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Original article

Benzofurazan derivatives as antifungal agents against phytopathogenic fungi

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ABSTRACT

A series of benzofurazan derivatives were prepared and evaluated for their biological activities against four important phytopathogenic fungi, namely, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Fusarium graminearum* and *Phytophthora capsici*, using the mycelium growth inhibition method. The structures of these compounds were characterized by ^{1}H NMR, ^{13}C NMR, and HRMS. *N*-(3-chloro-4-fluorophenyl)-7-nitrobenzo[c][1,2,5]oxadiazol-4-amine (**A3**) displayed the maximum antifungal activity against *R. solani* (IC_{50} = 1.91 μg/mL), which is close to that of the positive control Carbendazim (IC_{50} = 1.42 μg/mL). For other benzofurazan derivatives with nitro group at R^4 position (**A** series), 9 out of 30 compounds exhibited high antifungal effect against strain *R. solani*, with IC_{50} values less than 5 μg/mL. Most of the derivatives with substituents at R^2 and R^3 positions (**B** series) displayed moderate growth inhibition against *S. sclerotiorum* (IC_{50} < 25 μg/mL). Also, several benzofuran derivatives with nitro group at R^4 position and another conjugated aromatic ring at the R^1 position of the phenyl ring displayed high antifungal capability against strain *R. solani*. Compounds with substituents at R^2 and R^3 position had moderate efficacy against strain *S. sclerotiorum*.

1. Introduction

Pathogenic fungi and insect pests caused over 30% yield losses in major crops [1]. To increase yields and provide healthy crops, application of agrochemicals has become an established part of modern agriculture. For complicated plant disease situation, the combined use of different antifungal agents is common (ref). Consequently, fungicide-resistant pathogens have been selected and evolved. Therefore, there is a need for novel antifungal compounds with new mechanism of action and lower application dosage.

During screening of our in house compound library for biologically active components targeting plant pathogens, the benzofuroxan scaffold (Fig. 1) was identified to exhibit good *in vitro* activity against four important phytopathogenic fungi. Further structural modification revealed benzofuroxan derivatives with nitro at the R^4 position and small sized amino group at R^1 position resulted in candidates displaying significant *in vitro* and *in vivo* antifungal activity [2]. Unlike furoxan (Fig. 1), the benzofuroxans were considered to be devoid of the nitric oxide (NO) releasing capability [3]. Therefore, the reported antifungal activity should not relate to the NO biological function. Alternatively, the benzofuroxan system is highly electron deficient and nucleophile sensitive, especially when the phenyl R^4 position contains a nitro group. Nucleophiles like thiol or hydroxyl would readily attack the nitro *para* position to form the so called Meisenheimer-type complex intermediate, which quickly undergoes elimination reaction to provide a more stable benzofuroxan [4]. Previous studies have also established that substituents *ortho* to nitro would considerably diminish the Meisenheimer-type complex formation [5]. Whether the compound electrophilicity is involved in the antifungal activity should be explored and addressed. We anticipate that removal of the electron negative oxygen atom, transformation to benzofurazan (Fig. 1), will render the whole conjugated system less electron-deficient. Alternatively, the benzofurazan derivatives have been reported to possess a variety of bioactivity, such as anti-protozoa, antibacterial and calcium channel modulated property [6–10]. Given the structural similarity of benzofuroxan and benzofurazan,

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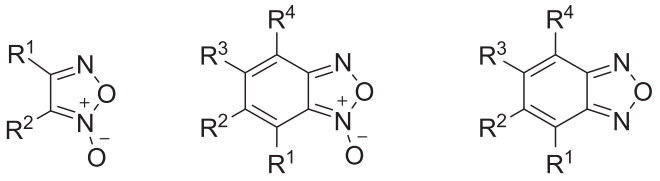


Fig. 1. Chemical structures of furoxan, benzofuroxan and benzofurazan.

we investigated the antifungal aspect of benzofurazan. In this paper, series of benzofurazan compounds were prepared, and the *in vitro* antifungal activities were evaluated against four important plant pathogen strains including *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Fusarium graminearum*, and *Phytophthora capsici*. These four strains of fungi are representative in their biological species, and caused a wide range of significant plant disease on different crops. For example, *F. graminearum*, also known by the name of teleomorph *Gibberella zeae*, is a plant pathogen that causes fusarium head blight on wheat and barley [11]; *R. solani* is a plant pathogenic fungus with a wide host range and worldwide distribution, also one of the fungi responsible for Brown patch (a turfgrass disease), as well as black scurf of potatoes, bare patch of cereals, root rot of sugar beet, belly rot of cucumber, sheath blight of rice, and many other pathogenic conditions [12].

2. Chemistry

To investigate whether the nitrogen–oxygen coordinate bond in benzofuroxan is necessary for antifungal activity, the corresponding benzofurazans were synthesized. Specifically, the benzene ring at the R^1 position (Fig. 1) was first chosen for modification (**A18**–**A26**, **B2**–**B4 and B10**). Further change with other conjugated heterocyclics, such as azoles and thiophene [13–16], which are common structural elements in compounds with antifungal activity were investigated. All new benzofurazan compounds were prepared using the synthetic routes shown in Schemes 1 and 2. 4-Chloro-7-nitrobenzo[c][1,2,5]oxadiazole(A1) was prepared from commercially available 2,6-dichloroaniline employing the reported synthesis procedure [17]. Then nucleophilic substitution reaction of compound **A1** with different amines was carried in a sealed tube at 100 °C to provide **A2**–**A6**. To prepare **A7**–**A8**, a nitrogen atmosphere and low temperature reaction condition was used for the pyrazole and 1,2,4-triazole substrates. Treating **A1** with thiols using sodium methoxide as base afforded **A9**–**A12** in good yield (>90%).

The key intermediate 5-chloro-4-fluoro-2-nitroaniline (**1**) was obtained from commercially available 3-chloro-4-fluoroaniline using the reported procedure [2]. Nucleophilic substitution reaction of compound **1** with sodium methoxide gave compound **2** in 90% yield. Based on ^{1}H NMR analysis, only the C5-substituted product was obtained. Treatment of compound **2** with sodium hypochlorite and 0.25% (w/v) KOH in ethanol afforded 5-ethoxy-6-methoxybenzofuroxan (**3**) in 53% yield. The other three benzofurazans (4-2, 4-3, 4-4) were prepared according to our previously reported procedure [2]. Reduction of these benzofuroxans with triphenylphosphine under reflux in dichloromethane afforded the target compounds **B1**–**B4**. The remaining listed compounds in Tables 1–3 (**A13**–**A30**, **B5**–**B10** and **C1**–**C5**) were prepared according to previously reported methods [18–24,27]. All compounds were analyzed by high-pressure liquid chromatography to ensure the purity (>95%) before submission for biological evaluation.

3. Antifungal activity

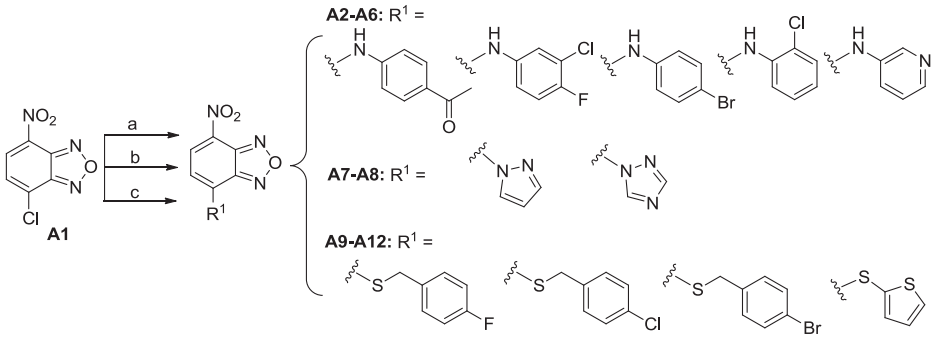
The antifungal activities of the synthetic molecules, expressed as IC_{50} (median inhibitory concentration) values, were determined using the mycelia growth inhibitory rate method. The results are shown in Tables 1–3.

The benzofurazan derivative **A3**, with the 3-chloro-4-fluoroaniline substituent at the R^1 position, showed a broad spectrum of antifungal activity against all four tested fungi phytopathogens. Its IC_{50} value against *R. solani* was 1.91 μg/mL, which was close to that of the positive control Carbendazim (IC_{50} = 1.42 μg/mL). Most of the other compounds with different aniline substitutions at the R^1 position also displayed high potency against *R. solani*. Especially, the 4-bromoaniline derivative **A4** (IC_{50} = 2.03 μg/mL) and corresponding chloro analogue **A28** (IC_{50} = 3.87 μg/mL) displayed inhibitory potency similar to that of carbendazim. Compounds **A20**–**A24**, with pyrrolidine, piperidine, piperazine and morpholine at the R^1 position, displayed weak antifungal activity with IC_{50} value higher than 25 μg/mL against all four tested fungi phytopathogens. Replacement of the above aliphatic amine with aromatic triazole and pyrazole (compounds **A7**–**A8**) improved the activity, with IC_{50} values 3.11–22.29 μg/mL against *R. solani*, *S. sclerotiorum* and *F. graminearum Sehw.* This suggested that the hetero-aromatic azole group has a great contribution on compound antifungal activity. Finally, **A10**–**A12** and **A19**, with different thiols at position 4 exhibited low IC_{50} (<5 μg/mL) against *R. solani*. These findings indicated that the presence of another conjugated system at the R^1 position is favorable for the antifungal activity of type **A** benzofurazan derivatives, and they may serve as new leads for the development of potentially useful antifungal agents against *R. solani*.

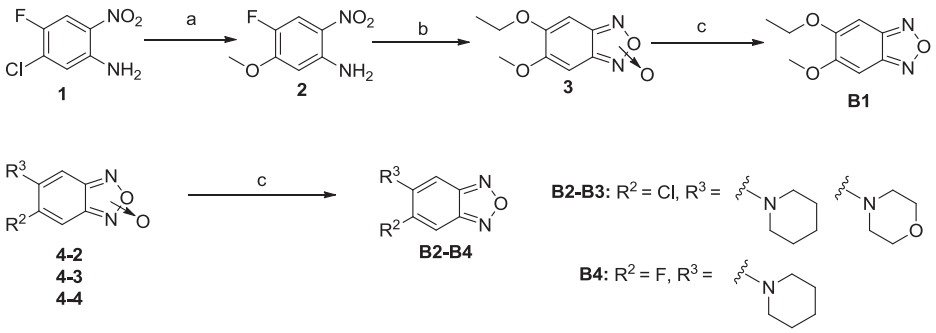
In the case of series **B** of benzofurazan derivatives, with substituents variation at the R^2 and R^3 positions, whereas both R^1 and R^4 positions are hydrogen atoms, seven out of ten compounds (**B2**–**B4** and **B6**–**B9**) exhibited potency against *S. sclerotiorum* (IC_{50} = 13.18–24.41 μg/mL), and their antifungal activity against the other three fungi phytopathogens was weak (IC_{50} > 25 μg/mL). Replacement of a chlorine atom at R^2 position with a fluorine atom increased the IC_{50} value from 8.54 μg/mL (for **B2**) to >25 μg/mL (for **B4**) against *R. solani*, indicating a chlorine atom at R^2 position was more appropriate than a fluorine atom. Moreover, the same trend was confirmed with **B3** (IC_{50} = 16.64 μg/mL) versus **B10** (IC_{50} > 25 μg/mL) or **B8** (IC_{50} = 15.71 μg/mL) versus **B9** (IC_{50} = 24.41 μg/mL) against *S. sclerotiorum*. In general, this series of compounds demonstrated better antifungal activity against *S. sclerotiorum* as compared with the other three fungi phytopathogens.

Series **C** benzofurazan derivatives exhibited weak antifungal activity (IC_{50} values >25 μg/mL). Compound **A13** had potency against *R. solani*, *S. sclerotiorum* and *F. graminearum Sehw* (IC_{50} = 10.07–20.82 μg/mL), while compound **C1** barely exhibited antifungal activity. When comparing the activity of **C4**–**C5** with the corresponding **A15** and **A18** (IC_{50} values >25 μg/mL), it was concluded that substitution of the R^4 nitro group with another electron-withdrawing benzenesulfonyl substituent significantly reduced the antifungal activity. The above results suggested that the nitro at R^4 position was highly important for the antifungal activity of the compounds.

To further understand the selectivity of these prepared compounds, the most potent compound **A3** was evaluated against invasive fungal pathogen *Candida albicans* and the human normal liver cell line HL-7702. At 25 μg/mL. The percentage of inhibition against *C. albicans* CMCC(F)98001 is 48.18 ± 2.53, and 84.8% of human normal liver cell HL-7702 was inhibited. Considering the IC_{50} of **A3** against phytopathogenic fungi *R. solani* is 1.91 ± 0.14 μg/mL, these results demonstrated that compound **A3** exhibited a good selectivity against the phytopathogenic fungi.



Scheme 1. Synthetic routes of compounds **A2**–**A12**. a (**A2**–**A6**): amines, CH_3CN, 100 °C in a sealed tube. b (**A7**–**A8**): 1,2,4-triazole or pyrazole, acetone, 60 °C under N_2 atmosphere. c (**A9**–**A12**): thiols, CH_3ONa, EtOH, room temperature.



Scheme 2. Synthetic route of compounds **B1**–**B4**. a: CH_3ONa, CH_3OH, 100 °C in sealed tube, 3 h, 90%. b: NaClO, 0.25% KOH (w/v), EtOH, 0 °C, 1 h, 53%. c: Ph_3P, CH_2Cl_2, reflux, 1 h.

4. Conclusion

In conclusion, we have synthesized a series of benzofurazan derivatives and evaluated their inhibitory ability against four phytopathogenic fungi. The selected benzofurazans were structurally related to our reported benzofurozan. Interestingly, removal of the nitrogen–oxygen coordination bond caused a decline of antifungal activity for most of compounds. However, an obvious activity improvement as compared to benzofurozans was observed.

Among these benzofurazan derivatives, the 3-chloro-4-fluoroaniline derivative **A3** displayed a broad spectrum of antifungal activity against all four tested fungi phytopathogens, the efficacy against *R. solani* is especially significant, with an IC_{50} values of 1.91 μg/mL. Eight other series **A** derivatives exhibited high antifungal effect against *R. solani*, with IC_{50} values less than 5.0 μg/mL. While the series **B** compounds, with substituents at the R^2 and R^3 positions of the phenyl ring, exhibited antifungal potency only against *S. sclerotiorum* (**B1**, **B5** and **B10** with IC_{50} higher than 25 μg/mL). Compounds **B2**–**B4** and **B6**–**B9** also displayed growth inhibition against *S. sclerotiorum* ($IC_{50} < 25$ μg/mL). None of these compounds displayed effective antifungal activity against this strain. Although the benzofurazan derivatives in general displayed weak antifungal activity compared to our previously reported benzofuroxan, we consider the benzofurazan scaffold as a more stable structural motif and less likely to act as a universal electrophilic reagent, and therefore possessing lower cytotoxicity. Additionally, results from activity evaluation against invasive fungal pathogen *C. albicans* and the human normal liver cell line HL-7702 demonstrated the compound good selectivity against phytopathogenic fungi. Hopefully, further exploration of the benzofurazan scaffold would lead to the discovery of even more potent agents against different phytopathogenic fungi.

5. Experimental section

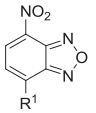
5.1. General

All reagents and solvents were of reagent grade or purified according to standard methods. Analytical thin layer chromatography (TLC) was performed with silica gel plates using silica gel 60 GF_{254} (Qingdao Haiyang Chemical Co., Ltd.). Column chromatography was performed over silica gel (200–300 mesh, Qingdao Marine Chemical Ltd.). The ^1H NMR and ^{13}C NMR spectra were recorded in deuterochloroform or other indicated solvents at ambient temperature using a Varian Mercury 400 M or 300 M NMR. The low resolution MS and HRMS were recorded with an Agilent1260LC and a Waters-GCT Premier, respectively. The melting points of the products were determined on a SGW X-4 apparatus (Shanghai Precision &Scientific Instrument Co., Ltd., Shanghai, China). PE, petroleum ether (bp 60–90 °C); EA, ethyl acetate; CH_2Cl_2, dichloromethane; MeOH, methanol. The compound purity was analyzed using Prominence UFLC from SHIMADZU. And the column was Inertsil® ODS-SP, 5 μm, 4.6 × 150 mm. HPLC condition for compound purity determination: methanol and water was used as mobile phase. Compound **A23** was eluted with 15% methanol; and the mobile phase of **A3**–**A4**, **A9**–**A12**, **A20**, **A28**, **B8**–**B10** and **C3**–**C5** was 60% methanol; and 75% methanol was used for compounds **B2**–**B4**, **B6**–**B7**. All the other compounds were analyzed using 50% methanol.

5.2. General synthetic procedure for the preparation of compounds *A2*–*A6*

4-Chloro-7-nitrobenzofurazan (**A1**) [15] (50 mg, 0.25 mmol) was dissolved in acetonitrile (5 mL). Then amine (2.5 mmol, 1-(4-aminophenyl)ethanone, 3-chloro-4-fluoroaniline, 4-bromoaniline,

Table 1
Antifungal activity of series A benzofurazan derivatives against four phytopathogens.[a]



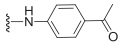
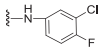
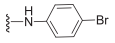
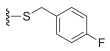
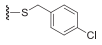
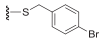
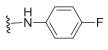
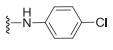
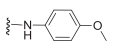
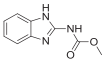
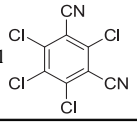
Compound	R^1	$IC_{50} \pm SD$ (μg/mL)[a]			
		Rhizoctonia solani	*Sclerotinia sclerotiorum*	*Fusarium graminearum*	*Phytophthora capsici*
A1	Cl	4.20 ± 0.97	9.13 ± 0.13	18.41 ± 0.31	>25
A2		>25	>25	>25	>25
A3		**1.91 ± 0.14**	8.99 ± 0.00	7.13 ± 0.15	11.41 ± 0.13
A4		**2.03 ± 0.17**	17.26 ± 0.05	22.64 ± 0.23	>25
A5		21.35 ± 0.12	>25	>25	>25
A6		>25	>25	>25	>25
A7		**3.11 ± 0.00**	21.38 ± 0.13	22.29 ± 0.15	>25
A8		4.43 ± 0.07	18.67 ± 0.18	13.51 ± 0.05	>25
A9		>25	>25	>25	>25
A10		4.49 ± 0.12	22.91 ± 0.17	>25	>25
A11		**3.45 ± 0.15**	>25	>25	>25
A12		**2.58 ± 0.07**	>25	>25	>25
A13	H	10.07 ± 1.38	20.82 ± 0.84	14.58 ± 0.07	>25
A14	N_3	>25	>25	>25	>25
A15	CH_3O	>25	>25	>25	>25
A16	C_2H_5O	21.65 ± 0.86	21.47 ± 0.62	>25	>25
A17	CH_3S	>25	22.64 ± 0.25	>25	>25
A18	$PhSO_2$	>25	>25	>25	>25
A19		4.63 ± 1.86	6.58 ± 0.14	>25	>25
A20		>25	>25	>25	>25
A21		>25	>25	>25	>25
A22		>25	>25	>25	>25
A23		>25	>25	>25	>25
A24		>25	>25	>25	>25
A25		6.74 ± 0.42	>25	>25	>25
A26		7.04 ± 0.41	>25	>25	>25
A27		7.41 ± 0.15	>25	24.79 ± 0.15	>25
A28		3.87 ± 0.07	15.30 ± 0.15	5.95 ± 0.05	>25
A29		7.13 ± 0.30	>25	21.32 ± 0.23	>25
A30		>25	>25	>25	>25

Table 1 (*continued*)

Compound	R^1	$IC_{50} \pm SD$ (μg/mL)[a]			
		Rhizoctonia solani	*Sclerotinia sclerotiorum*	*Fusarium graminearum*	*Phytophthora capsici*
Control 1	Carbendazim 	1.42 ± 0.14	0.15 ± 0.03	0.50 ± 0.08	–
Control 2	Chlorothalonil 	–	–	–	4.48 ± 0.40

[a] Values are the mean ± standard deviation (SD) of three replicates.

2-chloroaniline and pyridin-3-amine) was added. The solution was stirred at 100 °C in a sealed tube. The reaction was monitored by TLC, after completion, the solvent acetonitrile was removed in vacuo, and the residue was subjected to silica gel column chromatography (CH_2Cl_2/PE: 1/1 and then CH_2Cl_2) to give the desired products as red solids.

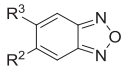
1-(4-((7-Nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino)phenyl)ethanone (**A2**): a red solid; 65 mg; yield 87%; R_f 0.5 (CH_2Cl_2); mp: 248–250 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.14 (br s, 1H), 8.57 (d, J = 8.8 Hz, 1H), 8.06 (d, J = 8.4 Hz, 2H), 7.62 (d, J = 8.4 Hz, 2H), 7.00 (d, J = 8.8 Hz, 1H), 2.59 (s, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 196.6, 145.3, 144.0, 142.6, 140.6, 137.1, 133.5, 129.8, 124.6, 122.2, 103.6, 26.6; HRMS (CI−) m/z calcd for $C_{14}H_{10}N_4O_4$ 298.0702, found 298.0709.

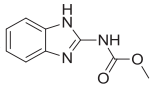
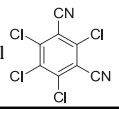
N-(3-chloro-4-fluorophenyl)-7-nitrobenzo[c][1,2,5]oxadiazol-4-amine (**A3**): a red solid; 70 mg; yield 91%; R_f 0.3 (PE/EA: 5/1); mp: 213–214 °C; ^{1}H NMR (400 MHz, acetone-d_6) δ 9.83 (brs, 1H), 8.55 (d, J = 8.8 Hz, 1H), 7.76 (dd, J = 6.4, 2.4 Hz, 1H), 7.68–7.56 (m, 1H), 7.49 (dd, J = 8.8 Hz, 1H), 6.89 (d, J = 8.8 Hz, 1H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 155.2 (d, J = 247.5 Hz), 144.9, 144.0, 141.9, 137.4, 135.1 (d, J = 3.0 Hz), 125.9, 124.7 (d, J = 7.1 Hz), 123.8, 120.3 (d, J = 18.2 Hz), 117.8 (d, J = 22.2 Hz), 102.3; HRMS (CI−) m/z calcd for $C_{12}H_6N_4O_3ClF$ 308.0112, found 308.0114.

N-(4-bromophenyl)-7-nitrobenzo[c][1,2,5]oxadiazol-4-amine (**A4**): a red solid; 76 mg; yield 91%; R_f 0.2 (CH_2Cl_2/PE: 1/1); mp: 213–214 °C; ^{1}H NMR (400 MHz, acetone-d_6) δ 9.85 (br s, 1H), 8.52 (d, J = 8.8 Hz, 1H), 7.69 (d, J = 8.0 Hz, 2H), 7.56 (d, J = 8.0 Hz, 2H), 6.92 (d, J = 8.8 Hz, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 145.0, 144.1, 141.7, 137.4, 137.3, 132.5, 125.7, 123.6, 118.4, 102.2; HRMS (CI−) m/z calcd for $C_{12}H_8N_4O_3$ ^{79}Br 334.9780 and $C_{12}H_8N_4O_3$ ^{81}Br 336.9759, found 334.9758 and 336.9756.

N-(2-chlorophenyl)-7-nitrobenzo[c][1,2,5]oxadiazol-4-amine (**A5**): a red solid; 70 mg; yield 96%; R_f 0.3 (PE/EA: 5/1); mp: 194–196 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.46 (d, J = 8.8 Hz, 1H), 7.76 (br s, 1H), 7.61 (dd, J = 14.0, 8.0 Hz, 2H), 7.44 (dd, J = 7.6 Hz, 1H), 7.32 (dd, J = 7.6 Hz, 1H), 6.67 (d, J = 8.8 Hz, 1H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 144.3, 144.1, 143.1, 137.3, 134.7, 130.7, 130.6, 129.6, 129.2,

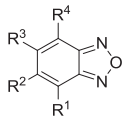
Table 2
Antifungal activity of series B benzofurazan derivatives against four phytopathogens.[a]



Compound	R^2	R^3	$IC_{50} \pm SD$ (μg/mL)[a]			
			Rhizoctonia solani	*Sclerotinia sclerotiorum*	*Fusarium graminearum Sehw*	*Phytophthora capsici*
B1	CH_3O	CH_3CH_2O	>25	>25	>25	>25
B2	Cl		8.54 ± 0.10	22.19 ± 0.07	>25	>25
B3	Cl		>25	16.64 ± 0.05	>25	>25
B4	F		>25	23.14 ± 0.25	>25	>25
B5	Cl	H	>25	>25	>25	>25
B6	Cl	Cl	15.99 ± 1.27	13.18 ± 0.76	>25	>25
B7	Cl	CH_3	>25	19.70 ± 0.23	>25	>25
B8	Cl	CH_3O	>25	15.71 ± 0.10	>25	>25
B9	F	CH_3O	>25	24.41 ± 0.08	>25	>25
B10	F		>25	>25	>25	>25
Control 1	Carbendazim 		1.42 ± 0.14	0.15 ± 0.03	0.50 ± 0.08	–
Control 2	Chlorothalonil 		–	–	–	4.48 ± 0.40

[a] Values are the mean ± standard deviation (SD) of three replicates.

Table 3
Antifungal activity of series C benzofurazan derivatives against four phytopathogens.[a]



Compound	R^1	R^2	R^3	R^4	$IC_{50} \pm SD$ (μg/mL)[a]			
					Rhizoctonia solani	*Sclerotinia sclerotiorum*	*Fusarium graminearum Sehw*	*Phytophthora capsici*
C1	H	H	H	H	>25	>25	>25	>25
C2	H	H	Cl	NO_2	>25	>25	>25	>25
C3	H	Cl	Cl	NO_2	>25	>25	>25	>25
C4	$PhSO_2$	H	H	$PhSO_2$	>25	>25	>25	>25
C5	CH_3O	H	H	$PhSO_2$	>25	>25	>25	>25

[a] Values are the mean ± standard deviation (SD) of three replicates.

128.7, 123.4, 102.4; HRMS (CI−) *m/z* calcd for $C_{12}H_7N_4O_3Cl$ 290.0207, found 292.0208.

7-Nitro-*N*-(pyridin-3-yl)benzo[c][1,2,5]oxadiazol-4-amine (**A6**): a red solid; 25 mg; yield 39%; R_f 0.6 (CH_2Cl_2/MeOH: 20/1); mp: 229–230 °C; ^{1}H NMR (400 MHz, acetone-d_6) δ 9.92 (br s, 1H), 8.82 (d, *J* = 1.6 Hz, 1H), 8.56 (m, 2H), 8.03 (d, *J* = 8.0 Hz, 1H), 7.56 (dd, *J* = 8.0, 4.8 Hz, 1H), 6.92 (d, *J* = 8.8 Hz, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 147.0, 145.2, 144.2, 142.1, 137.5, 135.1, 131.2, 124.3, 123.9, 102.4; HRMS (CI−) *m/z* calcd for $C_{11}H_7N_5O_3$257.0549, found 257.0545.

5.3. General synthetic procedure for compounds *A7–A8*

To a solution of 4-chloro-7-nitrobenzofurazan **A1** (100 mg, 0.50 mmol) in acetone (5 mL) was added 1,2,4-triazole and pyrazole (5.00 mmol) respectively. The reaction mixture was stirred under nitrogen atmosphere at 60 °C for 3 days and then concentrated in vacuo. The residue was purified by silica gel column chromatography (CH_2Cl_2) to give the desired products as yellow solids.

4-Nitro-7-(1*H*-pyrazol-1-yl)benzo[c][1,2,5]oxadiazole (**A7**): a yellow solid; 76 mg; yield 65%; R_f 0.7 (CH_2Cl_2); mp: 187–188 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.02 (s, 1H), 8.64 (d, *J* = 8.4 Hz, 1H), 8.22 (d, *J* = 8.4 Hz, 1H), 7.92 (s, 1H), 6.68 (s, 1H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 144.8, 144.3, 144.0, 134.0, 133.2, 132.5, 132.3, 116.1, 110.9; HRMS (CI−) *m/z* calcd for $C_9H_5N_5O_3$231.0392, found 231.0392.

4-Nitro-7-(1*H*-1,2,4-triazol-1-yl)benzo[c][1,2,5]oxadiazole (**A8**): a yellow solid; 80 mg; yield 69%; R_f 0.5 (CH_2Cl_2); mp: 156–158 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.63 (s, 1H), 8.68 (d, *J* = 8.4 Hz, 1H), 8.29 (d, *J* = 8.0 Hz, 1H), 8.27 (s, 1H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 153.9, 145.9, 144.3, 143.8, 134.1, 133.6, 130.1, 118.5; HRMS (CI−) *m/z* calcd for $C_8H_4N_6O_3$232.0345, found 232.0341.

5.4. General synthetic procedure for compounds *A9–A12*

To a solution of 4-chloro-7-nitrobenzofurazan (**A1**) (50 mg, 0.25 mmol) in ethanol (5 mL) was added different thiols, including thiophene-2-thiol, (4-fluorophenyl)methanethiol, (4-chlorophenyl)methanethiol, and (4-bromophenyl)methanethiol (0.38 mmol), respectively. Then sodium methoxide (20.3 mg, 0.38 mmol) in ethanol (0.5 mL) was added dropwise. The reaction mixture was stirred at room temperature for 1 h. The solution was then extracted with EtOAc (2 × 50 mL) and washed with brine. The combined organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was subjected to silica gel flash chromatography (PE/EA: 5/1 and then CH_2Cl_2) to afford the desired products as yellow solids.

4-((4-Fluorobenzyl)thio)-7-nitrobenzo[c][1,2,5]oxadiazole (**A9**): a yellow solid; 74 mg; yield 97%; R_f 0.4 (PE/EA: 5/1); mp: 154–156 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.36 (d, *J* = 8.0 Hz, 1H), 7.42 (dd, *J* = 8.0, 5.6 Hz, 2H), 7.19 (d, *J* = 8.0 Hz, 1H), 7.05 (dd, *J* = 8.4 Hz, 2H), 4.51 (s, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 161.66 (d, *J* = 245.4 Hz), 148.9, 142.5, 139.0, 132.4, 132.1, 131.1 (d, *J* = 8.1 Hz), 131.1, 122.7, 115.6 (d, *J* = 21.2 Hz), 34.1; MS (+ESI) *m/z* 327.6 $[M+Na]^+$.

4-((4-Chlorobenzyl)thio)-7-nitrobenzo[c][1,2,5]oxadiazole (**A10**): a yellow solid; 78 mg; yield 97%; R_f 0.3 (PE/EA: 5/1); mp: 157–159 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.35 (d, *J* = 7.6 Hz, 1H), 7.35 (dd, *J* = 21.6, 8.0 Hz, 4H), 7.18 (d, *J* = 7.6 Hz, 1H), 4.50 (s, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 148.9, 142.6, 138.8, 134.1, 132.5, 132.1, 130.9, 128.7, 122.8, 34.0; MS (+ESI) *m/z* 322.2 $[M+H]^+$.

4-((4-Bromobenzyl)thio)-7-nitrobenzo[c][1,2,5]oxadiazole (**A11**): a yellow solid; 85 mg; yield 93%; R_f 0.4 (PE/EA: 5/1); mp: 158–160 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.35 (d, *J* = 8.0 Hz, 1H), 7.49 (d, *J* = 8.0 Hz, 2H), 7.32 (d, *J* = 7.6 Hz, 2H), 7.18 (d, *J* = 7.6 Hz, 1H), 4.49 (s, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 148.9, 142.5, 138.8, 134.5, 132.5, 132.1, 131.6, 131.2, 122.8, 121.0, 34.1; MS (+ESI) *m/z* 366.0 $[M+H]^+$.

4-Nitro-7-(thiophen-2-ylthio)benzo[c][1,2,5]oxadiazole (**A12**): a yellow solid; 63 mg; yield 90%; R_f 0.5 (PE/EA: 5/1); mp: 174–176 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.29 (d, *J* = 7.6 Hz, 1H), 7.77 (d, *J* = 5.2 Hz, 1H), 7.51 (d, *J* = 2.8 Hz, 1H), 7.31–7.27 (m, 1H), 6.78 (d, *J* = 7.6 Hz, 1H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 148.0, 142.7, 140.0, 139.5, 135.8, 133.2, 132.4, 129.5, 123.4, 121.4; HRMS (CI−) *m/z* calcd for $C_{10}H_5N_3O_3S_2$278.9772, found 278.9774.

Synthesis of compounds **A13–A30:** in general, these compounds were prepared through reaction of **A1** with different nucleophiles according to previously reported methods [18–24,27].

4-Nitrobenzo[c][1,2,5]oxadiazole (**A13**): yellow solid; yield 60%; R_f 0.3 (PE/EA: 5/1); ^{1}H NMR (300 MHz, Acetone-d_6) δ 8.71 (d, *J* = 6.0 Hz, 1H), 8.53 (d, *J* = 9.0 Hz, 1H), 7.93 (dd, *J* = 6.0, 9.0 Hz, 2H).

4-Azido-7-nitrobenzo[c][1,2,5]oxadiazole (**A14**): yellow solid; yield 80%; R_f 0.4 (PE/EA: 5/1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.51 (d, *J* = 8.1 Hz, 1H), 7.06 (d, *J* = 8.1 Hz, 1H).

4-Methoxy-7-nitrobenzo[c][1,2,5]oxadiazole (**A15**): yellow solid; yield 61%; R_f 0.8 (CH_2Cl_2); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.56 (d, *J* = 8.1 Hz, 1H), 6.70 (d, *J* = 7.8 Hz, 1H), 4.24 (s, 3H).

4-Ethoxy-7-nitrobenzo[c][1,2,5]oxadiazole (**A16**): yellow solid; yield 60%; R_f 0.2 (PE/EA: 5/1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.54 (d, *J* = 8.4 Hz, 1H), 6.68 (d, *J* = 8.4 Hz, 1H), 4.48 (q, *J* = 7.0 Hz, 2H), 1.64 (t, *J* = 7.0 Hz, 3H).

4-(Methylthio)-7-nitrobenzo[c][1,2,5]oxadiazole (**A17**): yellow solid; yield 43%; R_f 0.4 (PE/EA: 5/1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.42 (d, *J* = 8.0 Hz, 1H), 7.11 (d, *J* = 8.0 Hz, 1H), 2.77 (s, 3H).

4-Nitro-7-(phenylsulfonyl)benzo[c][1,2,5]oxadiazole (**A18**): yellow solid; yield 30%; R_f 0.6 (DCM/PE: 2/1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.56 (s, 1H), 8.45 (s, 1H), 8.22 (s, 2H), 7.59 (s, 3H).

4-(Benzylthio)-7-nitrobenzo[c][1,2,5]oxadiazole (**A19**): yellow solid; yield 71%; R_f 0.4 (PE/EA: 5/1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.35 (s, 1H), 7.33 (t, J = 37.3 Hz, 6H), 4.53 (s, 2H).

N,N-dimethyl-7-nitrobenzo[c][1,2,5]oxadiazol-4-amine (**A20**): red solid; yield 97%; R_f 0.4 (PE/EA: 5/1); ^{1}H NMR (400 MHz, Acetone-d_6) δ 8.47 (d, J = 8.6 Hz, 1H), 6.38 (d, J = 8.4 Hz, 1H), 3.69 (s, 6H).

4-Nitro-7-(pyrrolidin-1-yl)benzo[c][1,2,5]oxadiazole (**A21**): red solid; yield 51%; R_f 0.2 (PE/EA: 5/1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.39 (d, J = 8.9 Hz, 1H), 5.99 (d, J = 9.0 Hz, 1H), 4.28 (s, 2H), 3.66 (s, 2H), 2.23 (s, 4H).

4-Nitro-7-(piperidin-1-yl)benzo[c][1,2,5]oxadiazole (**A22**): red solid; yield 56%; R_f 0.7 (CH_2Cl_2); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.42 (d, J = 9.0 Hz, 1H), 6.27 (d, J = 9.0 Hz, 1H), 4.11 (s, 4H), 1.84 (s, 6H).

4-Nitro-7-(piperazin-1-yl)benzo[c][1,2,5]oxadiazole (**A23**): red solid; yield 70%; R_f 0.7 (DCM/MeOH: 10/1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.43 (d, J = 9.0 Hz, 1H), 6.30 (d, J = 9.0 Hz, 1H), 4.09 (s, 4H), 3.13 (s, 4H).

4-Morpholino-7-nitrobenzo[c][1,2,5]oxadiazole (**A24**): red solid; yield 70%; R_f 0.8 (DCM/MeOH: 20/1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.45 (d, J = 8.9 Hz, 1H), 6.33 (d, J = 9.0 Hz, 1H), 4.15–4.05 (m, 4H), 4.03–3.91 (m, 4H).

7-Nitro-N-phenylbenzo[c][1,2,5]oxadiazol-4-amine (**A25**): red solid; yield 93%; R_f 0.4 (PE/EA: 5/1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.45 (d, J = 8.6 Hz, 1H), 7.81 (s, 1H), 7.51 (dd, J = 5.6, 3.4 Hz, 2H), 7.40 (m, 3H), 6.73 (d, J = 8.6 Hz, 1H).

7-Nitro-N-(p-tolyl)benzo[c][1,2,5]oxadiazol-4-amine (**A26**): red solid; yield 80%; R_f 0.4 (PE/EA: 5/1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.44 (d, J = 8.1 Hz, 1H), 7.76 (s, 1H), 7.31 (s, 4H), 6.64 (d, J = 8.2 Hz, 1H), 2.42 (s, 3H).

N-(4-fluorophenyl)-7-nitrobenzo[c][1,2,5]oxadiazol-4-amine (**A27**): red solid; yield 86%; R_f 0.4 (PE/EA: 5/1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.44 (d, J = 8.5 Hz, 1H), 7.73 (s, 1H), 7.41 (dd, J = 8.2, 4.6 Hz, 2H), 7.24 (dd, J = 15.2, 7.0 Hz, 2H), 6.57 (d, J = 8.5 Hz, 1H).

N-(4-chlorophenyl)-7-nitrobenzo[c][1,2,5]oxadiazol-4-amine (**A28**): red solid; yield 96%; R_f 0.4 (PE/EA: 5/1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.45 (d, J = 8.5 Hz, 1H), 7.80 (s, 1H), 7.49 (d, J = 8.4 Hz, 2H), 7.38 (d, J = 8.4 Hz, 2H), 6.69 (d, J = 8.5 Hz, 1H).

N-(2-fluorophenyl)-7-nitrobenzo[c][1,2,5]oxadiazol-4-amine (**A29**): red solid; yield 87%; R_f 0.3 (PE/EA: 5/1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.46 (d, J = 8.5 Hz, 1H), 7.66 (s, 1H), 7.56 (t, J = 7.3 Hz, 1H), 7.42–7.22 (m, 3H), 6.60 (d, J = 8.4 Hz, 1H).

N-(4-methoxyphenyl)-7-nitrobenzo[c][1,2,5]oxadiazol-4-amine (**A30**): red solid; yield 95%; R_f 0.3 (PE/EA: 5/1); ^{1}H NMR (400 MHz, Acetone-d_6) δ 9.81 (s, 1H), 8.52 (d, J = 8.7 Hz, 1H), 7.50 (d, J = 8.5 Hz, 2H), 7.10 (d, J = 8.5 Hz, 2H), 6.66 (d, J = 8.6 Hz, 1H), 3.87 (s, 3H).

5.5. Synthesis of 4-fluoro-5-methoxy-2-nitroaniline (*2*) [25]

A mixture of 5-chloro-4-fluoro-2-nitroaniline (200 mg, 1.05 mmol) and sodium methoxide (567 mg, 10.5 mmol) in methanol (10 mL) was heated at 100 °C in a sealed tube for 3 h. Then the solution was cooled to room temperature, diluted with 20 mL of water, extracted with EtOAc (3 × 50 mL) and washed with brine. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to give intermediate **2** as a yellow solid (176 mg, yield 90%): R_f 0.5 (CH_2Cl_2/MeOH: 50/1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.85 (d, J = 11.6 Hz, 1H), 6.21 (d, J = 7.2 Hz, 3H), 3.91 (s, 3H).

5.6. Synthesis of 5-ethoxy-6-methoxybenzofuroxan (*3*) [26]

4-Fluoro-5-methoxy-2-nitroaniline (compound **2**, 100 mg, 0.54 mmol) was dissolved in alcohol (10 mL) with 0.25% (w/v) KOH. The solution was cooled to 0 °C, and 5% aqueous NaClO solution was added dropwise until the red color disappeared. Then the reaction was stirred for another 0.5 h. The mixture was diluted with water (50 mL), then extracted with EtOAc (2 × 50 mL) and washed with brine. The combined organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was then subjected to silica gel flash chromatography (CH_2Cl_2/PE: 1/1) to afford the desired product as yellow solid (60 mg, 53%): R_f 0.4 (PE/EA: 5/1); mp: 199–200 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.62 (brs, 1H), 6.40 (br s, 1H), 4.14 (s, 2H), 3.95 (s, 3H), 1.53 (t, J = 6.8 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 92.7, 87.8, 65.3, 56.8, 14.1.

5.7. Synthesis of 5-ethoxy-6-methoxybenzo[c][1,2,5]oxadiazole (*B1*)

5-Ethoxy-6-methoxybenzofuroxan (**3**, 50 mg, 0.24 mmol) was treated with triphenylphosphine (93.6 mg, 0.36 mmol) at 50 °C for 1 h. Then the solvent was removed in vacuo, and the residue was purified by the preparation thin layer chromatography (CH_2Cl_2/PE: 1/1) to give the desired product as a white solid (33 mg, 71%): R_f 0.5 (CH_2Cl_2/PE: 1/1); mp: 173–175 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.86 (s, 1H), 6.84 (s, 1H), 4.18 (q, J = 6.8 Hz, 2H), 3.98 (s, 3H), 1.55 (t, J = 6.8 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.7, 154.8, 146.9, 146.8, 91.1, 90.7, 65.3, 56.7, 14.3; HRMS (CI−) m/z calcd for $C_9H_{10}N_2O_3$194.0691, found 194,0684.

5.8. General synthetic procedure for compounds B2–B4

Three different benzofuroxans (50 mg) in CH_2Cl_2 (2 mL) were treated with triphenylphosphine (1.5 equiv) respectively at 50 °C for 1 h. Then the solvent was removed in vacuo, and the residue was purified by the preparation of thin layer chromatography (PE/EA: 5/1) to give the desired products as yellow solids.

5-Chloro-6-(piperidin-1-yl)benzo[c][1,2,5]oxadiazole (**B2**): a yellow solid; 39 mg; yield 83%; R_f 0.8 (PE/EA: 5/1); mp: 77–79 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.85 (s, 1H), 7.07 (s, 1H), 3.05 (s, 4H), 1.92–1.69 (m, 4H), 1.69–1.45 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 153.5, 149.1, 146.9, 138.5, 116.8, 101.2, 53.4, 25.9, 24.0; HRMS (CI−) calcd for $C_{11}H_{12}ClN_3O$ 237.0669, found 237.0662.

5-Chloro-6-morpholinobenzo[c][1,2,5]oxadiazole (**B3**): a yellow solid; 40 mg; yield 85%; R_f 0.3 (PE/EA: 5/1); mp: 118–120 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.89 (s, 1H), 7.14 (s, 1H), 4.14–3.69 (m, 4H), 3.34–2.95 (m, 4H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 152.1, 148.7, 146.8, 137.5, 117.1, 101.9, 66.6, 52.2; HRMS (CI−) m/z calcd for $C_{10}H_{10}N_3O_2Cl$ 239.0462, found 232.0464.

5-Fluoro-6-(piperidin-1-yl)benzo[c][1,2,5]oxadiazole (**B4**): a yellow solid; 40 mg; yield 86%; R_f 0.5 (PE/EA: 5/1); mp: 66–68 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32 (d, J = 11.6 Hz, 1H), 6.92 (d, J = 7.6 Hz, 1H), 3.22–3.05 (m, 4H), 1.84–1.70 (m, 4H), 1.70–1.58 (m, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 160.5 (d, J = 265.6 Hz), 148.0, 147.8 (d, J = 16.2 Hz), 146.4 (d, J = 15.2 Hz), 99.3 (d, J = 27.3 Hz), 98.5 (d, J = 3.0 Hz), 52.1, 52.0, 25.9, 24.1; HRMS (CI−) m/z calcd for $C_{11}H_{12}N_3OF$ 221.0964, found 221.0963.

6. Antifungal bioassay

All phytopathogenic fungi were provided by the Laboratory of Plant Disease Control, Nanjing Agricultural University. The strains were retrieved from the storage tube of potato dextrose agar (PDA) slants to sterilized PDA Petri dishes and incubated at 25 °C in the dark for a week to produce new mycelia for the antifungal assay.

The antifungal activities of the synthetic compound were tested *in vitro* against four plant pathogenic fungi (*R. solani*, *S. sclerotiorum*, *F. graminearum* and *P. capsici*) using a mycelia growth inhibition method [27]. Before mixing with molten agar, a stock solution of 10 mg/mL in DMSO was prepared at room temperature. The

concentration for initial screening was 25 μg/mL. And the medium containing compounds was then poured into sterilized Petri dishes. After 2–4 days (different strains with different growth speed) incubation at 25 °C in the dark, the colony diameter of each strain was measured with the original mycelial disk diameter (5 mm) subtracted from this measurement. Percentage inhibition (I%) was calculated with the following equation: $I\% = (1 - a/b) \times 100$, where a is the colony diameter in Petri dishes with test compounds, and b is the mean colony diameter in Petri dishes without tested compounds. DMSO served as negative control, whereas commercially available agricultural fungicide, Carbendazim and Chlorothalonil, were used as positive controls. Compounds possessing good activities (inhibitory rate >50% at 25 μg/mL) were needed to be further evaluated using the above-mentioned method but with different concentrations. Each measurement consisted of at least three replicates. The concentration dependent curve was the logarithmic values of inhibition rates for the y axis against the test sample concentrations (μg/mL) for the X axis. The IC_{50} value was defined as the concentration required for 50% inhibition of mycelial growth, as shown in Tables 1–3.

Notes

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.04.058.

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