15 ± 0.76	68.63 ± 0.15	49.35 ± 0.42
a4	50	78.51 ± 0.18	87.81 ± 0.25	65.83 ± 0.54	10.89 ± 0.05
	25	72.72 ± 0.15	76.48 ± 0.66	35.02 ± 1.09	8.37 ± 0.06
a5	50	86.67 ± 0.56	99.22 ± 0.05	65.13 ± 0.88	52.86 ± 0.15
	25	74.71 ± 0.09	99.09 ± 0.03	63.18 ± 0.25	49.27 ± 0.23
a6	50	56.37 ± 1.01	89.63 ± 0.29	75.63 ± 0.74	10.95 ± 0.08
	25	40.55 ± 0.01	77.78 ± 0.85	59.84 ± 0.41	6.49 ± 0.43
a7	50	95.23 ± 0.77	94.33 ± 0.31	44.25 ± 0.45	53.6 ± 1.68
	25	90.15 ± 1.72	92.13 ± 0.39	35.75 ± 1.69	43.03 ± 0.41
a8	50	92.65 ± 0.71	89.00 ± 1.35	55.93 ± 0.43	35.12 ± 0.26
	25	84.48 ± 0.94	86.84 ± 1.38	38.95 ± 0.76	20.75 ± 0.44
a9	50	82.74 ± 0.48	87.88 ± 1.04	34.67 ± 1.11	6.19 ± 0.15
	25	74.17 ± 0.48	83.16 ± 0.68	30.53 ± 1.27	0 ± 0
a10	50	83.86 ± 0.72	98.03 ± 0.36	62.85 ± 0.66	30.74 ± 0.35
	25	75.93 ± 1.07	95.76 ± 0.07	50.54 ± 4.87	8.15 ± 0.69
a11	50	76.31 ± 0.25	88.02 ± 1.83	65.52 ± 0.52	38.35 ± 1.05
	25	67.92 ± 0.76	81.75 ± 1.34	51.49 ± 1.86	23.83 ± 1.75
a12	50	94.35 ± 0.82	100 ± 0	56.94 ± 0.99	29.68 ± 1.74
	25	89.65 ± 1.01	100 ± 0	24.58 ± 1.95	11.83 ± 0.79
a13	50	97.85 ± 0.54	99.58 ± 0.12	46.84 ± 1.07	20.78 ± 1.25
	25	96.69 ± 0.87	98.21 ± 0.32	27.04 ± 1.04	4.62 ± 1.62
a14	50	86.46 ± 1.54	96.45 ± 0.68	64.83 ± 2.19	57.56 ± 0.54
	25	83.57 ± 0.55	95.56 ± 0.46	45.59 ± 0.97	34.79 ± 0.79
a15	50	97.03 ± 0.36	97.09 ± 0.66	42.87 ± 1.55	47.65 ± 2.27
	25	95.55 ± 0.39	95.41 ± 0.33	9.65 ± 1.26	20.88 ± 0.34
a16	50	96.72 ± 0.16	95.06 ± 0.32	17.62 ± 2.76	33.78 ± 0.89
	25	94.13 ± 0.27	94.39 ± 0.08	0 ± 0	20.83 ± 1.32
a17	50	94.38 ± 0.44	99.30 ± 0.36	14.67 ± 1.27	37.64 ± 1.73
	25	93.31 ± 0.34	98.25 ± 0.05	0 ± 0	18.79 ± 0.71
a18	50	99.11 ± 0.28	100 ± 0	64.96 ± 2.45	47.72 ± 1.26
	25	97.33 ± 0.49	100 ± 0	48.12 ± 0.67	31.57 ± 0.57
a19	50	100 ± 0	99.33 ± 0.24	48.23 ± 2.46	32.07 ± 0.84
	25	100 ± 0	98.44 ± 0.44	32.61 ± 0.53	2.13 ± 1.44
a20	50	100 ± 0	100 ± 0	47.27 ± 1.93	13.15 ± 4.91
	25	100 ± 0	100 ± 0	28.22 ± 0.48	0 ± 0
a21	50	100 ± 0	100 ± 0	45.11 ± 0.54	13.15 ± 4.91
	25	100 ± 0	100 ± 0	23.43 ± 0.38	0 ± 0
a22	50	71.69 ± 0.28	99.40 ± 0.28	54.03 ± 1.64	53.79 ± 2.52
	25	59.48 ± 1.31	98.68 ± 0.21	39.25 ± 0.53	48.47 ± 1.86
a23	50	90.33 ± 0.32	98.44 ± 0.16	27.71 ± 0.93	32.53 ± 2.56
	25	84.91 ± 0.42	97.68 ± 0.09	18.66 ± 1.24	16.95 ± 0.99
a24	50	94.35 ± 0.82	100 ± 0	56.94 ± 0.99	29.68 ± 1.74
	25	89.65 ± 1.01	100 ± 0	24.58 ± 1.95	11.83 ± 0.79
b1	50	23.45 ± 0.36	38.41 ± 0.53	66.23 ± 1.56	40.44 ± 0.26
	25	8.69 ± 1.48	16.38 ± 0.65	48.78 ± 0.77	27.15 ± 0.93
b2	50	5.14 ± 1.14	0 ± 0	57.89 ± 0.78	34.44 ± 1.32
	25	0 ± 0	0 ± 0	44.49 ± 1.27	21.98 ± 1.11
b3	50	41.64 ± 0.09	34.87 ± 1.82	41.36 ± 1.37	27.45 ± 0.66
	25	14.05 ± 0.02	19.68 ± 1.35	14.63 ± 1.33	24.11 ± 0.94
c1	50	74.35 ± 3.36	99.36 ± 0.27	52.03 ± 1.65	39.17 ± 1.46
	25	68.99 ± 2.04	96.35 ± 0.21	40.02 ± 0.37	30.28 ± 0.64
c2	50	0 ± 0	58.45 ± 1.31	49.98 ± 1.98	48.01 ± 2.03
	25	0 ± 0	49.57 ± 0.93	25.69 ± 1.61	30.74 ± 0.94
c3	50	38.47 ± 0.51	50.34 ± 0.34	48.66 ± 0.27	47.23 ± 0.55

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Table 1. continued

compd	concn (μg/mL)	inhibition rate ± SD (%)			
		S. s.	*B. c.*	*F. g.*	*R. s.*
	25	24.29 ± 0.02	29.21 ± 0.58	19.43 ± 0.48	31.26 ± 1.62
d1	50	76.22 ± 0.66	92.53 ± 0.16	66.99 ± 0.54	84.86 ± 1.45
	25	43.74 ± 0.77	87.36 ± 1.92	57.85 ± 0.49	75.01 ± 1.49
d2	50	76.97 ± 0.53	96.15 ± 0.38	64.95 ± 0.54	82.42 ± 1.82
	25	66.81 ± 0.28	95.65 ± 0.52	50.22 ± 1.15	80.75 ± 0.14
d3	50	76.21 ± 0.46	97.33 ± 0.58	63.79 ± 1.49	75.11 ± 0.25
	25	44.04 ± 1.35	94.38 ± 0.44	51.30 ± 0.61	68.93 ± 2.13
d4	50	68.33 ± 0.46	92.28 ± 0.33	56.38 ± 0.35	67.89 ± 0.36
	25	47.11 ± 0.26	84.47 ± 1.18	42.11 ± 0.45	50.11 ± 0.32
e1	50	12.55 ± 0.05	20.39 ± 0.19	23.21 ± 0.37	0 ± 0
	25	0 ± 0	12.33 ± 0.63	14.84 ± 1.03	0 ± 0
e2	50	15.33 ± 0.26	56.92 ± 0.15	69.48 ± 0.31	17.05 ± 0.79
	25	7.33 ± 2.14	36.57 ± 0.11	47.68 ± 0.32	8.33 ± 0.36
e3	50	15.65 ± 0.15	58.95 ± 0.93	69.81 ± 0.37	14.9 ± 0.22
	25	0 ± 0	42.38 ± 0.35	50.25 ± 0.22	7.21 ± 0.20
e4	50	8.83 ± 0.12	40.95 ± 0.44	10.83 ± 0.09	0 ± 0
	25	0 ± 0	20.67 ± 0.11	0 ± 0	0 ± 0
f1	50	93.13 ± 0.01	100	70.35 ± 0.46	89.74 ± 0.40
	25	90.11 ± 0.09	95.93 ± 0.09	70.05 ± 2.07	85.93 ± 0.38
f2	50	88.23 ± 0.47	95.44 ± 0.34	65.22 ± 1.17	83.63 ± 0.03
	25	83.35 ± 0.16	92.12 ± 0.41	58.23 ± 0.47	77.21 ± 0.15
G1	50	0 ± 0	0 ± 0	6.55 ± 0.27	0 ± 0
	25	0 ± 0	0 ± 0	0 ± 0	0 ± 0
g2	50	7.29 ± 0.11	0 ± 0	6.89 ± 0.28	0 ± 0
	25	0 ± 0	0 ± 0	0 ± 0	0 ± 0
h1	50	96.58 ± 0.08	100 ± 0	64.99 ± 1.80	79.25 ± 0.56
	25	94.33 ± 0.41	98.06 ± 0.09	60.43 ± 0.74	70.58 ± 1.81
h2	50	86.83 ± 0.55	95.34 ± 0.28	53.11 ± 0.32	63.46 ± 0.62
	25	77.45 ± 0.65	91.32 ± 0.37	48.87 ± 0.26	57.56 ± 0.56
i1	50	14.21 ± 0.34	15.12 ± 1.21	21.43 ± 0.36	0 ± 0
	25	7.23 ± 0.35	6.67 ± 0.45	8.20 ± 0.75	0 ± 0
i2	50	20.65 ± 0.29	0 ± 0	10.35 ± 0.28	0 ± 0
	25	7.67 ± 0.16	0 ± 0	0 ± 0	0 ± 0
j1	50	71.22 ± 0.69	53.40 ± 3.25	64.95 ± 2.82	63.18 ± 0.58
	25	50.68 ± 0.54	36.34 ± 0.73	51.93 ± 1.66	32.11 ± 0.59
ASB	50	45.57 ± 0.29	47.88 ± 0.43	61.85 ± 0.06	50.47 ± 0.90
	25	30.43 ± 0.98	27.00 ± 0.23	57.32 ± 0.28	35.27 ± 0.87

[a] *S.s., Sclerotinia sclerotiorum*; *B.c., Botrytis cinerea*. *F.g., Fusarium graminearum*; *R.s., Rhizoctonia solani*. ASB: azoxystrobin.

China), the mycelia were incubated at 24 °C for 30 min in darkness and washed with PBS. Finally, the samples were photographed using an LSM-800 with Airyscan.

Effects on the Nuclear Morphology of *B. Cinerea*. After staining with Hoechst 33258 (Beyotime, China), the mycelia were incubated at 24 °C for 30 min in darkness and washed with PBS. Finally, the samples were photographed using an LSM-800 with Airyscan.

***In Vivo* Curative Activity Bioassay.** Based on the preceding test of *in vitro* antifungal activity, compound **a3** against *B. cinerea* was further tested on apple. The synthesized compound and positive control azoxystrobin in 0.1 mL of DMSO were dissolved in 10 mL of deionized water at a series of concentrations (200, 100, 50, and 25 μg/mL). Each apple was punctured with an inoculating needle, and then, the pathogen was inoculated. After 24 h, each sample measured in quadruplicates was sprayed evenly onto the apple, which had been already washed and treated with water and 75% aqueous ethyl alcohol. DMSO (1%) in 10 mL of water was set up as the blank control. All the treated samples were then placed into an illumination incubator at 25 °C and 100% relative humidity for 6 days. All assays were at least performed in triplicates by conventional methods, and results were presented as mean ± standard deviations.

Statistical Analysis. The statistical analysis was performed by SPSS 24.0. The EC_{50} values were obtained from the parameters in the regression curves, and 95% CI, regression equation, and R^2 are provided in the Supporting Information.

RESULTS AND DISCUSSION

Chemistry. The synthesis of the A-ring, D-ring, and A, D-ring substituted derivatives, bioisosterisms, ring-opened products, and methyl isomers of cryptolepine is outlined in Figures 2−5. ^{1}H and ^{13}C NMR and MS of compounds were applied to confirm their structures.

To investigate the influence of the electronic effects on the biological activities of cryptolepine derivatives, an available and flexible routine directed by Abe et al.[40] was employed to obtain target compounds **a1**−**a24** (Figure 2). Furthermore, a series of bioisosterisms were obtained by using anthranilic acid as the starting material[40] (Figure 3), and ring-opened derivatives were primarily gained through some enduring synthetic routes[42−44] (Figure 4). The methyl isomer **j1** of cryptolepine

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Table 2. EC_{50} Values of Cryptolepine Derivatives against Four Phytopathogenic Fungi[a]

	EC_{50} (μg/mL)			
compd	*S. s.*	*B. c.*	*F. g.*	*R. s.*
a1	5.507	0.050	21.838	>30
a2	1.246	0.037	13.728	>30
a3	1.038	0.027	10.912	>30
a4	3.136	3.954	>30	>30
a5	5.466	0.034	18.920	>30
a6	>30	3.078	20.815	>30
a7	3.248	0.876	>30	>30
a8	3.099	0.066	>30	>30
a9	6.306	1.027	>30	>30
a10	4.472	0.148	26.669	>30
a11	12.119	2.115	21.970	>30
a12	2.445	0.042	>30	>30
a13	1.852	0.065	>30	>30
a14	2.714	0.068	>30	>30
a15	2.520	0.131	>30	>30
a16	1.283	0.041	>30	>30
a17	1.193	0.087	>30	>30
a18	1.353	0.159	>30	>30
a19	1.424	0.037	>30	>30
a20	1.664	0.034	>30	>30
a21	1.148	0.037	>30	>30
a22	17.933	0.047	>30	>30
a23	2.177	0.056	>30	>30
a24	1.476	0.071	>30	>30
b1	>30	>30	>30	>30
b2	>30	>30	>30	>30
b3	>30	>30	>30	>30
c1	8.217	0.041	>30	>30
c2	>30	>30	>30	>30
c3	>30	>30	>30	>30
d1	24.250	3.970	14.069	6.170
d2	8.682	1.655	20.752	3.821
d3	21.263	1.198	24.000	9.395
d4	25.083	3.303	>30	22.449
e1	>30	>30	>30	>30
e2	>30	>30	23.996	>30
e3	>30	>30	21.892	>30
e4	>30	>30	>30	>30
f1	3.302	1.450	5.599	3.632
f2	3.226	1.513	14.712	5.321
g1	>30	>30	>30	>30
g2	>30	>30	>30	>30
h1	2.498	1.644	16.914	9.911
h2	6.706	1.938	>30	18.649
i1	>30	>30	>30	>30
i2	>30	>30	>30	>30
j1	19.421	>30	22.188	>30
ASB	>30	>30	27.436	>30

[a]*S.s., Sclerotinia sclerotiorum; B.c., Botrytis cinerea. F.g., Fusarium graminearum; R.s., Rhizoctonia solani.* ASB: azoxystrobin.

was easily obtained by adding CH_3I to a solution of KOH and **b1** in acetone (Figure 5).

***In Vitro* Activity and Structure–Activity Relationship.** An efficient and productive method was used to synthesize target compounds (**a1**–**a24**) to investigate the electronic impact on inhibitory activity. As shown in Tables 1 and 2, the data of cryptolepine and its ring-substituted derivatives against

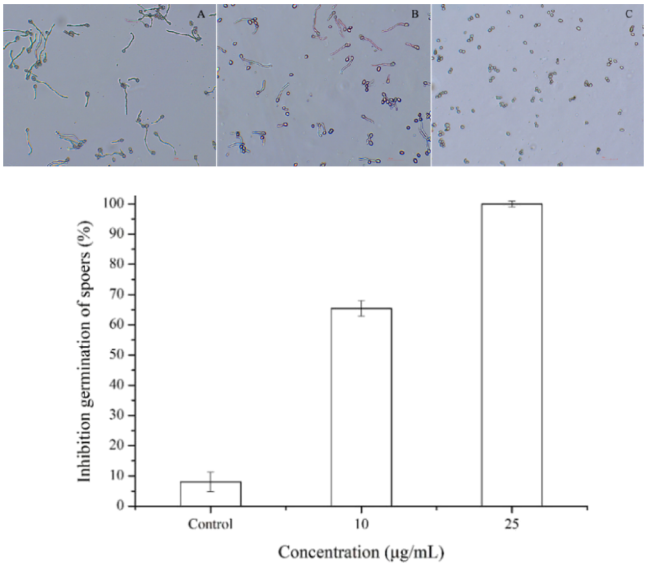

Figure 6. Spore germination inhibition assays of untreated control (A), 10 (B), and 25 μg/mL (C) of compound **a3**.

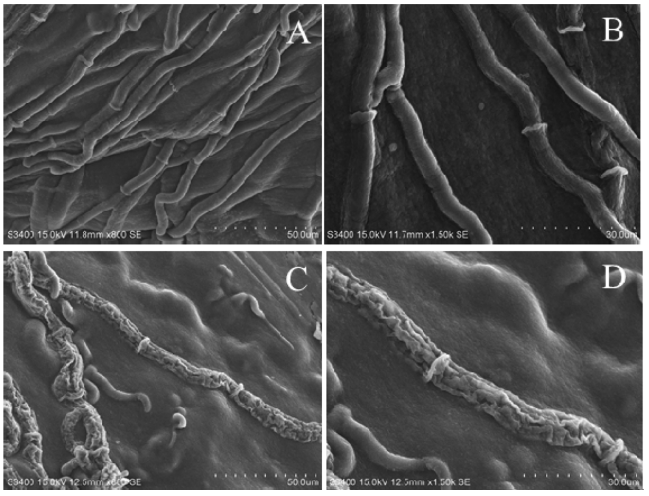

Figure 7. Scanning electron micrographs of *B. cinerea* hyphae in the untreated control (A,B) and 0.05 μg/mL (C,D) of compound **a3**.

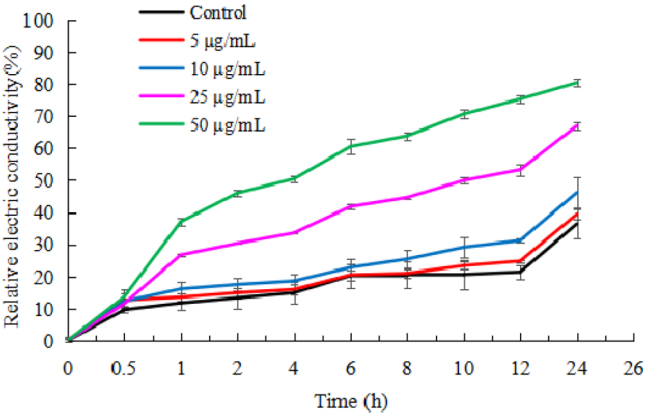

Figure 8. Effects of compound **a3** on the cell membrane permeability of *B. cinerea*.

R. solani, *B. cinerea*, *F. graminearum*, and *S. sclerotiorum* were demonstrated at 50 and 25 μg/mL. The inhibition activity of

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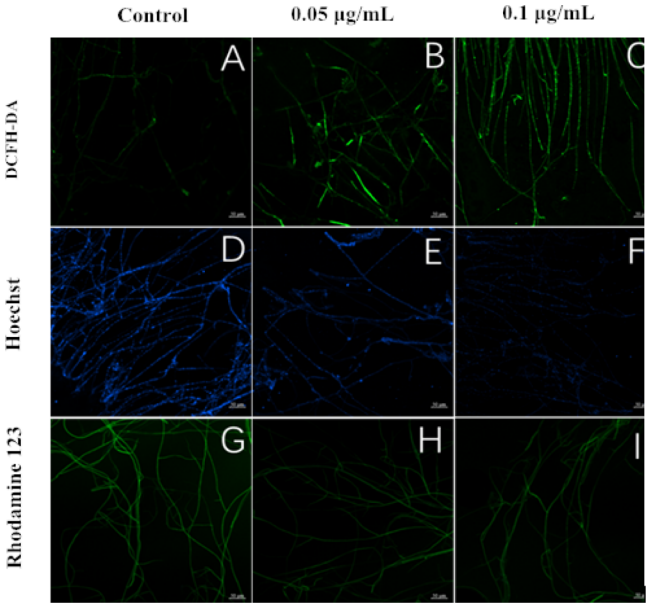

Figure 9. Effects of **a3** on the reactive oxygen species, cell nuclear, and MMP of *B. cinerea*.

the lead compound cryptolepine to *S. sclerotiorum* (EC_{50} = 5.507 μg/mL) and *B. cinerea* (EC_{50} = 0.050 μg/mL) was evidently better than other two fungi, prompting us to execute some ring-substituted modifications to further expand the range of antifungal spectrum and explore the structure−activity relationship (SAR).

Statistics showed that inhibitory rate of most A-ring derivatives (**a2**−**a12**) against *B. cinerea* at a concentration of 50 μg/mL exceeded 90%, which was superior to the positive control azoxystrobin. Especially compound **a3** (EC_{50} = 0.027 μg/mL) exhibited the unprecedented activity among them. Besides, the inhibitory rate of the majority of modifications against *S. sclerotiorum* was better than cryptolepine. Experimental results revealed that when some atoms or certain groups like chlorine, trifluoromethyl, methoxyl, and methyl were introduced into position C2 or position C3 of the A ring, the inhibition effects to *S. sclerotiorum* and *B. cinerea* were variably strengthened. Comparing **a3** (EC_{50} = 1.038 μg/mL) with **a10** (EC_{50} = 4.472 μg/mL) against *S. sclerotiorum* or **a7** (EC_{50} = 0.876 μg/mL) with **a11** (EC_{50} = 2.115 μg/mL) against *B. cinerea*, we found that antifungal activities of compounds modified at position C2 was better than that at position C3. However, the introduction of some larger atoms or groups like **a6** (bromine at C2) or **a11** (*tert*-butyl at C3) could evidently reduce the activity against *S. sclerotiorum* and *B. cinerea*. Most of the compounds showed reduced antifungal activity against *F. graminearum*, except for **a2** (EC_{50} = 13.728 μg/mL), **a3** (EC_{50} = 10.912 μg/mL), and **a5** (EC_{50} = 18.920 μg/mL) modified with a chlorine atom, methoxyl group, and methyl group, respectively. Nearly, all modified compounds did not show good activity against *R. solani*.

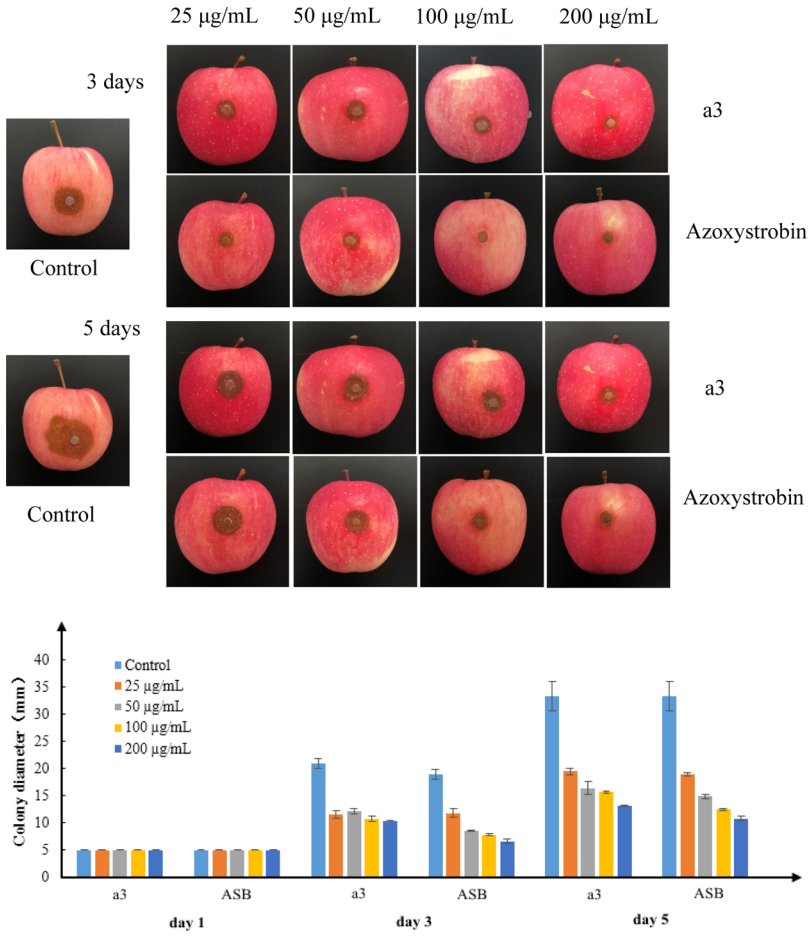

Figure 10. *In vivo* curative antifungal activities of compound **a3** against *B. cinerea*. ASB: azoxystrobin.

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Subsequently, a series of D-ring derivatives (**a13**−**a21**) were synthesized to further investigate their antifungal activities. Most of the D-ring compounds exhibited better antifungal activities with an EC_{50} of 1.148−2.714 μg/mL against *S. sclerotiorum* and an EC_{50} of 0.034−0.159 μg/mL against *B. cinerea*, which were almost equal with compound **a3** and prior to other A-ring derivatives. When the electron-donating group like methyl or methoxyl was introduced at position C7 or position C8 of the D-ring, the activity against *S. sclerotiorum* was better than compounds with the electron-withdrawing group. One thing worth noting was that the compound **a20** bearing a bromine at 8-position showed excellent activity against *B. cinerea* with an EC_{50} of 0.034 μg/mL, illustrating that the introduction of a large group into the A-ring might improve the antifungal effect. Compounds modified at A, D-rings together showed almost equal activity against *S. sclerotiorum* and *B. cinereal*, but all A-ring, D-ring, and A, D-ring derivatives showed poor activity against *F. graminearum* and *R. solani*. We speculated that introduction of different substitution groups at the A-ring or D-ring could be used to search for compounds with improved antifungal activity, but other modification methods were needed to expand the antifungal spectrum of lead cryptolepine.

According to the previous work reported by Ning et al.,[19] cryptolepine derivatives were prepared by replacing the nitrogen atom at position 10 with oxygen or sulfur to search for antimicrobial agents. Hence, a whole variety of bioisosterisms and salt forms of them were synthesized to explore their activities against plant pathogenic fungi.[23] From the activity data results, we found that the salt form **c1** of cryptolepine exhibited an improved activity with an EC_{50} of 0.041 μg/mL against *B. cinerea*. However, the antifungal activity of cryptolepine would become worse when the methyl at C5 was lacking. Bioisosterisms **b2**, **b3**, and their salt forms showed poor activity against four pathogenic fungi. Similar to A-ring, D-ring, and A, D-ring derivatives, most compounds did not show good activity against *F. graminearum* and *R. solani*. This implied that the integrity of the methyl group at 5-positon was vitally essential for antifungal activity of lead cryptolepine and the replacement of the nitrogen atom at position 10 could not effectively expand the antifungal spectrum.

The C-ring of cryptolepine was opened and then formed a series of quinolone derivatives decorated by heteroatoms linking with different substituted benzenes. Pleasantly, we found that compounds **d1**, **d2**, **d3**, **f1**, **f2**, and **h1** exhibited significantly improved activity against *F. graminearum* and *R. solani*; especially, **f1** possessed the most potent antifungal activity with an EC_{50} of 5.599 μg/mL against *F. graminearum* and an EC_{50} of 3.632 μg/mL against *R. solani*. Among them, compound **h1** displayed an outstanding inhibition effect against *S. sclerotiorum* with an EC_{50} of 2.498 μg/mL and compound **d3** possessed an EC_{50} of 1.198 μg/mL against *B. cinerea*. When nitrogen was replaced with oxygen (compounds **f1** and **f2**), the antifungal activity of formed derivatives against *R. solani* and *F. graminearum* was enhanced effectively. Compounds decorated with sulfur (compounds **h1** and **h2**) showed moderate enhancement to *R. solani*. Altogether, compounds replaced with oxygen performed better inhibition effects than compounds replaced with sulfur or nitrogen. The SAR study revealed that compounds bearing an electron-withdrawing group in the para position of the benzene ring was worse than the introduction of the electron-donating group or unmodified compounds. For ring-opened derivatives, inhibition effects would become vastly worse after methylation of the N of the quinoline ring. Lastly, the methyl isomer **j1** of cryptolepine was synthesized, but its antifungal activity was weaker than cryptolepine, implying that the introduction of methyl at N-10 was not a good strategy.

By systematic structural modification and ring-opened simplification, we have obtained compound **a3** (EC_{50} = 0.027 μg/mL against *B. cinerea*) with improved activity and compound **f1** (EC_{50} = 5.599 μg/mL against *F. graminearum* and EC_{50} = 3.632 μg/mL against *R. solani*) with an expanded antifungal spectrum, which was of great significance for the further research and exploration of cryptolepine.

Preliminary Mode of Action of Compound a3. *Effects of Compound **a3** on the Spore Germination.* *B. cinerea* is a saprophytic fungus that infects the host mainly by conidia, so the harm to the host plant could be reduced by inhibiting spore germination. As shown in Figure 6, the outcomes of spore germination inhibition assay indicated that compound **a3** could markedly inhibit the spore germination of *B. cinerea* by 65% and more than half of the spores did not germinate at a concentration of 10 μg/mL. Furthermore, the length of the germ tube formed by the germinated spores was shorter than the untreated group. Meanwhile, the data from the experiment indicated that effects of compound **a3** on the spore germination of *B. cinerea* were concentration-dependent.

*Scanning Electron Micrographs of Compound **a3** on the Hyphae Morphology.* To elucidate the effective pathway of compound **a3** against *B. cinerea*, the mycelia were treated with 0.05 μg/mL compound **a3** (Figure 7C,D) and we observed structural changes. We found that the tested mycelia were collapsed, shriveled, and crumpled compared with the regular, smooth, and homogeneous hyphae (Figure 7A,B). This indicated that compound **a3** might damage the cell membrane of mycelia, leading to spillage of cellular contents and barriers to the transport of the cellular material.

*Effects of Compounds **a3** on the Cell Membrane Permeability.* To determine whether compound **a3** acted on the membrane of *B. cinerea*, the test of changes in electrical conductivity of hyphae was executed. As shown in Figure 8, after treating the hyphae of *B. cinerea* with compound **a3**, the increase of conductivity was evidently rapid within 24 h and obviously concentration-dependent. These results suggested that compound **a3** could damage the cell membrane of hyphae in a dose-dependent manner, resulting in electrolyte leakage, thereby increasing conductivity.

*Effects of Compound **a3** on ROS Production.* From the fluorescence of Figure 9A−C, it was found that hyphae treated with **a3** at 0.05 and 0.1 μg/mL had an obvious fluorescence effect compared with untreated hyphae, and fluorescence intensity enhanced significantly with the increase of the compound concentration. Cells of *B. cinerea* were forced to practice oxidative stress response under the destructive effect of the compound **a3**, leading to changes of cell membrane permeability and hyphae morphology.

*Effects of Compound **a3** on the Nuclear Morphology.* The influence of **a3** at 0.05 and 0.1 μg/mL to the nucleus was shown at Figure 9D−F. The blue fluorescence signal of the drug application group was obviously weaker than the normal growing group, indicating that **a3** could cause severe damage to the cell nucleus of *B. cinerea* and induce mycelial death.

*Effects of Compound **a3** on the MMP.* The influence of **a3** to the MMP was shown at Figure 9G−I. The results showed that the fluorescence intensity of hyphae treated with **a3** was

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weaker than the untreated hyphae, and compound **a3** might cause mitochondrial dysfunction in mycelia by changing the MMP.

In Vivo Curative Effects against B. cinerea. Based on the bioassay data *in vitro*, compound **a3** was selected to further investigate *in vivo* activity against *B. cinerea*. As presented in Figure 10, the therapeutic effect of compound **a3** was gradually equal to azoxystrobin with the increase of dosage. The ulceration range in the administration group was markedly smaller than the blank control. Besides, the therapeutic effects at low concentrations of 25 and 50 μg/mL were similar to the positive control drug azoxystrobin on the 5th day, indicating that compound **a3** might have a longer curative time at low concentrations.

In conclusion, we synthesized a series of different types of compounds by structural modification and simplification of cryptolepine. Bioassay results showed that compound **a3** possessed the most potent antifungal activity against *B. cinerea* and ring-opened compound **f1** possessed an improved inhibition effect against *F. graminearum* and *R. solani*. The mechanism study of compound **a3** found that it could bring about adverse changes to spore germination, normal form of mycelium, and cell membrane of *B. cinerea*. Simultaneously, reactive oxygen, the normal cell structure, and MMP were badly produced, disrupted, and affected. In view of the above study on compound **a3**, it would be a helpful candidate to control crop disease. Further studies on the structural optimization cryptolepine and more detailed mechanisms of **a3** are in progress.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.0c06480.

***In vitro* EC_{50} values; physical properties of target compounds; and spectrograms** (PDF)

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Notes

The authors declare no competing financial interest.

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