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Novel Flavonol Derivatives Containing 1,3,4-Thiadiazole as Potential Antifungal Agents: Design, Synthesis, and Biological Evaluation

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Cite This: *ACS Omega* 2024, 9, 17297−17306

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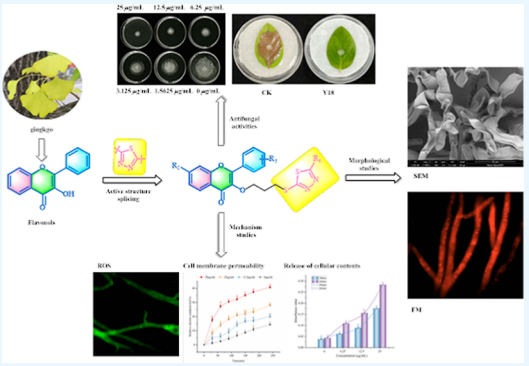
ABSTRACT: In order to discover novel compounds with excellent agricultural activities, novel flavonol derivatives containing 1,3,4-thiadiazole were synthesized and evaluated for their antifungal activities. The bioassay results showed that some of the target compounds had good antifungal activities against *Botrytis cinerea*, *Phomopsis* sp. and *Sclerotinia sclerotiorum* in vitro. It is worth noting that the half-effective concentration (EC_{50}) value of **Y18** against *B. cinerea* was 2.4 μg/mL, which was obviously superior to that of azoxystrobin (21.7 μg/mL). The curative activity of **Y18** at 200 μg/mL (79.9%) was better than that of azoxystrobin (59.1%), and its protective activity (90.9%) was better than that of azoxystrobin (83.9%). Morphological studies by using scanning electron microscopy and fluorescence microscopy revealed that **Y18** could affect the normal growth of *B. cinerea* mycelium. In addition, the mechanism of action studies indicated that **Y18** could affect the integrity of cell membranes by inducing the production of endogenous reactive oxygen species and the release of the malondialdehyde content, leading to membrane lipid peroxidation and the release of cell contents. The inhibitory activity of flavonol derivatives containing 1,3,4-thiadiazole on plant fungi is notable, offering significant potential for the development of new antifungal agents.



1. INTRODUCTION

Fungal plant pathogen infection causes significant losses to the global agricultural economy and poses a huge threat to human health and food security.[1] Another notable example of a plant disease is gray mold, caused by the pathogen *Botrytis cinerea*. This organism has a broad host range and is notorious as one of the most devastating plant fungal diseases.[2] This pathogen severely impairs the economic outcomes of various vital cash crops, such as grapes, strawberries, blueberries, and tomatoes. It can infect the roots, leaves, flowers, and fruits of plants, thereby reducing crop yields.[3,4] At present, the use of fungicides in crop cultivation is among the most reliable strategies for controlling plant diseases.[5] But the long-term and widespread use of chemical fungicides has led to an increase in the resistance of plant pathogenic fungi and posed severe threats to the environment.[6,7] Therefore, the discovery of novel, efficient, and ecofriendly fungicides is indispensable to tackling the aforementioned challenge.

Flavonols, 3-hydroxyl flavones, are a unique flavonoid mainly found in dicotyledonous plants, especially in the flowers and leaves of some woody plants, such as the ginkgo biloba, sea buckthorn, and sophora flowers.[8,9] Flavonoids, as important products of plant secondary metabolism, have various excellent physiological activities, extensive sources, minimal side effects, and exceptional safety.[10] Moreover, flavonols and their derivatives have good antiviral,[11,12] antibacterial,[13,14] insecticidal,[15,16] antioxidant,[17] anticancer,[18,19] and other biological activities,[20] and are widely used in the fields of pesticides and pharmaceuticals.

In addition, 1,3,4-thiadiazole and its derivatives, a category of five-membered heterocyclic compounds, possess good physical and chemical properties.[21] Their structural units contain the basic structural framework of carbon, nitrogen and sulfur, which may be chemically modified to obtain highly efficient and low-toxicity bioactive derivatives.[22,23] Among them, 1,3,4-thiadiazole derivatives have a wide variety of biological activities, such as antiviral,[24] antibacterial,[25,26] antifungal,[27,28] insecticidal,[29,30] and other biological activities.[31,32] Their vast applications in pesticides have been acknowledged widely. Currently, among the various successfully developed products are the bactericides bismerthiazol, thiodiazole copper, and the herbicide fluthiamide (Figure 1).

Received: December 23, 2023
Revised: March 14, 2024
Accepted: March 22, 2024
Published: April 5, 2024

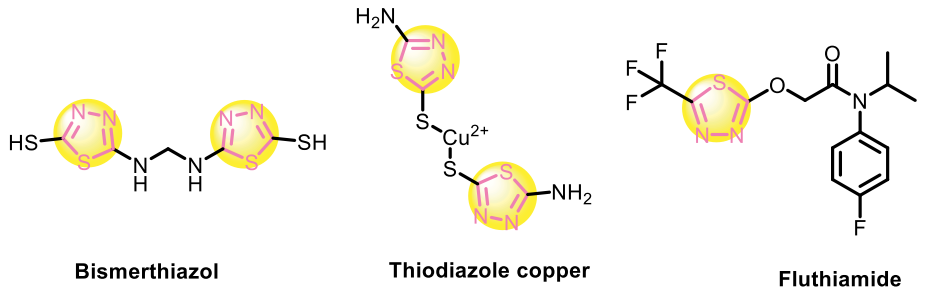


Figure 1. Chemical structures of 1,3,4-thiadiazole fragments with biological activities.

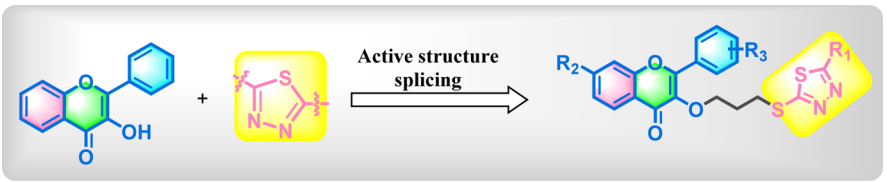
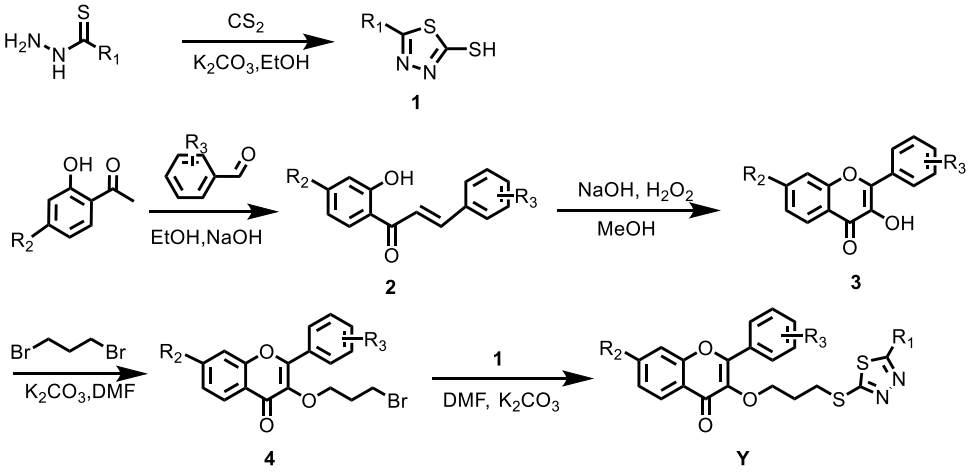


Figure 2. Design of target compounds.

Scheme 1. Synthetic Route of the Target Compounds Y1−Y22



Y1: R_1=NH_2, R_2=H, R_3=4-CH_3
Y2: R_1=NH_2, R_2=H, R_3=4-OCH_3
Y3: R_1=NH_2, R_2=H, R_3=2-OCH_3
Y4: R_1=NH_2, R_2=H, R_3=4-Cl
Y5: R_1=NH_2, R_2=H, R_3=4-Br
Y6: R_1=NH_2, R_2=H, R_3=4-F
Y7: R_1=NH_2, R_2=H, R_3=3-F
Y8: R_1=NH_2, R_2=OCH_3, R_3=4-CH_3
Y9: R_1=NH_2, R_2=OCH_3, R_3=4-OCH_3
Y10: R_1=NH_2, R_2=OCH_3, R_3=2-OCH_3
Y11: R_1=NH_2, R_2=OCH_3, R_3=4-F

Y12: R_1=H, R_2=H, R_3=4-CH_3
Y13: R_1=H, R_2=H, R_3=4-OCH_3
Y14: R_1=H, R_2=H, R_3=2-OCH_3
Y15: R_1=H, R_2=H, R_3=4-Cl
Y16: R_1=H, R_2=H, R_3=4-Br
Y17: R_1=H, R_2=H, R_3=4-F
Y18: R_1=H, R_2=H, R_3=3-F
Y19: R_1=H, R_2=OCH_3, R_3=4-CH_3
Y20: R_1=H, R_2=OCH_3, R_3=4-OCH_3
Y21: R_1=H, R_2=OCH_3, R_3=2-OCH_3
Y22: R_1=H, R_2=OCH_3, R_3=4-F

In summary, we propose to introduce substituting 1,3,4-thiadiazole groups into the structure of flavonols by active substructure splicing to synthesize a series of flavonol derivatives containing 1,3,4-thiadiazole. Following biological activity screening and mechanism studies, the compound **Y18** was discovered to possess superior antifungal activity compared to azoxystrobin, which provides significant potential for the development of novel antifungal drugs (Figure 2).

2. RESULTS AND DISCUSSION

2.1. Synthesis. Scheme 1 outlines the design and synthesis of 22 flavonol derivatives containing 1,3,4-thiadiazole (**Y1**−**Y22**). First, by using substituted thionylhydrazine and CS_2 as raw materials, intermediates **1** were obtained in the reflux reaction. Utilizing substituted 2-hydroxyacetophenone and substituted benzaldehyde as raw materials, EtOH was used as a solvent to obtain intermediates **2** by a hydroxyl aldehyde condensation reaction under alkaline conditions at room temperature. Then, using intermediates **2** as the raw material, MeOH as the solvent, and H_2O_2 as the oxidant, intermediate **3** was obtained by cyclization reaction under alkaline conditions. 1,3-dibromopropane reacted with intermediates **3** to synthesize intermediates **4** at room temperature. Finally, intermediates **4** and **1** were stirred at 25 °C for 8 h with K_2CO_3 as a weak acid binder to yield flavonol derivatives containing 1,3,4-thiadiazole. The structures of these compounds were verified by ^{1}H, ^{13}C, and ^{19}F nuclear magnetic resonance (NMR) and high-

https://doi.org/10.1021/acsomega.3c10294
ACS Omega 2024, 9, 17297−17306

Table 1. Inhibition Effect of Target Compounds against Ten Plant Phytopathogenic Fungi at 100 μg/mL

compounds	inhibition rates (%)[a]									
	Bc[b]	*Ps*	*Cg*	*Rs*	*Pc*	*Ss*	*Fcu*	*Fg*	*Ab*	*Fca*
Y1	55.5 ± 1.2	32.2 ± 3.0	47.7 ± 1.7	54.0 ± 3.9	30.0 ± 3.7	40.6 ± 1.1	20.5 ± 1.1	33.2 ± 3.0	44.4 ± 1.6	44.4 ± 0.6
Y2	61.3 ± 3.8	40.4 ± 4.1	46.0 ± 1.2	55.5 ± 3.0	38.4 ± 1.2	38.2 ± 1.1	18.9 ± 1.1	36.5 ± 2.2	46.0 ± 2.9	33.9 ± 1.1
Y3	65.1 ± 3.3	37.2 ± 1.1	47.7 ± 1.7	59.3 ± 4.0	34.5 ± 3.2	63.6 ± 1.7	32.3 ± 1.7	24.9 ± 0.9	51.7 ± 1.3	48.8 ± 0.9
Y4	36.6 ± 0.9	34.1 ± 1.1	42.7 ± 1.4	38.6 ± 3.0	41.8 ± 3.8	43.8 ± 3.4	35.0 ± 1.2	38.5 ± 2.4	51.0 ± 4.1	40.3 ± 1.1
Y5	38.7 ± 2.8	60.2 ± 1.1	41.8 ± 2.2	43.8 ± 4.9	36.3 ± 2.2	44.6 ± 2.7	32.7 ± 1.2	31.6 ± 3.6	49.8 ± 1.6	42.3 ± 0.9
Y6	60.1 ± 3.9	51.7 ± 1.3	32.2 ± 2.0	48.5 ± 2.4	28.7 ± 4.4	10.7 ± 1.4	41.7 ± 1.1	32.8 ± 3.3	35.6 ± 1.3	42.3 ± 2.6
Y7	74.6 ± 2.7	36.8 ± 1.1	30.1 ± 2.6	55.3 ± 1.0	32.1 ± 2.7	51.2 ± 3.7	29.1 ± 1.4	35.7 ± 1.7	32.6 ± 1.1	39.1 ± 1.7
Y8	62.2 ± 3.5	53.5 ± 2.8	35.6 ± 1.9	51.5 ± 0.8	31.2 ± 4.9	52.6 ± 2.3	30.7 ± 1.1	28.3 ± 2.6	42.9 ± 0.9	42.7 ± 1.8
Y9	54.4 ± 0.9	55.0 ± 4.6	36.0 ± 1.2	52.2 ± 1.0	49.4 ± 2.9	53.8 ± 1.6	31.1 ± 1.6	31.6 ± 3.8	39.8 ± 0.9	47.6 ± 1.1
Y10	56.7 ± 2.2	37.2 ± 1.1	38.9 ± 1.2	47.1 ± 1.8	51.9 ± 2.9	45.0 ± 1.7	30.3 ± 2.2	33.2 ± 4.3	28.7 ± 1.3	41.5 ± 2.6
Y11	33.3 ± 4.7	27.1 ± 1.7	24.3 ± 1.7	57.4 ± 1.8	35.7 ± 1.2	45.7 ± 1.7	46.5 ± 2.6	33.5 ± 1.7	36.8 ± 1.7	47.6 ± 1.1
Y12	91.7 ± 1.2	59.6 ± 1.9	36.0 ± 1.2	55.5 ± 2.0	24.5 ± 2.7	54.2 ± 1.4	37.8 ± 1.1	35.7 ± 1.7	39.5 ± 1.1	45.6 ± 3.0
Y13	83.2 ± 2.4	42.8 ± 1.1	41.8 ± 1.7	54.0 ± 2.0	34.2 ± 3.8	47.0 ± 1.4	46.5 ± 1.1	35.7 ± 1.7	39.8 ± 1.6	52.4 ± 1.1
Y14	69.1 ± 1.9	73.8 ± 1.7	54.0 ± 1.2	65.6 ± 1.6	16.8 ± 3.4	71.5 ± 1.2	40.9 ± 1.4	20.2 ± 1.6	47.9 ± 1.1	50.8 ± 1.8
Y15	58.8 ± 2.4	59.0 ± 0.9	36.0 ± 1.2	40.1 ± 0.8	32.9 ± 3.8	57.4 ± 0.9	39.4 ± 1.1	51.6 ± 3.8	34.5 ± 1.1	47.6 ± 1.1
Y16	50.4 ± 4.2	50.2 ± 2.2	23.9 ± 1.2	40.8 ± 2.4	32.5 ± 4.5	48.8 ± 1.8	39.4 ± 1.1	31.2 ± 2.0	30.3 ± 1.1	51.6 ± 2.0
Y17	87.3 ± 1.1	66.1 ± 0.5	46.4 ± 1.2	65.2 ± 2.0	22.4 ± 4.4	65.3 ± 2.8	34.3 ± 1.6	28.4 ± 1.7	48.3 ± 1.1	50.4 ± 1.2
Y18	96.2 ± 1.7	65.3 ± 1.1	45.2 ± 1.7	63.4 ± 2.4	22.4 ± 2.9	58.7 ± 2.3	29.5 ± 0.9	18.0 ± 1.6	47.1 ± 1.3	51.2 ± 0.9
Y19	60.1 ± 4.3	49.4 ± 2.9	41.0 ± 5.7	45.6 ± 1.0	28.3 ± 4.0	47.0 ± 1.4	41.3 ± 0.9	32.0 ± 2.3	40.2 ± 1.9	46.4 ± 1.7
Y20	65.9 ± 4.1	42.8 ± 3.5	34.3 ± 1.7	45.2 ± 2.4	49.8 ± 3.4	45.0 ± 09	26.0 ± 1.1	26.6 ± 2.2	31.4 ± 1.6	46.4 ± 0.9
Y21	58.4 ± 2.6	72.8 ± 0.9	45.2 ± 4.6	46.3 ± 1.6	46.8 ± 2.0	66.9 ± 3.4	35.4 ± 1.1	44.3 ± 3.3	42.5 ± 1.3	48.4 ± 1.1
Y22	52.7 ± 4.3	38.0 ± 4.3	37.7 ± 0.9	52.8 ± 1.0	52.7 ± 4.3	41.7 ± 3.2	28.7 ± 0.9	37.7 ± 0.9	36.8 ± 1.7	44.4 ± 1.2
AZ[c]	80.7 ± 1.2	58.1 ± 1.1	56.1 ± 2.4	75.7 ± 0.3	75.5 ± 1.9	71.9 ± 2.3	48.0 ± 1.9	36.6 ± 3.4	24.5 ± 0.9	60.9 ± 0.9

[a]Average of three replicates. [b]*Rs* (*Rhizoctonia solani*), *Bc* (*B. cinerea*), *Fg* (*Fusarium graminearum*), *Cg* (*Colletotrichum gloeosporioides*), *Ss* (*S. sclerotiorum*), *Pc* (*Phytophthora capsica*), *Ab* (*Alternaria brassicae*), *Fcu* (*Fusarium oxysporum f.* sp. *cucumerinum*), *Fca* (*Fusarium oxysporum f.* sp. *capsicum*), and *Ps* (*Phomopsis* sp.). [c]**AZ** (azoxystrobin).

resolution mass spectrometry (HRMS) data. Refer to the Supporting Information for detailed synthesis steps.

2.2. Evaluation of Antifungal Activity. *2.2.1. Antifungal Activity In Vitro.* Preliminary antifungal activity results of **Y1**−**Y22** against ten types of pathogenic fungi in vitro are presented in Table 1. The data illustrated that some compounds exhibit excellent antifungal activities. The inhibition rates of **Y12**, **Y13**, **Y17**, and **Y18** against *B. cinerea* were 91.7, 83.2, 87.3, and 96.2%, respectively, which were superior to that of azoxystrobin (80.7%). The inhibition rates of **Y14** and **Y21** against *Phomopsis* sp. were 73.8 and 72.8%, respectively, which were superior to those of azoxystrobin (58.1%). The antifungal efficacy of **Y14** against *Sclerotinia sclerotiorum* was 71.5%, which was similar to that of azoxystrobin (71.9%).

By evaluating the EC_{50} values of compounds with good activity against three fungal strains, the antifungal potential of these synthetic compounds was further assessed (Table 2 and Figure 3). It is noteworthy that the EC_{50} value of **Y18** against *B. cinerea* was 2.4 μg/mL, which was obviously superior to that of azoxystrobin (21.7 μg/mL), and the EC_{50} value of **Y14** against *Phomopsis* sp. was 21.2 μg/mL and better than that of azoxystrobin (29.2 μg/mL).

Table 2. EC_{50} Values of Several Target Compounds[a]

fungi	compounds	EC_{50} (μg/mL)	r^2	regression equation
Bc	**Y12**	3.5	0.9826	$y = 1.2119x + 4.3468$
	Y13	4.8	0.9966	$y = 0.7208x + 4.5068$
	Y17	7.2	0.9691	$y = 1.0206x + 4.1271$
	Y18	2.4	0.9841	$y = 1.0797x + 4.5941$
	AZ	21.7	0.9727	$y = 1.0730x + 3.5651$
Ss	**Y14**	45.9	0.9661	$y = 1.1608x + 3.0713$
	AZ	43.6	0.9666	$y = 0.8361x + 3.6291$
Ps	**Y14**	21.2	0.9503	$y = 0.9175x + 3.7831$
	Y21	25.5	0.9514	$y = 0.9474x + 3.6677$
	AZ	29.2	0.9953	$y = 0.5431x + 4.2039$

[a]Average of three replicates.

2.2.2. Structure−Activity Relationship. First, the antifungal activity was higher when the R_1 group was substituted with −H rather than $-NH_2$. Second, compounds with electron-withdrawing groups on the phenyl substituent of the flavonol structure had better antifungal activity, specifically, **Y18** (3-F) > **Y12** (4-CH_3) > **Y13** (4-OCH_3). Finally, the antifungal activity was greater when R_3 was 3-F compared to when R_3 was 4-F. In summary, the antifungal activity of **Y18** (R_1 = H, R_2 = H, and R_3 = 3-F) was significantly superior to other target compounds and azoxystrobin.

*2.2.3. Antifungal Activity of **Y18** and **Y14** In Vivo.* **Y18** was chosen to further explore the antifungal activity against *B. cinerea* on blueberry leaves in vivo. As depicted in Table 3 and Figure 4, compared with the untreated negative control group, the leaf spot length of blueberries treated with **Y18** was smaller and the leaf decay was lighter, indicating that **Y18** obviously had inhibitory activity against *B. cinerea*. The curative activity of **Y18** (79.9%) at 200 μg/mL was superior to that of azoxystrobin (59.1%), and the protective activity (90.9%) was better than that of azoxystrobin (83.9%). The experimental results suggested that **Y18** could be potentially utilized in crop protection.

Y14 was selected to evaluate its antifungal activity against *Phomopsis* sp. on kiwifruit further in vivo. As can be seen in Table 4 and Figure 5, the protective activity of **Y14** against *Phomopsis* sp. was 71.8% at 200 μg/mL, and its curative activity was 60.2%, which was superior to those of azoxystrobin (60.2

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ACS Omega 2024, 9, 17297−17306

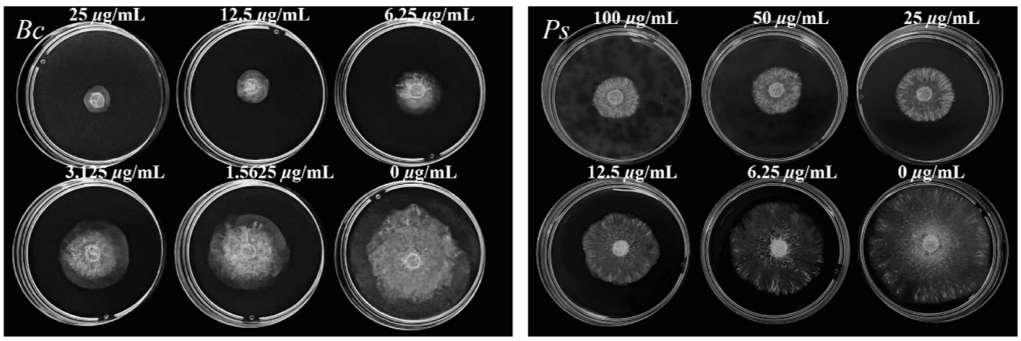

Figure 3. Antifungal activities of **Y18** against *B. cinerea* (*Bc*) and **Y14** against *Phomopsis* sp. (*Ps*) in vitro.

Table 3. Curative and Protective Activities of Y18 against *B. cinerea* In Vivo[a]

treatment	concentration (μg/mL)	curative activity		protective activity	
		lesion length (mm)	control efficacy (%)	lesion length (mm)	control efficacy (%)
Y18	200	10.0 ± 1.7[c]	79.9	7.2 ± 0.8[c]	90.9
AZ	200	15.2 ± 3.0[b]	59.1	8.8 ± 1.0[b]	83.9
negative control		29.8 ± 4.8[a]		28.8 ± 4.2[a]	

[a]Values are the mean ± SD of three replicates. Statistical analysis was conducted using SPSS 27.0 software. Different letters indicate significant differences at $p < 0.05$ in the same group.

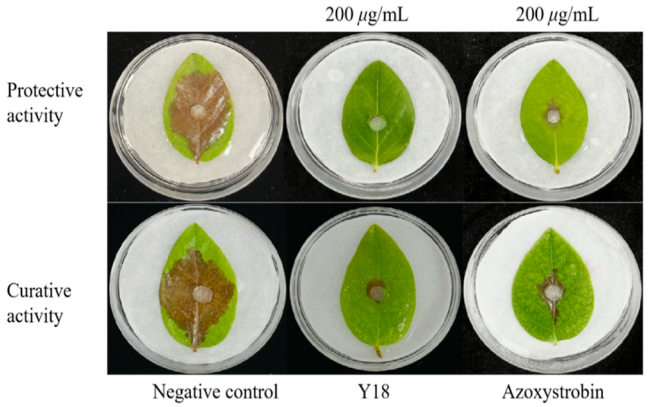

Figure 4. Curative and protective activity of **Y18** and azoxystrobin against *B. cinerea* on blueberry leaves.

and 59.9%, respectively). It can be shown that **Y14** has excellent antifungal activity against *Phomopsis* sp. in vivo.

2.3. Morphological Analysis by Scanning Electron Microscopy and Fluorescence Microscopy. The effect of **Y18** on the *B. cinerea* mycelium was assessed by scanning electron microscopy (SEM), which revealed morphological changes. As depicted in Figure 6, the mycelium of the untreated negative control group had a full shape, smooth surface, and vigorous growth, whereas it was wrinkled, collapsed, and severely deformed after being treated with 50 and 100 μg/mL **Y18**, respectively. The study revealed that with increasing **Y18** concentrations, the damage to the mycelium became more severe, resulting in adverse effects on its normal growth.

Propyl iodide (PI) is a unique nuclear dye that emits red fluorescence when embedded in double-stranded DNA, and is primarily used for DNA staining. When more and brighter red fluorescence appears, it indicates more severe cell membrane damage.[33] As depicted in Figure 7, the blank control group displayed almost no fluorescence, indicating that the mycelium within this group was undamaged. However, with the increase of concentration of **Y18**, more red fluorescence was present, indicating that **Y18** can damage the integrity of the mycelial cell membrane.

2.4. Reactive Oxygen Species Assay of *B. cinerea*. Reactive oxygen species (ROS) is important to the regulation of a normal cellular process. ROS imbalance can cause the body's oxidation-antioxidant imbalance, resulting in cell membrane destruction and cell death.[34,35] As depicted in Figure 8, the untreated negative control group demonstrated almost no green fluorescence. However, after treatment with various concentrations of **Y18**, it was observed that the fluorescence intensity rose with increasing concentration,

Table 4. Curative and Protective Activities of Y14 against *Phomopsis* sp. In Vivo[a]

treatment	concentration (μg/mL)	curative activity		protective activity	
		lesion length (mm)	control efficacy (%)	lesion length (mm)	control efficacy (%)
Y14	100	34.8 ± 1.2[c]	37.0	25.8 ± 0.8[c]	57.5
	200	23.8 ± 0.8[d]	60.2	18.8 ± 1.7[d]	71.8
AZ	100	38.5 ± 1.1[b]	29.2	32.5 ± 1.1[b]	43.9
	200	24.0 ± 1.3[d]	59.9	24.5 ± 0.8[c]	60.2
negative control		52.3 ± 3.4[a]		54.0 ± 3.4[a]	

[a]Values are mean ± SD of three replicates. Statistical analysis was conducted using SPSS 27.0 software. Different letters indicate significant differences at $p < 0.05$ in the same group.

https://doi.org/10.1021/acsomega.3c10294
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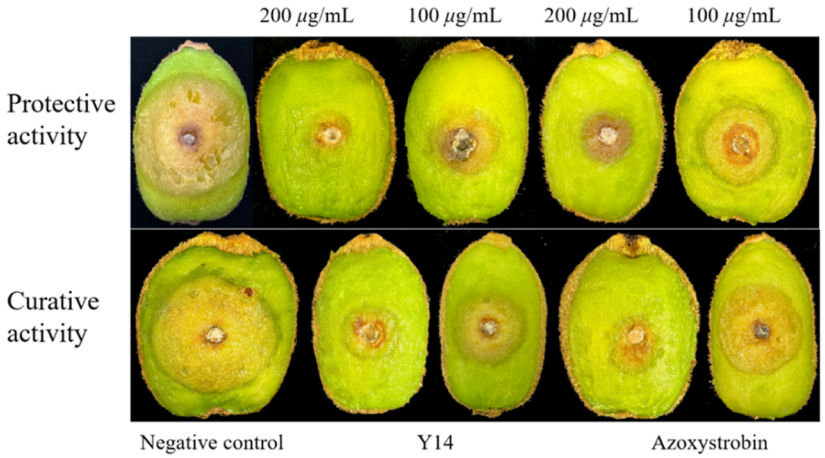

Figure 5. Curative and protective activity of **Y14** and azoxystrobin against *Phomopsis* sp. on kiwifruit.

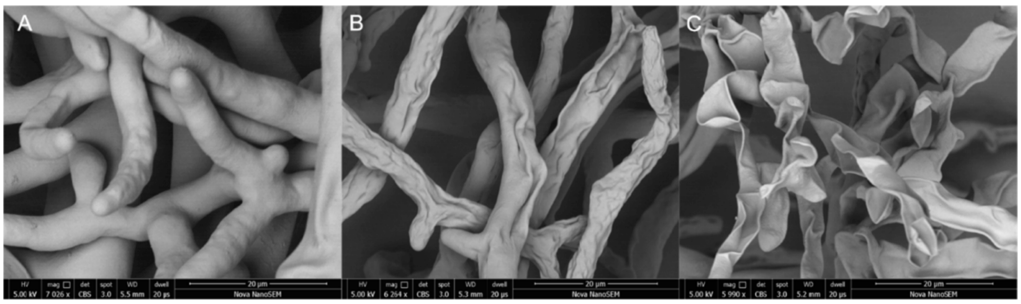

Figure 6. SEM images of the hyphae of *B. cinerea* after treatment with different concentrations of **Y18**. (A) 0 μg/mL, (B) 50 μg/mL, and (C) 100 μg/mL. Scale for 20 μm.

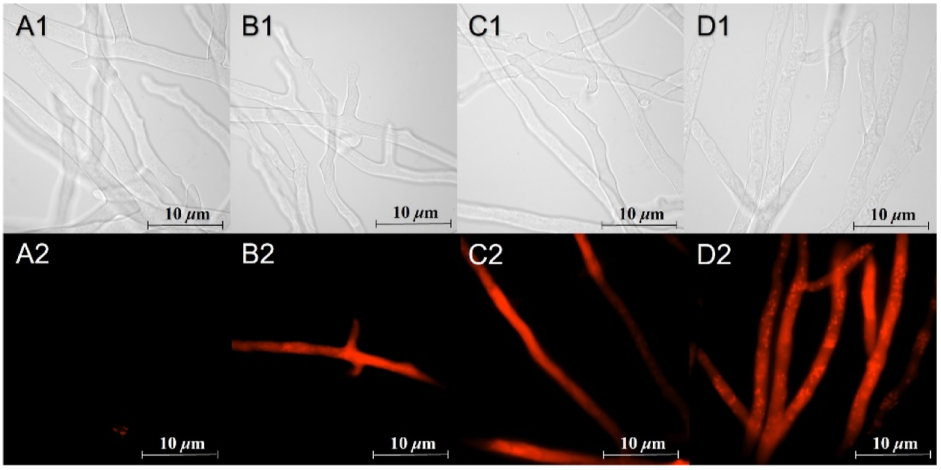

Figure 7. Morphological observation of *B. cinerea* after treatment with **Y18** at different concentrations by fluorescence microscopy (FM). (A1−D1) Under bright field, (A2−D2) Under a fluorescence field, (A1,A2) 0 μg/mL, (B1,B2) 25 μg/mL, (C1,C2) 50 μg/mL, and (D1,D2) 100 μg/mL. Magnification 100 × 10. Scale for 10 μm.

showing that **Y18** can stimulate the production of ROS and cause an oxidation-antioxidant imbalance in the body.

2.5. Spore Germination Assay of *B. cinerea*. The inhibitory effect on *B. cinerea* spore growth can substantially reduce damage to the host plant. As depicted in Figure 9, the relative inhibition rates of **Y18** on spore germination at concentrations of 200, 100, 50, 25, and 12.5 μg/mL were 98.2, 92.7, 73.7, 67.3, and 26.7%, respectively, and the EC_{50} value was 21.1 μg/mL. The results revealed that **Y18** was capable of inhibiting the germination of spores effectively.

2.6. Cell Membrane Permeability Assay of *B. cinerea*. Cell membranes are crucial for the maintenance of cell shape, structural integrity, and physiological function. The permeation of cell membranes can be assessed through the determination of relative conductivity.[36] As depicted in Figure 10, when **Y18** was applied to the mycelium, the relative conductivity of the mycelium exhibited a significant increase, positively correlating with the increasing drug concentration. The upward trend in relative conductivity was notably greater than that of the untreated negative control group. The experimental results indicated that **Y18** was capable of destroying the cell

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ACS Omega 2024, 9, 17297−17306

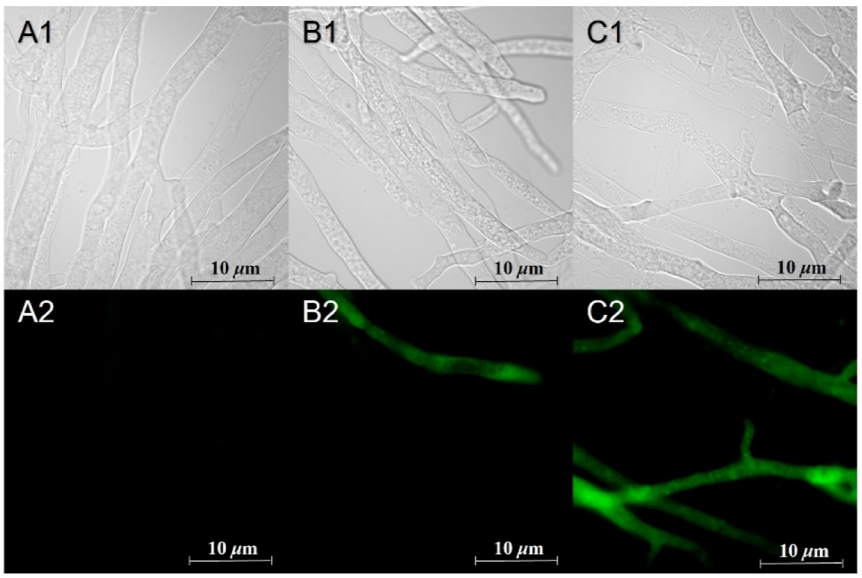

Figure 8. Effects on the ROS of *B. cinerea* treated with **Y18** at different concentrations. (A1−C1) Under a bright field, (A2−C2) under a fluorescence field, (A1,A2) 0 μg/mL, (B1,B2) 12.5 μg/mL, and (C1,C2) 25 μg/mL. Magnification 100 × 10. Scale for 10 μm.

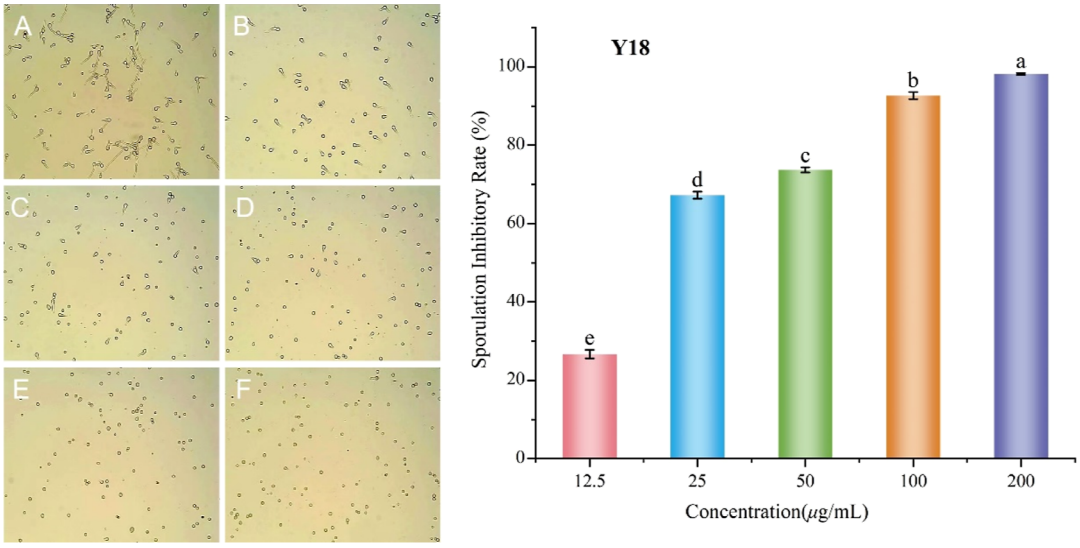

Figure 9. Effects of **Y18** on spore germination of *B. cinerea* for different concentrations. (A) 0 μg/mL, (B) 12.5 μg/mL, (C) 25 μg/mL, (D) 50 and μg/mL, (E) 100 μg/mL, and (F) 200 μg/mL. Magnification 10 × 10. Scale for 10 μm. Statistical analysis was conducted using SPSS 27.0 software. Different letters indicate significant differences at $p < 0.05$ in the same group.

membrane's structure, enhancing its permeability, and culminating in the death of mycelia.

2.7. Cytoplasmic Leakage Assay of *B. cinerea*. The absorbance of mycelium suspensions at 260 and 280 nm was measured using an ultraviolet−visible spectrophotometer to investigate the leakage of cytoplasmic substances such as nucleic acids and proteins.[37] From Figure 11, it is clear that the absorbance values after treatment with varying concentrations of **Y18** increased significantly with increasing drug concentration as compared to that of the untreated negative control group. The conclusion drawn from this is that **Y18** caused a notable release of nucleic acids and proteins from the mycelium.

2.8. Malondialdehyde Content Assay of *B. cinerea*. The malondialdehyde (MDA) content is a crucial metabolite in the peroxidation process of biological cell membranes. An increase in MDA levels, an indicator of oxidative damage, may suggest the extent of membrane damage and thus the severity of the cell damage.[38] When compared with the untreated negative control group, it can be seen that the level of the MDA content gradually increased as the **Y18** concentration increased following treatment with varying concentrations, as depicted in Figure 12. Consequently, **Y18** is capable of notably increasing the level of lipid peroxidation in the cell membrane of the *B. cinerea* mycelium, thereby inducing cell membrane damage, which is in line with the previous experimental analysis.

3. CONCLUSIONS

In summary, 22 flavonol derivatives containing 1,3,4-thiadiazole were designed and synthesized, and the structures of all the target compounds were determined by NMR and HRMS. The results of antifungal experiments in vitro showed that **Y18** had good antifungal activity against *B. cinerea*, and its

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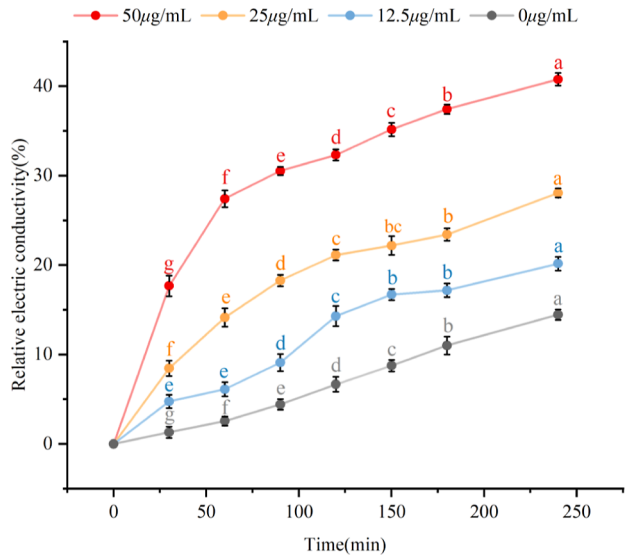

Figure 10. Changes in cell membrane permeability of **Y18** against *B. cinerea*. Statistical analysis was conducted using SPSS 27.0 software. Different letters indicate significant differences at $p < 0.05$ in the same group.

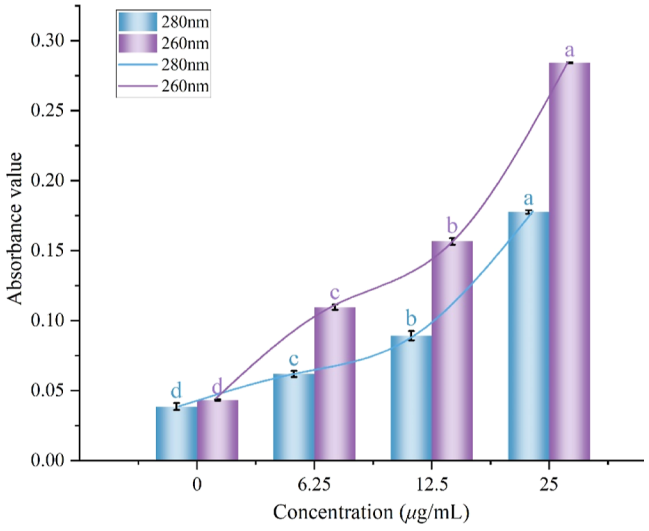

Figure 11. Release of cellular contents from *B. cinerea* after treatment with **Y18**. Statistical analysis was conducted using SPSS 27.0 software. Different letters indicate significant differences at $p < 0.05$ in the same group.

EC_{50} value was 2.4 μg/mL, which was obviously superior to that of azoxystrobin (21.7 μg/mL). Moreover, the activity experiment results in vivo showed that the curative activity of **Y18** (79.9%) at 200 μg/mL was better than that of azoxystrobin (59.1%), and the protective activity (90.9%) was better than that of azoxystrobin (83.9%). **Y18** also can effectively inhibit conidial germination to reduce damage to the host plant. Preliminary studies on the mechanism indicated that **Y18** could affect the integrity of cell membranes by inducing endogenous ROS production, causing lipid peroxidation of cell membranes, and releasing cell contents, and the specific mechanism of action of **Y18** with *B. cinerea* is under further exploration.

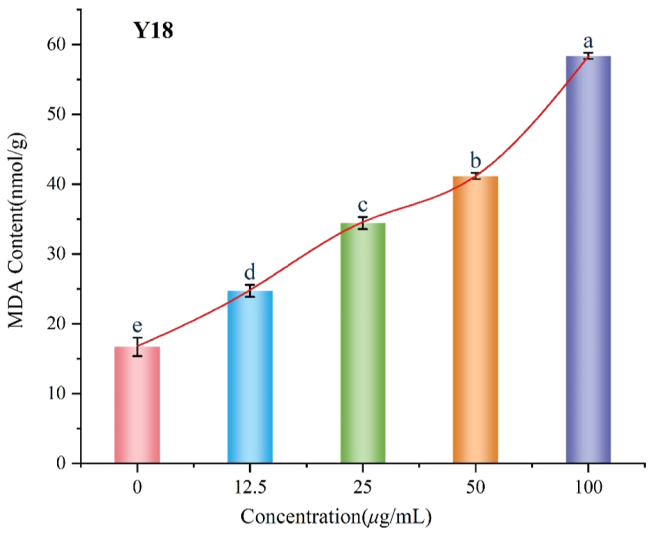

Figure 12. MDA contents of *B. cinerea* treated with **Y18**. Statistical analysis was conducted by SPSS 27.0 software. Different letters indicate significant differences at $p < 0.05$ in the same group.

4. MATERIALS AND METHODS

4.1. Instruments and Chemicals. ^{1}H, ^{13}C, and ^{19}F NMR spectra were determined by a Bruker 400 NMR spectrometer (Bruker Corporation, Germany). HRMS data were obtained by a Thermo Scientific Q Exactive instrument (Thermo Scientific, America). SEM data were measured on an FEI Nova Nano 450 (Hillsboro, OR, America). An Olympus-BX53 fluorescence microscope and a CX21FS1 microscope were obtained from Olympus Ltd., Japan. The reagents and solvents utilized in this study are of analytical grade, and the kits employed are manufactured by Beijing Solaibao Technology Co., Ltd.

4.2. Fungi. *R. solani*, *B. cinerea*, *F. graminearum*, *C. gloeosporioides*, *S. sclerotiorum*, *P. capsica*, *A. brassicae*, *Fusarium oxysporum f.* sp. *cucumerinum* (*F.* sp. *cucumerinum*), *Fusarium oxysporum f.* sp. *capsicum* (*F.* sp. *capsicum*), and *Phomopsis* sp. were selected for the experiment. These fungi were cultivated on potato dextrose agar plates at 25 ± 1 °C and preserved at 4 °C.

4.3. Synthesis. *4.3.1. General Procedure for the Synthesis of Intermediates 1−4.* Intermediates **1**−**4** were synthesized according to the reported procedures.[39−42] Refer to the Supporting Information for detailed synthesis steps.

4.3.2. General Procedure for the Synthesis of Target Compounds Y1−Y22. Intermediates **1** (1.61 mmol), K_2CO_3 (2.01 mmol), and 20 mL of DMF were added sequentially to a 50 mL round-bottomed flask and stirred at room temperature for 30 min. Then, intermediates **4** (1.34 mmol) were added slowly and reacted for 10−12 h at 25 °C. The reaction was monitored by TLC (petroleum ether/ethyl acetate = 2:1, *v*/*v*), and when the reaction was completed, the system was extracted with ethyl acetate. The organic layer was obtained by layering, and the solvent was removed to obtain the crude product. Finally, the target compounds **Y1**−**Y22** were isolated by column chromatography, where the crude product was eluted with a (petroleum ether/ethyl acetate = 5:1, *v*/*v*) gradient.

4.4. Bioassays. *4.4.1. Antifungal Activity In Vitro.* The inhibitory activity of **Y1**−**Y22** against ten phytopathogenic fungi was tested according to refs 43−46. Refer to the

https://doi.org/10.1021/acsomega.3c10294
ACS Omega 2024, 9, 17297−17306

Supporting Information for detailed steps. Based on the preliminary test results, the compounds with promising antifungal activity were identified by the EC_{50} value.

4.4.2. Antifungal Activity In Vivo. The in vivo antifungal test was performed according to the results of the in vitro antifungal activity assay. The test method is determined according to the methods reported in the literature.[47] Refer to the Supporting Information for detailed steps.

4.5. Antifungal Mechanism Study. *4.5.1. Effects of Y18 Treatment on Cell Membrane Integrity. 4.5.1.1. Morphological Analysis by SEM.* First, the potato dextrose broth (PDB) medium containing the mycelia of *B. cinerea* was cultured at 28 °C and 180 rpm for 24 h. Then, **Y18** solution (50 and 100 μg/mL) was added and incubated with mycelium at 28 °C and 180 rpm. Subsequently, mycelium was washed with 0.01 mol/L phosphate-buffered saline (PBS) and then maintained for 24 h by adding 1.5 mL of a 2.5% glutaraldehyde solution. Finally, the mycelium was washed with ethanol and freeze-dried to make samples.[48]

4.5.1.2. Morphological Analysis by FM. As abovementioned, the mycelium was cleaned three times with PBS, and then 10 μL of PI solution (10 mg/L) was added to stain the mycelium. The mycelium was incubated at 37 °C for 25 min and then washed with PBS three times. Some mycelium was taken on a slide, then a droplet of glycerin was added, and the cover slide was covered to make a sample and to be observed.[49,50]

4.5.2. Determination of ROS. The mycelium of *B. cinerea* was prepared by following the procedure outlined in Section 4.5.1. **Y18** solution (12.5 and 25 μg/mL) was added and incubated with mycelium for 24 h, and the mycelium was washed with PBS three times. Subsequently, the hyphae were treated with 0.1 mL of DCFH-DA (25 μmol/L) and then incubated in a dark environment at 37 °C for 60 min. This was followed by three washes with PBS. Finally, samples were prepared and observed by FM.[51]

4.5.3. Determination of Spore Germination. The mycelium of *B. cinerea* was prepared following the procedure outlined in Section 4.5.1. The PDB medium containing *B. cinerea* was cultured at 28 °C for 7 days. After a large number of spores were formed, a spore suspension (1.5×10^5 spores/mL) was produced with 0.1% Tween 80 solution.[52] Refer to the Supporting Information for detailed steps.

4.5.4. Determination of Cell Membrane Permeability. The mycelium of *B. cinerea* was prepared following the procedure outlined in Section 4.5.1. First, the mycelium was washed with sterile water and then filtered. Then, 200 mg of mycelium was weighed and treated with **Y18** (12.5, 25, and 50 μg/mL). Then, the conductivity was measured for 0, 30, 60, 90, 120, 150, 180, and 240 min. Finally, after boiling the mycelium for 1 h, the conductivity was measured after cooling.[53]

4.5.5. Determination of Cytoplasmic Content Leakage. The mycelium of *B. cinerea* was prepared following the procedure outlined in Section 4.5.1. 100 mg of mycelium was weighed and treated with **Y18** (6.25, 12.5, and 25 μg/mL), cultured at 25 °C for 10 h. Finally, the absorbance of the supernatant was measured at 260 and 280 nm on an ultraviolet−visible spectrophotometer.[54]

4.5.6. Determination of MDA Contents. The literature indicates that the MDA contents can reflect the extent of cell membrane damage by oxidative stress.[55] The mycelium of *B. cinerea* was prepared following the procedure outlined in Section 4.5.1. **Y18** (0, 12.5, 25, 50, and 100 μg/mL) was added to the cultured mycelium and incubated at 28 °C. After 24 h, the mycelium was washed with sterile water and then filtered. Finally, the mycelium was freeze-dried for 2 h, and the MDA contents were determined according to the kit instructions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c10294.

Characterization data, ^1H, ^{13}C, and ^{19}F NMR spectra, and HRMS of title compounds **Y1−Y22** (PDF)

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https://doi.org/10.1021/acsomega.3c10294
ACS Omega 2024, 9, 17297−17306

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was completed with the support of the National Natural Science Foundation of China (no. 32072446) and the Science Foundation of Guizhou Province (no. 20192452).

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https://doi.org/10.1021/acsomega.3c10294
ACS Omega 2024, 9, 17297−17306