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Synthesis and Biological Evaluation of Benzofuroxan Derivatives as Fungicides against Phytopathogenic Fungi

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Supporting Information

ABSTRACT: Forty-four benzofuroxan derivatives were designed and prepared as antifungal agents. Their structures were characterized by ^{1}H NMR, ^{13}C NMR, and HRMS. Their antifungal activities were tested in vitro with four important phytopathogenic fungi, namely, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Fusarium graminearum* and *Phytophthora capsici*, using the mycelium growth inhibition method. Compound **A5** displayed the maximum antifungal activity against *F. graminearum* (IC_{50} = 1.1 μg/mL, which is about 2-fold higher than that of the well-known positive control carbendazim (IC_{50} = 0.5 μg/mL). **A14** exhibited high antifungal effect against both *S. sclerotiorum* and *F. graminearum* Sehw., with IC_{50} values of 2.52 and 3.42 μg/mL, respectively. Among 14 benzofuroxan derivatives with substitutions at the R^2 and R^3 positions of the phenyl ring (**B** series), 7 compounds displayed strong growth inhibition against *R. solani* ($IC_{50} \leq 3.0$ μg/mL). Analysis of the structure−activity relationship data of these compounds revealed that (1) introduction of an electron-donating amino group to the R^2 position of the phenyl ring favors antifungal activity against *R. solani* and (2) the presence of a nitro group at the R^4 position and substituent variation at the R^1 position of the phenyl ring can result in good antifungal candidates against *F. graminearum* Sehw. Overall, the benzofuroxan was discovered as a novel scaffold for the development of fungicides. Significantly, **A14** was demonstrated to successfully suppress disease development in *S. sclerotiorum* infected cole in vivo.

KEYWORDS: *antifungal activity, phytopathogenic fungi, benzofuroxan derivative, Rhizoctonia solani, Sclerotinia sclerotiorum, Fusarium graminearum*

INTRODUCTION

Plant pathogenic fungi remain a serious and global problem for food security and human health. For example, fungus infection can cause severe crop yield reduction and results in dramatic economic losses in agriculture.[1] In addition, the threat of fungus-produced mycotoxins, such as aflatoxins, fumonisins, and trichothecenes, can last for many years.[2,3] Although fungus-resistant crop varieties and other biological methods have been employed to deal with the fungus problem, fungicidal chemicals remain the principal control tool. However, like all other microorganisms, plant pathogenic fungi exhibit a remarkable ability to change and adapt. More virulent and fungicide-resistant pathogenic strains continually arise and can no longer be controlled by chemicals that were once effective.[4,5] As a result, it is necessary to develop novel and effective fungicidal agents to effectively control those agricultural diseases.

Recently, compounds containing furoxan (1,2,5-oxadiazole *N*-oxide, Figure 1) have increasingly attracted medicinal chemists' attention due to their broad and interesting bioactivities, such as anticancer, antiparasite, antiaggregatory, and vasodilating.[6−11] In the meantime, it was also demonstrated that benzofuroxan derivatives had fungicidal activity even though benzofuroxan derivatives as antifungal agents have not been systematically investigated.[12] In this study, 44 benzofuroxan derivatives with modification on the R^1−R^4 positions of the phenyl ring were synthesized. Their fungicidal activities were evaluated against four important plant pathogen strains including *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Fusarium graminearum*, and *Phytophthora capsici* in vitro, and the in vivo efficacies of **A14** and **A15** against *S. sclerotiorum* infected cole leave were evaluated.

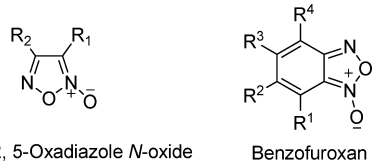

Figure 1. Chemical structures of 1,2,5-oxadiazole *N*-oxide and benzofuroxan.

MATERIALS AND METHODS

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 ^{1}H NMR and ^{13}C NMR spectra were recorded in deuterochloroform at ambient temperature using a Varian Mercury 400. The ESIMS and ESI-HRMS were recorded with an Agilent

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Table 1. Antifungal Activity of Series A Benzofuraxan Derivatives against Four Phytopathogens[a]

Compound	R^1	R^2	R^3	R^4	$IC_{50} \pm SD$ (μg/mL)*			
					Rhizoctonia solani	*Sclerotinia sclerotiorum*	*Fusarium graminearum, Sehw*	*Phytophthora capsici*
A1	CH_3CH_2O	H	H	NO_2	> 25	> 25	> 25	> 25
A2	CH_3S	H	H	NO_2	> 25	18.27±0.10	> 25	> 25
A3		H	H	NO_2	24.97±0.71	16.79±0.83	15.77±0.37	22.50±0.63
A4		H	H	NO_2	6.60±0.82	> 25	> 25	> 25
A5		H	H	NO_2	> 25	> 25	**1.1±0.27**	> 25
A6		H	H	NO_2	14.2±0.72	> 25	13.11±0.14	> 25
A7		H	H	NO_2	11.41±0.28	> 25	7.01±0.33	> 25
A8		H	H	NO_2	14.37±0.62	> 25	9.65±0.25	> 25
A9		H	H	NO_2	**3.02±0.56**	> 25	**2.28±0.08**	24.06±0.11
A10	Cl	H	H	NO_2	> 25	16.71±1.46	> 25	> 25
A11	N_3	H	H	NO_2	22.56±0.45	> 25	> 25	> 25
A12	$PhSO_2$	H	H	NO_2	> 25	> 25	> 25	> 25
A13		H	H	NO_2	> 25	> 25	> 25	> 25
A14		H	H	NO_2	7.40±0.21	**2.52±1.64**	**3.42±1.03**	9.07±0.72
A15		H	H	NO_2	6.15±1.42	6.73±0.94	8.40±0.74	23.04±0.90
A16	CH_3O	H	H	NO_2	> 25	> 25	> 25	> 25
A17		H	H	NO_2	8.88±0.56	16.27±0.93	5.10±0.72	15.20±0.75
A18		H	H	NO_2	> 25	> 25	> 25	> 25
A19		H	H	NO_2	9.22±0.61	25.1±0.13	9.15±0.13	> 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.

1260LC and an Agilent Technologies 6220 Accurate-Mass TOF LC/MS, respectively. The melting points of the products were determined on an XT5 apparatus (Beijing Keyielec-opti Instrument Factory, Beijing, China). PE, petroleum ether (bp 60−90 °C); EA, ethyl acetate; DMAP, *N*,*N*-dimethylaminopyridine; NBS, *N*-bromosuccinimide; BPO, benzoyl peroxide; DCM, dichloromethane.

Plant Pathogenic Fungi. All strains of fungi were provided by the Laboratory of Plant Disease Control, Nanjing Agricultural University. The strains were retrieved from the storage tube and incubated in PDA at 25 °C for a week to get new mycelia for the antifungal assay.

Antifungal Bioassay. The fungicidal activities of the synthetic compounds were tested in vitro against four plant pathogenic fungi (*R. solani*, *S. sclerotiorum*, *F. graminearum*, and *P. capsici*) using a mycelia growth inhibition method.[13] The synthesized compounds were dissolved in DMSO to prepare the 10 mg/mL stock solution before mixing with molten agar below 60 °C. The medium containing compounds at a concentration of 25 μg/mL for the initial screening was then poured into sterilized Petri dishes. After appropriate days at 25 °C, the colony diameter of each strain was measured with the original mycelial disk diameter (5 mm) subtracted from this measurement. Percentage inhibition was calculated as $(1 - a/b) \times 100$, where a is the colony diameter in Petri dishes with 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 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, shown in Tables 1−3.

In Vivo Testing on Cole Leaves Infected by *S. sclerotiorum*. Strain *S. sclerotiorum* and susceptible cole leaves collected from Pailou Experimental Centre of Nanjing Agricultural University were used to measure the efficacy of compounds in vivo. Healthy cole leaves were sprayed with compounds (0.5 mg/mL) and subsequently cultivated at 20 °C for 24 h before artificial inoculation with strain *S. sclerotiorum*. Results were observed as diameters of symptoms after cultivation at 20 °C for 36 h. Carbendazim (50% WP, Jiangsu Rotam Lanfeng Biochemical Co., Ltd., China) was coassayed as the positive control. The efficacy of disease control was calculated as $(1 - c/d) \times 100$, where c is the diameter of the treatment and d is the diameter of the negative control. The disease control picture is shown in Figure 2.

General Synthetic Procedure for the Key Target Molecules. Intermediates **1**−**5** were synthesized from commercially sourced chemicals using the reported procedure.[14−16] New benzofuroxan compounds were prepared using the synthetic routes shown in Schemes 1−3.

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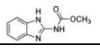
Table 2. Antifungal Activity of Series B Benzofuraxan Derivatives against Four Phytopathogens[a]

Compound	R^1	R^2	R^3	R^4	$IC_{50} \pm SD$ (μg/mL)*			
					Rhizoctonia solani	*Sclerotinia sclerotiorum*	*Fusarium graminearum, Sehw*	*Phytophthora capsici*
B1	H	Cl	CH_3	H	4.34±0.28	17.85±0.48	17.58±0.83	> 25
B2	H	Cl	$BrCH_2$	H	7.32±1.63	4.13±1.71	13.56±2.85	11.11±1.49
B3	H		Cl	H	6.98±0.23	> 25	> 25	> 25
B4	H		Cl	H	**1.81±0.32**	20.86±0.21	7.43±0.16	22.65±0.51
B5	H		Cl	H	**2.67±0.21**	20.86±0.34	> 25	> 25
B6	H		Cl	H	> 25	> 25	> 25	> 25
B7	H	CH_3O	Cl	H	**2.10±0.14**	23.06±0.04	24.45±0.18	> 25
B8	H		F	H	**1.76±0.47**	> 25	22.7±0.35	> 25
B9	H		F	H	**2.58±0.31**	> 25	> 25	> 25
B10	H		F	H	> 25	> 25	> 25	> 25
B11	H		F	H	3.59±0.39	> 25	> 25	> 25
B12	H	Cl	F	H	**3.09±0.82**	9.24±0.64	13.77±1.27	> 25
B13	H	C_2H_5O	Cl	H	**3.05±0.04**	> 25	> 25	> 25
B14	H	H	H	H	6.84±1.02	> 25	> 25	> 25
B15	H	Cl	H	H	4.85±0.38	23.6±1.25	24.58±0.65	> 25
B16	H	Cl	Cl	H	4.50±0.11	10.06±1.11	11.23±1.07	> 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 Benzofuraxan Derivatives against Four Phytopathogens[a]

Compound	R^1	R^2	R^3	R^4	$IC_{50} \pm SD$ (μg/mL)*			
					Rhizoctonia solani	*Sclerotinia sclerotiorum*	*Fusarium graminearum, Sehw*	*Phytophthora capsici*
C1	Cl	Cl	H	NO_2	> 25	> 25	> 25	> 25
C2		Cl	H	NO_2	> 25	> 25	> 25	> 25
C3		Cl	H	NO_2	> 25	> 25	> 25	> 25
C4		Cl	H	NO_2	21.1±0.48	> 25	> 25	> 25
C5	CH_3SO_2	H	H	CH_3SO_2	> 25	> 25	> 25	> 25
C6	$PhSO_2$	H	H	$PhSO_2$	> 25	> 25	> 25	> 25
C7	CH_3O	H	H	$PhSO_2$	> 25	> 25	> 25	> 25
C8	NO_2	H	NO_2	Cl	> 25	> 25	> 25	> 25
C9	NO_2	Cl	H	H	5.91±0.37	17.32±1.04	> 25	> 25
C10	H	NO_2	H	NO_2	> 25	> 25	> 25	> 25

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

*Synthesis of 7-Ethoxy-4-nitrobenzofuroxan (**A1**).* To a solution of 4-chloro-7-nitrobenzofuroxan (100 mg, 0.46 mmol) in ethanol (10 mL) was added at 0 °C with 0.5 M CH_3CH_2ONa (1.1 mL). The reaction mixture was stirred for 2 h. Then the reaction mixture was concentrated in vacuo, the residue was subjected to silica gel column chromatography (eluant: PE/EA 5:1 and then CH_2Cl_2) for purification to afford the desired product as a yellow solid (63 mg, yield 60%): R_f 0.2 (PE/EA 5:1); mp 94−96 °C; ^{1}H NMR (400 MHz,

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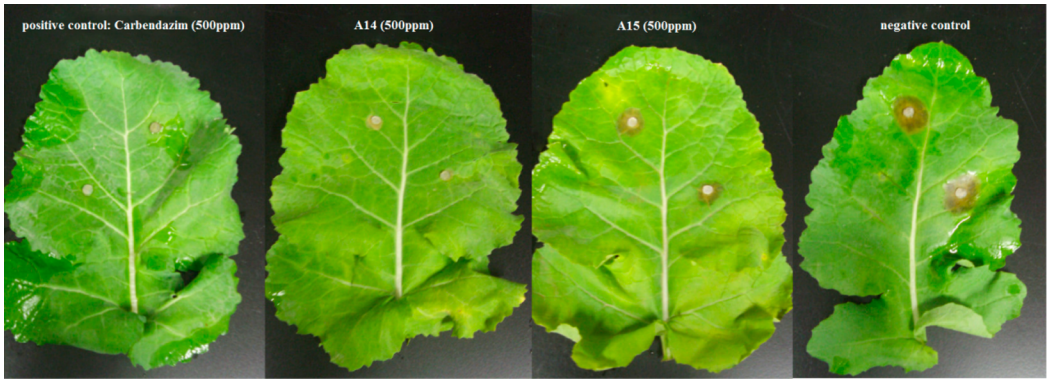
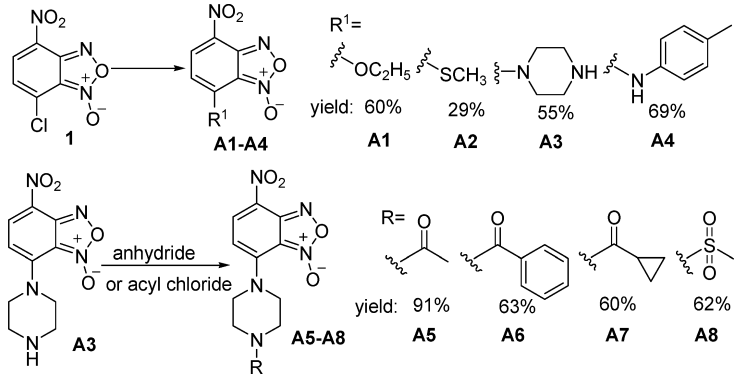
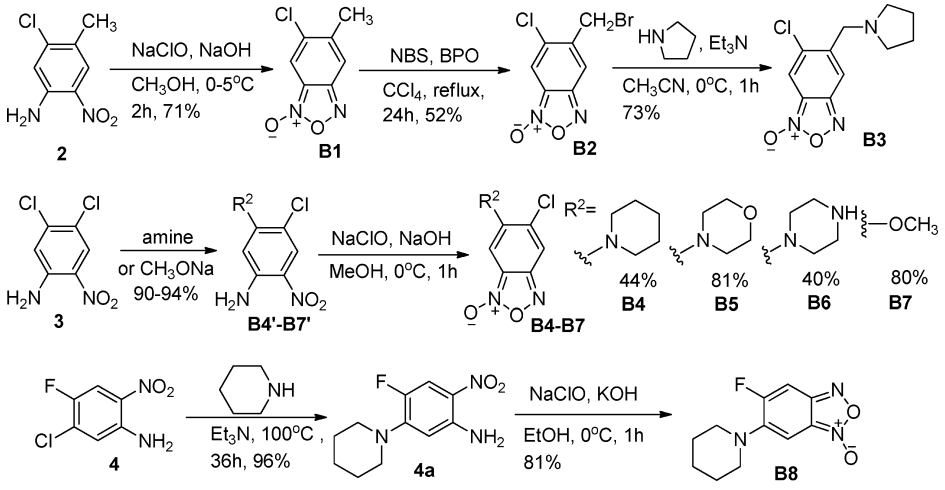


Figure 2. Effects of compounds **A14** and **A15** against *Sclerotinia sclerotiorum* infected cole leaves.

Scheme 1. Synthetic Route of Benzofuroxan Derivatives A1−A8



Scheme 2. Synthetic Route of Benzofuroxan Derivatives B1−B8



$CDCl_3$) δ 8.39 (d, J = 8.5 Hz, 1H, ArH), 6.42 (d, J = 8.5 Hz, 1H, ArH), 4.38 (q, J = 7.0 Hz, 2H, $-CH_2-$), 1.56 (t, J = 7.0 Hz, 3H, Me−H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 153.7 (ArC), 146.0 (ArC), 135.3 (ArC), 129.3 (ArC), 109.2 (ArC), 101.6 (ArC), 67.5 ($-CH_2-$), 13.9 (Me−C); HRMS (ESI) m/z calcd for $C_8H_7N_3NaO_5$[M + Na] 248.0283, found 248.0273.

*Synthesis of 7-(Methylthio)-4-nitrobenzofuroxan (**A2**).* To a solution of 4-chloro-7-nitrobenzofuroxan (50 mg, 0.23 mmol) in ethanol/0.1 M sodium phosphate buffer (1:1 v/v, 5 mL) was added 15% sodium methanethiolate (130.1 mg, 0.28 mmol), and the reaction mixture was stirred for 1.5 h. The solvent ethanol was removed in vacuo. The aqueous phase was then extracted with EtOAc (25 mL × 2), dried over anhydrous Na_2SO_4, and concentrated in vacuo. The residue was subjected to silica gel column chromatography (eluant: PE/EA 5:1 and then CH_2Cl_2) to give the desired product as a red solid (15 mg, yield 29%): R_f 0.8 (CH_2Cl_2); mp 180−182 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.32 (d, J = 8.1 Hz, 1H, ArH), 6.76 (d, J = 8.1 Hz, 1H, ArH), 2.66 (s, 3H, Me−H); ^{13}C NMR (75 MHz, DMSO) δ 145.6 (ArC), 141.3 (ArC), 133.0 (ArC), 131.3 (ArC), 117.9 (ArC), 115.3 (ArC), 13.9 (Me−C); HRMS (ESI) m/z calcd for $C_7H_5N_3NaO_4S$ [M + Na] 249.9899, found 249.9896.

*Synthesis of 4-Nitro-7-(piperazin-1-yl)benzofuroxan (**A3**).* To a solution of 4-chloro-7-nitrobenzofuroxan (50 mg, 0.23 mmol) in ethanol/0.1 M sodium phosphate buffer (1:1 v/v, 5 mL) was added piperazine (199.8 mg, 2.32 mmol), and the reaction was stirred for 0.5 h. The solvent ethanol was removed in vacuo. The aqueous phase was then extracted with EtOAc (50 mL × 2), dried over anhydrous Na_2SO_4, and concentrated in vacuo. The residue was subjected to silica

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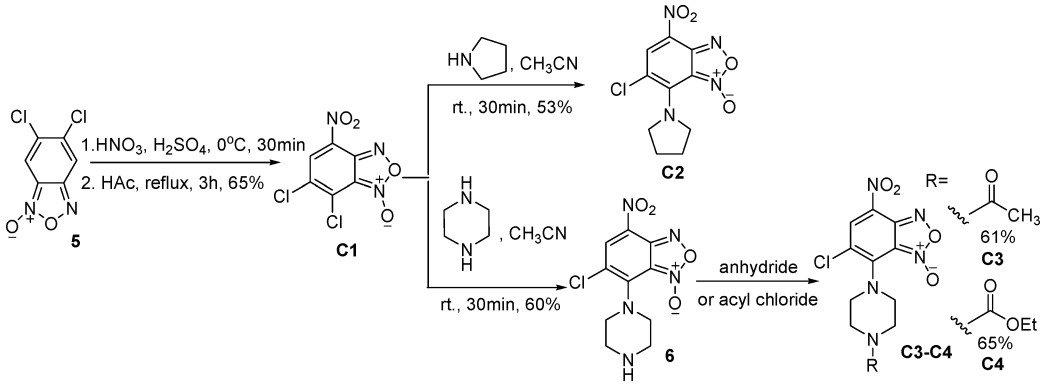
Scheme 3. Synthetic Route of Benzofuroxan Derivatives C1−C4



gel flash chromatography (DCM/MeOH = 10:1) to afford the title compound as a red solid (34 mg, yield 55%): R_f 0.6 (DCM/MeOH = 10:1); mp 149−150 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.31 (d, J = 8.6 Hz, 1H, ArH), 6.24 (d, J = 8.6 Hz, 1H, ArH), 3.47−3.32 (m, 4H, $-NCH_2-$), 3.13−3.05 (m, 4H, $-NCH_2-$); ^{13}C NMR (75 MHz, DMSO) δ 147.6 (ArC), 146.9 (ArC), 136.0 (ArC), 124.0 (ArC), 111.7 (ArC), 105.6 (ArC), 52.8($-NCH_2-$ × 2), 45.3 ($-NCH_2-$ × 2); HRMS (ESI) m/z calcd for $C_{10}H_{11}N_5NaO_4$ $[M + Na]^+$ 288.0709, found 288.0700.

*Synthesis of 4-Nitro-7-(p-tolylamino)benzofuroxan (**A4**).* To a solution of 4-chloro-7-nitrobenzofuroxan (50 mg, 0.23 mmol) in CH_3CN/0.1 M sodium phosphate buffer (1:1 v/v, 5 mL) was added paratoluidine (248.6 mg, 2.32 mmol), and the reaction mixture was stirred for 0.5 h. The methyl cyanide was removed in vacuo. The resulting aqueous phase was then extracted with CH_2Cl_2 (25 mL × 2), and the organic layer was dried over anhydrous Na_2SO_4 and concentrated in vacuo. The residue was purified by silica gel column chromatography (eluant: PE/EA = 10:1 and then DCM/PE = 2:1) to give **A4** as a red solid (46 mg, yield 69%): R_f 0.4 (PE/EA 10:1); mp 183−185 °C; ^{1}H NMR (400 MHz, DMSO) δ 9.95 (br s, 1H, NH), 8.34 (d, J = 9.0 Hz, 1H, ArH), 7.48−7.19 (m, 4H, ArH), 6.22 (d, J = 9.0 Hz, 1H, ArH), 2.35 (s, 3H, Me−H); ^{13}C NMR (75 MHz, DMSO) δ 150.1 (ArC), 147.9 (ArC), 147.0 (ArC), 142.8 (ArC), 137.9 (ArC), 138.0 (ArC), 136.9 (ArC), 134.4 (ArC), 134.0 (ArC), 130.3 (ArC), 130.1 (ArC), 126.5 (ArC), 125.1 (ArC), 122.9 (ArC), 122.1 (ArC), 121.5 (ArC), 113.0 (ArC), 112.0 (ArC), 109.9 (ArC), 99.9 (ArC), 20.7 (Me−C); HRMS (ESI) m/z calcd for $C_{13}H_{10}N_4NaO_4$ $[M + Na]^+$ 309.0600, found 309.0608.

*Synthesis of 7-(4-Acetylpiperazin-1-yl)-4-nitrobenzofuroxan (**A5**).* To a solution of 4-nitro-7-(piperazin-1-yl)benzofuroxan (20 mg, 0.08 mmol) in ethanol/pyridine (1:1 v/v, 1 mL) was added acetic anhydride (23.1 mg, 0.23 mmol) at room temperature, the reaction mixture was stirred for 30 min and then concentrated in vacuo, and the residue was subjected to silica gel column chromatography (CH_2Cl_2/MeOH 100:1) to give **A5** as a red solid (21 mg, yield 91%): R_f 0.6 (CH_2Cl_2/MeOH 10:1); mp 211−212 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.34 (d, J = 8.5 Hz, 1H, ArH), 6.29 (d, J = 8.5 Hz, 1H, ArH), 3.81 (d, J = 52.0 Hz, 4H, $-NCH_2-$), 3.38 (d, J = 19.5 Hz, 4H, $-NCH_2-$), 2.17 (s, 3H, Me−H); ^{13}C NMR (75 MHz, DMSO) δ 168.7 (−C═O), 147.5 (ArC), 146.6 (ArC), 136.1 (ArC), 125.3 (ArC), 112.1 (ArC), 106.4 (ArC), 51.2 ($-NCH_2-$), 50.5 ($-NCH_2-$), 45.0 ($-NCH_2-$), 21.3 (Me−C); HRMS (ESI) m/z calcd for $C_{12}H_{13}N_5NaO_5$ $[M + Na]^+$ 330.0814, found 330.0816.

General Synthetic Procedure for the Preparation of Compounds A6−A8. To a solution of 4-nitro-7-(piperazin-1-yl)benzofuroxan (50 mg, 0.19 mmol) in CH_2Cl_2 (4 mL) was added Et_3N (19.1 mg, 0.19 mmol) at 0 °C. Then three different acyl chlorides including benzoyl chloride, cyclopropanecarbonyl chloride, and methanesulfonyl chloride (0.19 mmol) were added dropwise. The reaction mixture was stirred for another 15 min and concentrated in vacuo, and the residue was subjected to silica gel column chromatography (CH_2Cl_2/MeOH 100:1) to give **A6−A8** as red solids.

*7-(4-Benzoylpiperazin-1-yl)-4-nitrobenzofuroxan (**A6**):* 44 mg; yield 63%; R_f 0.7 (CH_2Cl_2/MeOH 10:1); mp 188−189 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.32 (d, J = 8.4 Hz, 1H, ArH), 7.45 (s, 5H, ArH), 6.29 (d, J = 8.5 Hz, 1H, ArH), 3.96 (t, J = 77.6 Hz, 4H, $-NCH_2-$), 3.39 (s, 4H, $-NCH_2-$); ^{13}C NMR (75 MHz, DMSO) δ 169.3 (−C═O), 147.5 (ArC), 146.5 (ArC), 136.1 (ArC), 135.5 (ArC), 129.8 (ArC × 2), 128.5 (ArC × 2), 127.1 (ArC), 125.5 (ArC), 112.1 (ArC), 106.6 (ArC), 50.8 ($-NCH_2-$); HRMS (ESI) m/z calcd for $C_{17}H_{15}N_5NaO_5$ $[M + Na]^+$ 392.0971, found 392.0965.

*7-(4-(Cyclopropanecarbonyl)piperazin-1-yl)-4-nitrobenzofuroxan (**A7**):* 38 mg; yield 60%; R_f 0.8 (CH_2Cl_2/MeOH 10:1); mp 194−196 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.33 (d, J = 8.4 Hz, 1H, ArH), 6.29 (d, J = 8.4 Hz, 1H, ArH), 3.92 (d, J = 23.8 Hz, 4H, $-NCH_2-$), 3.41 (d, J = 28.8 Hz, 4H, $-NCH_2-$), 1.83−1.67 (m, 1H, CH), 1.03 (s, 2H, $-CH_2-$), 0.84 (d, J = 5.0 Hz, 2H, $-CH_2-$); ^{13}C NMR (75 MHz, DMSO) δ 171.4 (−C═O), 147.5 (ArC), 146.5 (ArC), 136.0 (ArC), 125.2 (ArC), 112.0 (ArC), 106.4 (ArC), 55.0 ($-NCH_2-$), 51.4 ($-NCH_2-$), 50.5 ($-NCH_2-$), 44.3 ($-NCH_2-$), 10.4 (CH), 7.3 ($-CH_2-$ × 2); HRMS (ESI) m/z calcd for $C_{14}H_{15}N_5NaO_5$ $[M + Na]^+$ 356.0971, found 356.0965.

*7-(4-(Methylsulfonyl)piperazin-1-yl)-4-nitrobenzofuroxan (**A8**):* 40 mg; yield 62%; R_f 0.6 (CH_2Cl_2/MeOH 10:1); mp 199−201 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.44 (d, J = 8.6 Hz, 1H, ArH), 6.65 (dd, J = 8.7, 4.2 Hz, 1H, ArH), 3.64−3.59 (m, 4H, $-NCH_2-$), 3.49−3.44 (m, 4H, $-NCH_2-$), 2.95 (s, 3H, Me−H); ^{13}C NMR (75 MHz, DMSO) δ 147.4 (ArC), 146.4 (ArC), 136.0 (ArC), 126.1 (ArC), 112.2 (ArC), 107.2 (ArC), 50.4 ($-NCH_2-$ × 2), 44.9 ($-NCH_2-$ × 2), 34.4 (Me−C); HRMS (ESI) m/z calcd for $C_{11}H_{13}N_5NaO_6S$ $[M + Na]^+$ 366.0484, found 366.0487.

*Synthesis of 6-Chloro-5-methylbenzofuroxan (**B1**).* The suspension of sodium hydroxide (70.7 mg, 1.77 mmol) in methanol (20 mL) was stirred until the solid was completely dissolved and then 5-chloro-4-methyl-2-nitroaniline (300 mg, 1.61 mmol) was added. The brown solution was cooled to 0 °C, and then 5% aqueous NaClO solution (4.6 mL, 3.22 mmol) was added dropwise until the red color disappeared. The mixture was diluted with water (200 mL), and the precipitate was filtered and purified by column chromatography on silica gel (eluant: PE/EA 50:1) to give **B1** as a yellow solid (210 mg, yield 71%): R_f 0.7 (PE/EA 5:1); mp 118−120 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.51 (br s, 1H, ArH), 7.27 (br s, 1H, ArH), 2.42 (s, 3H, Me−H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 140.0 (br m, ArC), 116.1 (br m, ArC), 112.4 (br m, ArC), 21.4 (Me−C;. MS (+ESI) m/z 186.0 $[M + H]^+$.

*Synthesis of 5-(Bromomethyl)-6-chlorobenzofuroxan (**B2**).* To a solution of 6-chloro-5-methyl-benzofuroxan (200 mg, 1.08 mmol) in CCl_4 (15 mL) was added *N*-bromosuccinimide (192.9 mg, 1.08 mmol) and benzoyl peroxide (262.5 mg, 1.08 mmol). The reaction mixture was stirred under nitrogen atmosphere at 78 °C for 24 h and then concentrated in vacuo. The residue was purified by silica gel column chromatography (eluant: PE/EA 200:1) to give **B2** as a yellow solid (150 mg, yield 52%): R_f 0.3 (PE/EA 40:1); mp 58−60 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.63 (br s, 2H, ArH), 4.56 (s, 2H,

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$BrCH_2-$); ^{13}C NMR (101 MHz, $CDCl_3$) δ 29.5 ($BrCH_2-$); MS (ESI) m/z 262.6 $[M - H]^-$.

*Synthesis of 6-Chloro-5-(pyrrolidin-1-ylmethyl)benzofuroxan (**B3**).* To a solution of 5-(bromomethyl)-6-chlorobenzofuroxan (50 mg, 0.19 mmol) in acetonitrile (1 mL) was added Et_3N (0.5 mL). The solution was cooled to 0 °C, and pyrrolidine (20.3 mg, 0.29 mmol) was added dropwise. After 1 h, the reaction mixture was then diluted with EtOAc (25 mL × 2) and washed with brine, and the organic layer was dried over anhydrous Na_2SO_4 and concentrated in vacuo. The residue was purified by silica gel column chromatography (eluant: CH_2Cl_2 /MeOH 100:1) to give **B3** as a yellow solid (35 mg, yield 73%): R_f 0.4 (PE/EA 5:1); mp 82−83 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 7.59 (br d, J = 33.0 Hz, 2H, ArH), 3.73 (s, 2H, $-NCH_2-$), 2.66 (s, 4H, $-NCH_2-$ × 2), 1.85 (s, 4H, $-CH_2-$ × 2); ^{13}C NMR (101 MHz, $CDCl_3$) δ 57.5 ($-NCH_2-$), 54.4 ($-NCH_2-$ × 2), 23.9 ($-CH_2-$ × 2); HRMS (ESI) m/z calcd for $C_{11}H_{13}ClN_3O_2$ $[M + H]^+$ 254.0696, found 254.0598.

*Synthesis of 4-Chloro-2-nitro-5-(piperidin-1-yl)aniline (**B4′**).* 4,5-Dichloro-2-nitroaniline (100 mg, 0.48 mmol) was dissolved in piperidine (0.5 mL). The solution was stirred at 100 °C for 2 h. Then the solution was cooled to room temperature and diluted with 10 mL of water; the solution was then extracted with EtOAc (25 mL × 2) and washed with brine. The organic combination was dried over Na_2SO_4 and concentrated in vacuo. The residue was subjected to silica gel flash chromatography (eluant: PE/EA 10:1) to afford the compound as **B4′** (112 mg, yield 91%): R_f 0.3 (PE/EA 5:1); ^1H NMR (400 MHz, $CDCl_3$) δ 8.09 (s, 1H, ArH), 6.21 (s, 1H, ArH), 6.16 (s, 2H, NH_2), 3.14−3.05 (m, 4H, $-NCH_2-$ × 2), 1.72 (d, J = 4.3 Hz, 4H, $-CH_2-$ × 2), 1.67−1.58 (m, 2H, $-CH_2-$).

*Synthesis of 4-Chloro-5-morpholino-2-nitroaniline (**B5′**).* A solution of 4,5-dichloro-2-nitroaniline (100 mg, 0.48 mmol) in morpholine (0.5 mL) was stirred at 100 °C for 3 h. Then it was cooled to room temperature and diluted with 10 mL of water, and the mixture was then extracted with EtOAc (25 mL × 2) 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 (eluant: CH_2Cl_2) to afford **B5′** as a yellow solid (117 mg, yield 94%): R_f 0.3 (CH_2Cl_2); ^1H NMR (400 MHz, $CDCl_3$) δ 8.12 (s, 1H, ArH), 6.24 (s, 1H, ArH), 6.13 (s, 2H, NH_2), 3.86 (s, 4H, $-CH_2O-$ × 2), 3.14 (s, 4H, $-NCH_2-$ × 2).

*Synthesis of 4-Chloro-2-nitro-5-(piperazin-1-yl)aniline (**B6′**).* To a solution of 4,5-dichloro-2-nitroaniline (50 mg, 0.24 mmol) in Et_3N (3 mL) was added piperazine (208.04 mg, 2.42 mmol). The reaction mixture was stirred at 100 °C for 48 h. Then the solution was cooled to room temperature and diluted with 10 mL of water. The solution was then extracted with EtOAc (25 mL × 2) and washed with brine. The combined organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to give **B6′** as a yellow solid (56 mg, 90%): R_f 0.2 (CH_2Cl_2/MeOH 10:1); ^1H NMR (400 MHz, $CDCl_3$) δ 8.14 (s, 1H, ArH), 6.22 (s, 1H, ArH), 6.13 (s, 2H, NH_2), 3.35 (s, 8H, $-NCH_2-$ × 4).

*Synthesis of 4-Chloro-5-methoxy-2-nitroaniline (**B7′**).* A solution of 4,5-dichloro-2-nitroaniline (100 mg, 0.48 mmol) in methanol (5 mL) with 25% (w/v) sodium methoxide was heated under reflux for 3 h. Then the solution was cooled to room temperature, diluted with 20 mL of water, and extracted with CH_2Cl_2; the organic layer was dried over Na_2SO_4 and concentrated to give **B7′** as a yellow solid (90 mg, yield 92%): R_f 0.2 (PE/EA 5:1); ^1H NMR (300 MHz, $CDCl_3$) δ 8.18 (s, 1H, ArH), 6.21 (d, J = 14.4 Hz, 3H, ArH, NH_2), 3.92 (s, 3H, Me−H).

General Synthetic Procedure for Compounds B4−B7. The suspension of sodium hydroxide (11.10 mg, 0.28 mmol) in methanol (5 mL) was stirred until the solid was completely dissolved. Then four different anilines including 4-chloro-2-nitro-5-(piperidin-1-yl) aniline, 4-chloro-5-morpholino-2-nitroaniline, 4-chloro-2-nitro-5-(piperazin-1-yl)aniline, and 4-chloro-5-methoxy-2-nitroaniline (0.25 mmol) were added. The solution was cooled to 0 °C, and 5% aqueous NaClO solution was added dropwise until the red color disappeared. The mixture was diluted with water (50 mL), and the precipitate was filtered and subjected to silica gel column chromatography (CH_2Cl_2) to give the desired product as a yellow solid.

*5-Chloro-6-(piperidin-1-yl)benzofuroxan (**B4**):* 29 mg; yield 44%; R_f 0.6 (PE/EA 15:1); mp 73−75 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 7.52 (br s, 1H, ArH), 6.67 (br s, 1H, ArH), 3.02 (s, 4H, $-NCH_2-$ × 2), 1.75 (s, 4H, $-CH_2-$ × 2), 1.62 (s, 2H, $-CH_2-$); ^{13}C NMR (101 MHz, $CDCl_3$) δ 53.2 ($-NCH_2-$ × 2), 25.7 ($-CH_2-$ × 2), 23.8 ($-CH_2-$); HRMS (ESI) m/z calcd for $C_{11}H_{12}ClN_3NaO_2$ $[M + Na]^+$ 276.0516, found 276.0515.

*5-Chloro-6-morpholinobenzofuroxan (**B5**):* 52 mg; yield 81%; R_f 0.7 (CH_2Cl_2); mp 126−127 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 7.59 (br s, 1H, ArH), 6.76 (br s, 1H, ArH), 3.90 (d, J = 3.9 Hz, 4H, $-CH_2O-$ × 2), 3.11 (s, 4H, $-NCH_2-$ × 2); ^{13}C NMR (101 MHz, $CDCl_3$) δ 66.4 ($-CH_2O-$ × 2), 52.0 ($-NCH_2-$ × 2); MS (+ESI) m/z 256.6 $[M + H]^+$.

*5-Chloro-6-(piperazin-1-yl)benzofuroxan (**B6**):* 25 mg; yield 40%; R_f 0.6 (CH_2Cl_2); mp 125−127 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 7.58 (br s, 1H, ArH), 6.76 (br s, 1H, ArH), 3.37 (s, 4H, $-NCH_2-$ × 2), 3.24 (s, 4H, $-NCH_2-$ × 2); ^{13}C NMR (101 MHz, $CDCl_3$) δ 61.3 ($-NCH_2-$), 51.7 ($-NCH_2-$); HRMS (ESI) m/z calcd for $C_{10}H_{11}ClN_4O_2$ $[M + H]^+$ 254.0571, found 255.0555.

*5-Chloro-6-methoxybenzofuroxan (**B7**):* 40 mg; yield 80%; R_f 0.6 (PE/EA 5:1); mp 90−91 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 7.55 (br d, J = 82.4 Hz, 1H, ArH), 6.62 (br d, J = 85.2 Hz, 1H, ArH), 3.98 (s, 3H, Me−H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 57.1 (Me−C); MS (+ESI) m/z 223.2 $[M + Na]^+$.

*Synthesis of 4-Fluoro-2-nitro-5-(piperidin-1-yl)aniline (**4a**).* To a solution of 5-chloro-4-fluoro-2-nitroaniline (55 mg, 0.29 mmol) and Et_3N (0.5 mL) was added piperidine (0.5 mL). The reaction mixture was stirred at 100 °C for 24 h. Then the solution was cooled to room temperature and diluted with 10 mL of water. The precipitate was filtered and then subjected to purification by silica gel column chromatography (PE/EA 10:1) to give the desired product as a yellow solid (66 mg, yield 96%): R_f 0.5 (CH_2Cl_2); ^1H NMR (400 MHz, $CDCl_3$) δ 7.73 (d, J = 14.1 Hz, 1H, ArH), 6.08 (s, 2H, NH_2), 6.03 (d, J = 7.7 Hz, 1H, ArH), 3.23−3.18 (m, 4H, $-NCH_2-$ × 2), 1.70 (d, J = 4.7 Hz, 4H, $-NCH_2-$ × 2), 1.63 (d, J = 4.8 Hz, 2H, $-CH_2-$).

*Synthesis of 5-Fluoro-6-(piperidin-1-yl)benzofuroxan (**B8**).* 4-Fluoro-2-nitro-5-(piperidin-1-yl)aniline (**4a**, 62 mg, 0.25 mmol) was dissolved in alcohol (5 mL) with 25% (w/v) KOH. The solution was cooled to 0 °C, and 5% aqueous NaClO solution was added dropwise until the red color disappeared. The mixture was diluted with water (50 mL), and the precipitate was filtered and then subjected to purification by column chromatography (PE/EA 100:1) to give **B8** as a yellow solid (49 mg, yield 80%): R_f 0.8 (PE/EA 5:1); mp 89−91 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 7.07 (br s, 1H, ArH), 6.42 (br s, 1H, ArH), 3.15−3.04 (m, 4H, $-NCH_2-$ × 2), 1.78−1.69 (m, 4H, $-CH_2-$ × 2), 1.67−1.60 (m, 2H, $-CH_2-$); ^{13}C NMR (101 MHz, $CDCl_3$) δ 51.8 ($-NCH_2-$), 51.8 ($-NCH_2-$), 25.6 ($-CH_2-$ × 2), 23.8 ($-CH_2-$); HRMS (ESI) m/z calcd for $C_{11}H_{12}FN_3NaO_2$ $[M + Na]^+$ 260.0811, found 260.0812.

*Synthesis of 6,7-Dichloro-4-nitrobenzofuroxan (**C1**).* A solution of 5,6-dichlorobenzofuroxan (0.450 mg, 2.20 mmol.) in concentrated sulfuric acid (6 mL) was treated with fuming nitric acid (152.2 mg, 2.41 mmol) at 0 °C for 20 min. The solution was then treated with ice−water to form the precipitation as the crude nitro product. This crude product was dissolved in acetic acid (6 mL). The temperature was raised to 110 °C, and the solution was stirred for 3 h. The reaction mixture was extracted with EtOAc (50 mL × 3), and the combined organic layer was dried over anhydrous Na_2SO_4 and concentrated in vacuo. The residue was then subjected to flash chromatography (PE/EA = 30:1) to afford the title compound as a yellow solid (360 mg, 65%): R_f 0.7 (PE/EA 5:1); mp 143−145 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 8.40 (s, 1H, ArH); ^{13}C NMR (101 MHz, acetone) δ 145.7 (ArC), 136.4 (ArC), 134.4 (ArC), 131.1 (ArC), 126.3 (ArC), 115.3 (ArC); MS (+ESI) m/z 250.8 $[M + H]^+$.

*Synthesis of 6-Chloro-4-nitro-7-(pyrrolidin-1-yl)benzofuroxan (**C2**).* To a solution of 6,7-dichloro-4-nitrobenzofuroxan (125 mg, 0.50 mmol) in acetonitrile (3 mL) was added pyrrolidine (106.68 mg, 1.50 mmol). The reaction was stirred for 15 min and then diluted with

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100 mL of water, and the precipitation was filtered to give **C2** as a red solid (75 mg, yield 53%): R_f 0.5 (PE/EA 5:1); mp 119−121 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.03 (s, 1H, ArH), 4.22 (s, 4H, $-NCH_2-\times 2$), 2.06 (s, 4H, $-CH_2-\times 2$); ^{13}C NMR (101 MHz, $CDCl_3$) δ 149.7 (ArC), 141.4 (ArC), 136.3 (ArC), 135.5 (ArC), 120.3 (ArC), 107.1 (ArC), 106.7 (ArC), 55.5 (t, J = 17.3 Hz, $-NCH_2-$), 26.6 − 25.1 (m, $-CH_2-$); HRMS (ESI) m/z calcd for $C_{10}H_9ClN_4NaO_4$ $[M + Na]^+$ 307.0210, found 307.0207.

Synthesis of 6-Chloro-4-nitro-7-(piperazin-1-yl)benzofuroxan. A solution of 6,7-dichloro-4-nitrobenzofuroxan (100 mg, 0.40 mmol) in methyl cyanide (5 mL) was treated with pyrrolidine (172.3 mg, 2.0 mmol). The reaction mixture was stirred for 15 min. The solution was concentrated in vacuo, and the residue was subjected to silica gel column chromatography (CH_2Cl_2/MeOH 100:1 to 20:1) to give the desired product as a red solid (72 mg, yield 60%): R_f 0.2 (CH_2Cl_2/MeOH 10:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.32 (s, 1H, ArH), 3.51 (d, J = 4.6 Hz, 4H, $-NCH_2-\times 2$), 3.10 (s, 4H, $-NCH_2-\times 2$).

*Synthesis of 7-(4-Acetylpiperazin-1-yl)-6-chloro-4-nitrobenzofuroxan (**C3**).* A solution of 6-chloro-4-nitro-7-(piperazin-1-yl)-benzofuroxan (20 mg, 0.07 mmol) in ethanol/pyridine (1:1 v/v, 1 mL) was treated with acetic anhydride (10.22 mg, 0.10 mmol) at room temperature for 15 min. The solution was concentrated in vacuo. and the residue was subjected to silica gel column chromatography (PE/EA = 1:1 to 1:2) to give **C3** as a red solid (14 mg, yield 61%): R_f 0.7 (CH_2Cl_2/MeOH 10:1); mp 163−165 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.29 (s, 1H, ArH), 3.83 (s, 2H, $-NCH_2-$), 3.73−3.69 (m, 2H, $-NCH_2-$), 3.52−3.47 (m, 4H, $-NCH_2-\times 2$), 2.16 (s, 3H, Me−H); ^{13}C NMR (75 MHz, DMSO) δ 168.5 (−C═O), 146.3 (ArC), 141.7 (ArC), 137.6 (ArC), 127.4 (ArC), 114.9 (ArC), 114.2 (ArC), 51.9 ($-NCH_2-$), 51.5 ($-NCH_2-$), 46.3 ($-NCH_2-$), 41.4 ($-NCH_2-$), 21.3 (Me−C); HRMS (ESI) m/z calcd for $C_{12}H_{12}ClN_5NaO_5$ $[M + Na]^+$ 364.0425, found 364.0424.

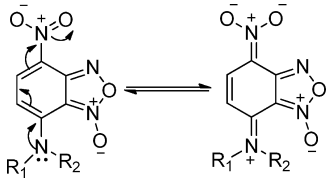
*Synthesis of 6-Chloro-7-(4-(ethoxycarbonyl)piperazin-1-yl)-4-nitrobenzofuroxan (**C4**).* A solution of 6-chloro-4-nitro-7-(piperazin-1-yl)benzofuroxan (20 mg, 0.07 mmol) in CH_2Cl_2 (2 mL) was treated with ethyl carbonochloridate (10.9 mg, 0.10 mmol) at room temperature for 15 min. The solution was concentrated in vacuo, and the residue was subjected to silica gel column chromatography (PE/EA = 1:1 to 1:2) to give **C4** as a red solid (16 mg, yield 65%): R_f 0.8 (CH_2Cl_2/MeOH 10:1); mp 167−169 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.31 (s, 1H, ArH), 4.21−4.16 (m, 2H, $-CH_2O-$), 3.71 (d, J = 5.0 Hz, 4H, $-NCH_2-\times 2$), 3.48 (s, 4H, $-NCH_2-\times 2$), 1.29 (t, J = 7.1 Hz, 3H, Me−H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.3 (−C═O), 145.7 (ArC), 141.2 (ArC), 137.2 (ArC), 133.0 (ArC), 128.8 (ArC), 116.4 (ArC), 113.4 (ArC), 61.9 ($-NCH_2-$), 52.1 ($-NCH_2-$), 51.2 ($-NCH_2-$), 44.1 ($-NCH_2-$), 14.7 (Me−C); HRMS (ESI) m/z calcd for $C_{13}H_{14}ClN_5NaO_6$ $[M + Na]^+$ 394.0530, found 394.0541.

RESULTS AND DISCUSSION

Chemistry. The synthetic routes for target molecules **A1**−**A8**, **B1**−**B8**, and **C1**−**C4** are shown in Schemes 1−3. Compounds **1**−**5** were the key intermediates, which were prepared according to reported procedures.[14−16] Nucleophilic substitution reaction of compound **1** with nucleophiles such as sodium ethoxide, sodium methanethiolate, piperazine, and paratoluidine afforded the target molecules **A1**−**A4**. On the other hand, treatment of **A3** with anhydride or acyl chloride under basic condition gave compounds **A5**−**A8** in moderate to good yields. It is worth mentioning that the ^{1}H NMR spectra of compounds **A3**−**A8** showed the coexistence of two sets of signals, which comes from the resonance forms for these benzofuroxan derivatives (Scheme 4).

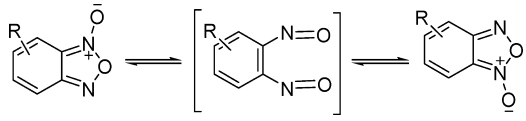
The synthesis of target molecules **B1**−**B8** is outlined in Scheme 2. Reaction of **2** with sodium hypochlorite in methanol gave **B1** in 70% yield, which was then treated with NBS to introduce a bromine atom at the benzylic position, affording **B2** in 52% yield. Subsequent substitution reaction between **B2** and pyrrolidine provided **B3** in 73% yield. Nucleophilic substitution

Scheme 4. Two Resonance Structures for Compounds A3−A8



reaction of **3** with corresponding amine or sodium methoxide gave **B4′**−**B7′** in >90% yields, in which only a C5-substituted product was observed on the basis of the ^{1}H NMR assignment. Again, the formation of the benzofuroxan was accomplished by treatment of **B4′**−**B7′** with sodium hypochlorite, affording **B4**−**B7**. Common features of the ^{1}H NMR spectra of compounds **B1**−**B8** were that both hydrogen signals of the phenyl ring were broad peaks. Furthermore, the ^{13}C NMR signals of their aromatic carbon could barely be observed. This phenomenon was reported in other benzofuroxan derivatives as well.[17,18] This was due to the fast tautomeric equilibrium between the two benzofuroxan tautomers of benzofuroxan via *o*-dinitroso intermediates (Scheme 5), which caused the

Scheme 5. Rearrangement Occurring in Benzofuroxan



coalescence of the individual signals into a very broad multiplet. High-concentration (200 mg/mL in $CDCl_3$) ^{13}C NMR was attempted for **B1**, and again, the aromatic carbons originated some broad multiplets (**B1**: δ 140.0, 116.1, 112.4) because of the very fast tautomeric equilibrium.

Compound **C1** was prepared by reaction of **5** with fuming nitric acid in concentrated sulfuric acid, which gave 6-dichloro-4-nitrobenzofuroxan first. Refluxing of this nitro product in acetic acid for 3 h caused rearrangement to give the more stable **C1**. Nucleophilic substitution reaction of **C1** with pyrrolidine or piperazine afforded **C2** and **6**. Intermediate **6** is unstable and could quickly decompose upon exposure to air. Therefore, **6** was immediately treated with acetic anhydride or ethyl carbonochloridate to gave target molecules **C3** and **C4**, respectively, which were stable. The remaining compounds listed in Tables 1−3 were prepared according to the literature reported methods.[19−27] Before submission for biological evaluation, all compounds were analyzed by high-pressure liquid chromatography to ensure >95% purity.

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

For series **A** benzofuroxan derivatives, all compounds with amino substitution at the nitro para-position exhibited antifungal effect with IC_{50} < 25 μg/mL. Specifically, compound **A9** displayed very good fungicidal activity against *R. solani* and *F. graminearum* Sehw. with IC_{50} values of 3.02 and 2.28 μg/mL, respectively. **A5** gave IC_{50} of 1.1 μg/mL against *F. graminearum* Sehw., which was half-fold less active than the positive control carbendazim (IC_{50} = 0.5 μg/mL), but had no antifungal effects

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on other fungal pathogens (IC_{50} > 25 μg/mL). With the pyrrolidine substitution at the R^1 position, **A14** was found to show high antifungal activity against *S. sclerotiorum* and *F. graminearum* Sehw. with IC_{50} = 2.52 and 3.42 μg/mL, respectively. Although these three compounds are the most potent derivatives among the **A** series, their IC_{50} values were similar to that of the positive control carbendazim and lower than that of another positive control, chlorothalonil. All other compounds in this series of benzofuraxan derivatives show moderate to weak antifungal activities, as illustrated by their high IC_{50} values. In addition, the following five compounds show >25 μg/mL IC_{50} values against all four tested fungi phytopathogens: **A1**, **A12**, **A13**, **A16**, and **A18**, with methoxyl, phenylsulfonyl, benzylmethiolate, ethoxyl, and phenyl amino groups at the R^1 position, respectively. These findings indicated that the presence of an amino group at the R^1 position and formation of resonance-stabilized structures may be essential for the antifungal activity of type **A** benzofuraxan derivatives. Meanwhile, in general, this series of compounds have demonstrated better fungicidal activity against *R. solani* and *F. graminearum* Sehw. as compared to the other two fungi phytopathogens, *S. sclerotiorum* and *P. capsici*.

In the case of series **B** benzofuraxan derivatives, the substituent varies at the R^2 and R^3 positions, whereas both R^1 and R^4 positions are hydrogen atoms. **B6** and **B10**, with piperazyl group at the R^2 position, displayed >25 μg/mL IC_{50} values against all four fungi phytopathogens. The other compounds exhibited high potency only against *R. solani*. Especially, **B4** (IC_{50} = 1.81 μg/mL) and **B8** (IC_{50} = 1.76 μg/mL) showed inhibitory potency similar to that of carbendazim (IC_{50} = 1.42 μg/mL). Significantly, all compound IC_{50} values are <8.0 μg/mL. These results suggest that benzofuroxan derivatives with variation at the phenyl R^2 and R^3 positions may serve as new leads for the development of potentially useful antifungal agents against *R. solani*. In contrast, the series **B** derivatives did not generally exhibit good antifungal activity against the other three plant phytopathogens. However, **B2** showed a low IC_{50} value against all fungus strains. The presence of a benzyl bromide in **B2** may be the reason for it to act as a universal nucleophilic attack acceptor to exert its cytotoxicity against plant fungi.

Most series **C** benzofuroxan derivatives showed weak antifungal activity, as illustrated with their higher than 25 μg/mL IC_{50} values. Replacement of a hydrogen atom at the R^2 position with a chlorine atom leads to the IC_{50} value increase from 2.52 μg/mL (for **A14**) to >25 μg/mL (for **C2**) against *S. sclerotiorum* and *F. graminearum* Sehw. This suggested that the introduction of an electron-withdrawing group at the R^2 position is unfavorable for the compound antifungal activity toward the tested phytopathgenic fungi; the same trend was confirmed with **C3** (IC_{50} > 25 μg/mL) versus **A5** (IC_{50} = 1.10 μg/mL) or **C4** (IC_{50} > 25 μg/mL) versus **A19** (IC_{50} = 9.15 μg/mL) against *F. graminearum* Sehw. Substitution of the R^4 nitro group with methanesulfonyl or benzenesulfonyl caused significant loss of fungicidal activity, **C5**, **C6**, and **C7** all having >25 μg/mL IC_{50} values, which indicates that the strongly electron-withdrawing and bulky sulfonyl group at the R^4 position appeared to be less favorable for the antifungal activity. The remaining four compounds, **C1**, **C8**, **C9**, and **C10**, displayed >25 μg/mL IC_{50} values. Overall, the low activity of series **C** benzofuroxan derivatives suggested that the presence of more than two electron-withdrawing groups on the phenyl ring would cause antifungal activity decrease.

In Vivo Testing on Cole Leaves Infected by *S. sclerotiorum*. Among the compounds tested for antifungal activity in vitro, **A14** and **A15** were found to be potent against *S. sclerotiorum*. Therefore, they were further evaluated in the greenhouse for the control of *S. sclerotiorum* infected cole. The efficacy of the treatment is shown in Figure 2. The untreated negative control (pathogen only) resulted in 100% disease incidence (0% healthy plant standard) 24 h after transplantation. Treatment with 0.5 mg/mL benzofuroxan derivative **A14** resulted in 46.1% healthy plant standard after 36 h of treatment. Accordingly, **A15** resulted in 11.9% disease incidence (Table 4). In contrast, leaves treated with carbendazim at the same concentration resulted in 71.1% healthy plant standard. Thus, significant differences existed among the treated and untreated groups for disease control experiments in the greenhouse cole leaves.

Table 4. In Vivo Efficacy of 500 μg/mL Compounds on Cole Leaves Infected by *Sclerotinia sclerotiorum*[a]

	diameter of lesions (mm)	protection efficacy (%)
A14	9.0 ± 0.8	46.1
A15	14.8 ± 1.3	11.9
carbendazim	4.9 ± 0.5	71.1
negative control	16.8 ± 1.7	

[a]Statistical analysis of the data was performed by analysis of variance (one-way ANOVA). A probability value of $p \leq 0.05$ was considered to denote a statistically significance difference.

In conclusion, we have synthesized a series of benzofuroxan derivatives and evaluated their inhibitory ability against four phytopathogenic fungi. Most compounds displayed significant antifungal activity against certain strains of phytopathogenic fungi. Significantly, the antifungal activity of **A5** was half-fold that of the well-known commercial fungicide carbendazim and was identified as the most potent compound against *F. graminearum* Sehw. **B8** displayed strong antifungal activity against *R. solani* with an IC_{50} value of 1.76 μg/mL, which is comparable with that of carbendazim (1.42 μg/mL). **A14** was found to have the lowest IC_{50} (2.52 μg/mL) against *S. sclerotiorum*. However, among the benzofuroxan derivatives reported herein, none was found to possess strong antifungal activity against *P. capsici*.

Overall, the series **B** compounds, with substituents at the R^2 and R^3 positions of the phenyl ring, exhibited strong fungicidal potency against *R. solani*. Several series **A** benzofuroxan derivatives were revealed to display strong antifungal effect against *F. graminearum* Sehw. Additionally, **A9** was the only compound that displayed high potency and selectivity to both *R. solani* and *F. graminearum* Sehw. The benzofuroxan structural scaffold with variations at different phenyl positions could be further explored for the discovery of more potent antifungal agents against different pyhtopathogenic fungi.

■ ASSOCIATED CONTENT

Supporting Information

^{1}H NMR, ^{13}C NMR, and HRMS spectra of new synthetic compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

REFERENCES

(1) Wilson, R. A.; Talbot, N. J. Fungal physiology − a future perspective. *Microbiology (Reading, U.K.)* **2009**, *155*, 3810−3815.

(2) Solomon, S. P. Assessing the mycotoxigenic threat of necrotrophic pathogens of wheat. *Mycotoxin Res.* **2011**, *27*, 231−237.

(3) Woloshuk, P. C.; Shim, B. W. Aflatoxins, fumonisins, and trichothecenes: a convergence of knowledge. *FEMS Microbiol. Rev.* **2013**, *37*, 94−109.

(4) Shima, Y.; Ito, Y.; Hatabayashi, H.; Koma, A.; Yabe, K. Five carboxin-resistant mutants exhibited various responses to carboxin and related fungicides. *Biosci., Biotechnol., Biochem.* **2011**, *75*, 181−184.

(5) Dodds, P. N.; Rathjen, J. P. Plant immunity: Towards an integrated view of plant pathogen interactions. *Nat. Rev. Genet.* **2010**, *11*, 539−548.

(6) Cerecetto, H.; Porcal, W. Pharmacological properties of furoxans and benzofuroxans: recent developments. *Mini-Rev. Med. Chem.* **2005**, *5*, 57−71.

(7) Cerecetto, H.; González, M. Benzofuroxan and furoxan. chemistry and biology. *Top. Heterocycl. Chem.* **2007**, *10*, 265−308.

(8) Sartini, S.; Cosconati, S.; Marinelli, L.; Barresi, E.; Maro, D. S.; Simorini, F.; Taliani, S.; Salerno, S.; Marini, M. A.; Settimo, D. F.; Novellino, E.; Motta, L. C. Benzofuroxane derivatives as multi-effective agents for the treatment of cardiovascular diabetic complications. synthesis, functional evaluation, and molecular modeling studies. *J. Med. Chem.* **2012**, *55*, 10523−10531.

(9) Schiefer, T. I.; VandeVrede, L.; Fa', M.; Arancio, O.; Thatcher, R. J. G. Furoxans (1,2,5-oxadiazole-*N*-oxides) as novel NO mimetic neuroprotective and procognitive agents. *J. Med. Chem.* **2012**, *55*, 3076−3087.

(10) Hwang, J. K.; Park, C. Y.; Kim, J. H.; Lee, H. J. Synthesis and antifungal activities of furoxan designed as novel fungicide. *Biosci., Biotechnol., Biochem.* **1998**, *62* (9), 1693−1697.

(11) Sayed, A. A.; Simeonov, A.; Thomas, C. J.; Inglese, J.; Austin, C. P.; Williams, D. L. Identification of oxadiazoles as new drug leads for the control of schistosomiasis. *Nat. Med.* **2008**, *14*, 407−412.

(12) Yusupova, L. M.; Garmonov, Y. S.; Zakharov, M. I.; Bykov, R. A.; Falyakhov, F. I.; Garipov, V. T. Fungicidal and toxicological properties of functionally substituted nitrobenzofuroxans. *Pharm. Chem. J.* **2008**, *42* (4), 183−185.

(13) Xiao, Y.; Li, H. X.; Li, C.; Wang, J. X.; Li, J.; Wang, M. H.; Ye, Y. H. Antifungal screening of endophytic fungi from *Ginkgo biloba* for discovery of potent anti-phytopathogenic fungicides. *FEMS Microbiol. Lett.* **2013**, *339*, 130−136.

(14) Boulton, J. A.; Gripper Gray, C. A.; Katritzky, R. A. Heterocyclic rearrangements. Part IV: furazanobenzofuroxan. *J. Chem. Soc.* **1965**, 5958−5964.

(15) Dixon, B. R. Preparation of flavin derivatives to be used as riboswitch ligands or anti-infective agents. PCT Int. Appl. WO 2010019208 A1 20100218, 2010.

(16) Sheremetev, A. B.; Aleksandrova, N. S.; Ignat'ev, N. V.; Schulte, M. Straightforward one-pot synthesis of benzofuroxans from o-halonitrobenzenes in ionic liquids. *Mendeleev Commun.* **2012**, *22*, 95−97.

(17) de Carvalho, P. S.; Marostica, M.; Gambero, A.; Pedrazzoli, J. Synthesis and pharmacological characterization of a novel nitric oxide-releasing diclofenac derivative containing a benzofuroxan moiety. *Eur. J. Med. Chem.* **2010**, *45*, 2489−2493.

(18) Gasco, A.; Boulton, A. J. Furoxans and benzofuroxans. *Adv. Heterocycl. Chem.* **1981**, *29*, 251−340.

(19) Huo, H.; Wang, B. O.; Liu, Q.; Luo, Y. F.; Zhang, Y. G. Synthesis and characterizat ion of 7-chloro-4,6-dinitro-benzofuroxans. *Chin. J. Explos. Propellants* **2009**, *32* (4), 31−33.

(20) Belton, G. J.; McElhinney, S. R. Nucleophilic attack on 4,7-disubstituted benzofurazans and their *N*-oxides: synthesis of tetrazolo-[1,5-*a*]azepines. *J. Chem. Soc., Perkin Trans. 1* **1988**, *2*, 145−150.

(21) Belton, J. G. Novel N→S oxygen migration in 2,1,3-benzoxadiazole systems. *Proc. R. Irish Acad., Sect. B: Biol., Geol. Chem. Sci.* **1974**, *74* (14), 185−192.

(22) Edwards, R.; Cummins, I.; Steel, P. Method for overcoming multiple herbicide resistance in weeds with benzodiazoles. PCT Int. Appl. WO 2009034396 A2 20090319, 2009.

(23) Wang, B. Z.; Huo, H.; Li, J. Z.; Fan, X. Z.; Liu, Q. Synthesis and characterization of 4,6-dinitro-5,7-diamino benzenfuroxan (CL-14). *Chin. J. Org. Chem.* **2011**, *31* (1), 132−135.

(24) Kotovskaya, K. S.; Romanova, A. S.; Charushin, N. V.; Kodess, I. M.; Chupakhin, N. O. 5(6)-Fluoro-6(5)-*R*-benzofuroxans: synthesis and NMR ^{1}H, ^{13}C and ^{19}F studies. *J. Fluorine Chem.* **2004**, *125*, 421−428.

(25) El-Abadelah, M. M.; Nazer, Z. M.; El-Abadla, S. N.; Awadallah, M. A. Synthesis and properties of some new 5-fluoro-6-(heterocyclyl) benzofuroxans. *Asian J. Chem.* **1999**, *11* (4), 1463−1468.

(26) Kotovskaya, K. S.; Romanova, A. S.; Charushin, N. V.; Kodess, I. M. Fluorine-containing heterocycles: XI. 5(6)-Fluoro-6(5)-X-benzofuroxans: synthesis, tautomerism, and transformations. *Russ. J. Org. Chem.* **2004**, *40* (8), 1167−1174.

(27) Belton, G. J.; Conalty, L. M.; O'Sullivan, F. J. Anticancer agents. XI. antitumor activity of 4-amino-7-nitrobenzofuroxans and related compounds. *Proc. R. Soc. London, Ser. B.* **1976**, *76* (9), 133−149.