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Synthesis and antifungal activity of novel sulfoxide derivatives containing trimethoxyphenyl substituted 1,3,4-thiadiazole and 1,3,4-oxadiazole moiety

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Abstract—Selective oxidation of sulfides **7** or **8** to sulfoxides **9** or **10** is achieved by *m*CPBA. The structures of the compounds **9** or **10** are confirmed by elemental analysis, IR, and ^{1}H NMR. The bioassay results showed that title compound **10a** possess high antifungal activities with EC_{50} values ranging from 19.91 to 63.97 μg/mL. The mechanism of action of **10a** against *Sclerotinia sclerotiorum* was studied. After treating with compound **10a** at 100 μg/mL for 12 h, the mycelial reducing sugar, D-GlcNAc, soluble protein and pyruvate content, chitinase activity showed declining tendency.

1. Introduction

Sulfoxide derivatives represent one of the most active class of compounds possessing a wide spectrum of biological activity. They are widely used in pharmaceuticals and agrochemicals,[1–4,10] as fungicide,[5,6] herbicide,[7] and antitumor agent.[8,9] Some compounds with broad spectrum of bioactivity have been commercialized, for example, Fipronil which is highly effective against *Myzus persicae*, *Empoasca*, *larvae of Lepidoptera*, *Musca domestica*, and *Hymenoptera pests*.[10] Thus, their synthesis has been of great interest in the elaboration of biologically active heterocyclic compounds. Recently, we reported the fungicidal activity of novel sulfone derivatives, some of which were found to possess good fungicidal bioactivity.[11]

Fascinated by these findings and in an attempt to evaluate the modification of the fungicidal profile induced by the change of the substituents at the alkyl group, we designed and synthesized a series of sulfoxide derivatives with various substituents and measured their fungicidal activities. The synthetic route is shown in Scheme 1. Starting from the key intermediate 5-(3,4,5-trimethoxyphenyl)-1,3,4-thiadiazole-2-thiol (**4**) or the oxadiazole analogue (**5**), the title compounds **9** and **10** were synthesized in two steps. Thioetherification reaction of **4** or **5** with organohalide catalyzed by indium or indium tribromide first affords appropriate sulfides **7** or **8**, which were then converted into title compounds **9** or **10** by *m*CPBA oxidation in dichloromethane. The structures of **9** and **10** were firmly established by well-defined IR, ^{1}H NMR, ^{13}C NMR, and elemental analysis. Preliminary bioassay tests showed that some compounds possess certain degree of antifungal activity against three phytopathogenic fungi at 50 mg/L in vitro as shown in Table 5, however, with a degree of variation. The bioassay results showed that title compound **10a** possessed high antifungal activities against nine kinds of fungi and the EC_{50} values ranging from 19.91 to 63.97 μg/mL. After

Abbreviations: DCM, dichloromethane; DCE, 1,2-dichloroethane; [bmim]PF_6, 1-butyl-3-methylimidazolium hexafluorophosphate; ^{13}C NMR, ^{13}C nuclear magnetic resonance; *m*CPBA, *meta*-chloroperoxybenzoic acid; IR, infra-red; ^{1}H NMR, ^{1}H nuclear magnetic resonance; PDA, potato dextrose agar culture; DNS, 3,5-dinitrosalicylic acid; DMAB, 3,2′-dimethyl-4-aminobiphenyl; EC_{50}, 50% effective concentration; LDH, lactate dehydrogenase; *G. zeae*, *Gibberella zeae*; *C. mandshurica*, *Cytospora mandshurica*; *F. oxysporium*, *Fusarium oxysporium*; *P. infestans*, *Phytophthora infestans*; *R. solani*, *Rhizoctonia solani*; *T. cucumeris*, *Thanatephorus cucumeris*; *C. gloeosporioides*, *Colletotrichum gloeosporioides*; *B. cinerea*, *Botrytis cinerea*; *S. sclerotiorum*, *Sclerotinia sclerotiorum*.

Keywords: Sulfoxides; Thioether; Antifungal bioactivity; Mechanism of action.

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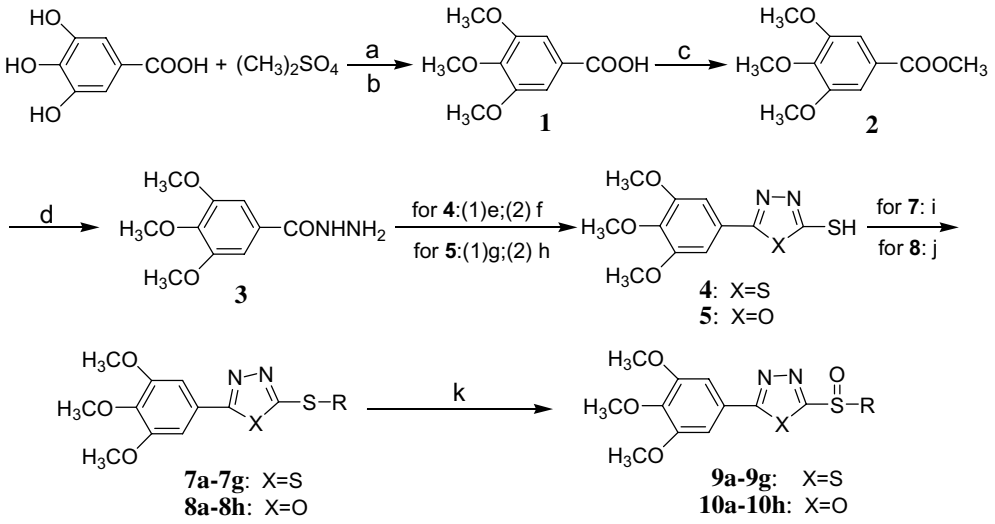


Scheme 1. Synthetic route to title compounds **9** and **10**. Reagents and conditions: (a) $(CH_3)_2SO_4$, 10% NaOH; (b) 35% HCl; (c) CH_3OH, 98% H_2SO_4, reflux; (d) $NH_2NH_2{\cdot}H_2O$, CH_3OH, reflux for 5 h; (e) KOH, CS_2, C_2H_5OH, rt; (f) 98% H_2SO_4, 0–5 °C; (g) KOH, CS_2, C_2H_5OH, reflux for 6 h; (h) 5% HCl, ice-bath; (i) In, 3% NaOH, H_2O, RX **(6)**, rt; (j) $InBr_3$, 3% NaOH, H_2O, RX **(6)**, rt; (k) *m*CPBA, dichloromethane, 4 °C, 2 h and then rt, 5 h.

treating *Sclerotinia sclerotiorum* with compound **10a** at 100 μg/mL, only 5.4% of its spore bourgeoned, the permeability of the cell membrane increased and most of its hyphal became distorted, malformed, and got entangled.

2. Results and discussion

2.1. Chemistry

The synthetic route designed for the sulfoxide analogues **9** and **10** is summarized in Scheme 1. Following the reported method,[12] 5-(3,4,5-trimethoxyphenyl)-1,3,4-thiadiazole-2-thiol **4** was synthesized from gallic acid in five steps including etherification, esterification, hydrazidation, salt formation, and cyclization. 5-(3,4,5-Trimethoxyphenyl)-1,3,4-oxadiazole-2-thiol **5** was easily prepared by the reaction of 5-trimethoxyphenylhydride **3**, potassium hydroxide, and carbon disulfide in water-free ethanol under reflux condition. Then, 5-(3,4,5-trimethoxyphenyl)-1,3,4-thiadiazole-2-thiol **4** and the oxadiazole analogue **5** were converted to thioether derivatives containing thiadiazole **7** or oxadiazole moiety **8** by a thioetherification reaction with halide **(RX)** catalyzed by indium or indium tribromide. Treatment of sulfides **7** and **8** with *m*CPBA in dichloromethane afforded the heterocyclic sulfoxides **9** and **10** with moderate yields.[13–15]

Recently, the oxidation of sulfides to sulfoxides by hydrogen peroxide or *m*CPBA has proved to be traditional methods. Over the past few years, the importance of hydrogen peroxide and its derivatives as oxidizing agents has become an emerging part recently. In contrast to other commonly employed oxidizing agents, for example, hypochlorite, hypobromite, potassium permanganate, and potassium chromate, hydrogen peroxide is easy to handle, store, transport, and is relatively cheap. Also, Hydrogen peroxide is a kind of pro-nuclear oxidant and the oxidation of sulfides to sulfoxides without catalysts is generally slow. So a number of new, atom-efficient and waste-free methods for sulfoxide synthesis are known and numerous methods for sulfide oxidation by H_2O_2 in the presence of transition metals as catalysts have been developed. In order to oxidize sulfide to its sulfoxide selectively, we need to select suitable catalyst to prevent sulfoxide from overoxidizing to sulfone. The synthesis of **10a** was carried out under several different synthesis methods, and the results are summarized in Table 1.

As could be observed from Table 1 that the oxidation of sulfides to sulfoxides by hydrogen peroxide or potassium periodate has proved to be unsuccessful methods. The oxidation of sulfides to sulfoxides by *m*CPBA as oxidant and dichloromethane as solvents has proved to be the best oxidation method. So we tested this method.

In order to optimize the reaction conditions for the preparation of **9** and **10**, the synthesis of **9a** was carried out under several different conditions. We took *m*CPBA as the oxidant and varied the reaction conditions. The effects of reaction time, reaction temperature, and oxidant amount on the reaction were investigated, and the results are summarized in Table 2.

As can be seen from Table 2, treatment of **7a** with 1.1 equiv of *m*CPBA (with respect to the substrate) at 4 °C for 2 h followed by warming to room temperature and stirring for another 5 h gave the highest yield (43.7%). Further investigation indicated that the addition of *m*CPBA in excess to 1.1 equiv resulted in the formation of sulfone. On the other hand, addition of less than 1.1 equiv of *m*CPBA led to poor conversion of the substrate into product **9a**. Addition of different catalysts in the reaction mixture had little or negligible effect in terms of improving the product yield. Under the optimized conditions as described above, different sulfides were converted by *m*CPBA oxidation into their corresponding sulfoxides and the results are depicted in Tables 3 and 4.

Table 1. The effect of yield in different methods

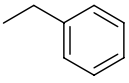
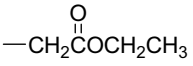
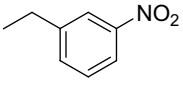
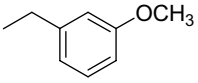
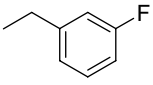
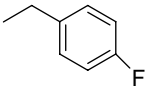
Entry	Oxidant (mmol)	Catalyst (mmol)	Temperature (°C)	Reaction time (h)	Solvents	Yield (%)
1	KIO_4 (1)	—	0	7	Methanol	0
2	H_2O_2 (5)	Acetic acid (5)	0	12	DCE	0
3	H_2O_2 (10)	$InBr_3$ (10%)	19	30	DCM	0
4	H_2O_2 (1.5)	$(NH_4)_6Mo_7O_{24}$ (2%) and [bmim]PF_6 (20)	10	2	Ethanol	0
5	H_2O_2 (2)	Lactic acid (20%) and [bmim]PF_6 (20)	20	16	DCM	0
6	*m*CPBA (1.1)	—	4	2	DCM	17.4

Table 2. The yield of **9a** under different reaction conditions

Entry	*m*CPBA (mmol)	Reaction temperatures (°C)	Reaction times (h)	Yield (%)
1	1.0	−10	0.5	0
2	1.0	0	0.5	8.7
3	1.0	4	0.5	12.4
4	1.0	20	0.5	10.9
5	1.1	4	0.5	24.6
6	1.1	4	1	31.5
7	1.1	4	2	38.4
8	1.1	4	3	38.0
9[a]	1.1	4	2 + 5	43.7
10[a]	1.2	4	2 + 5	36.6
11[a]	1.5	4	2 + 5	27.0

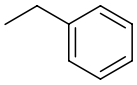
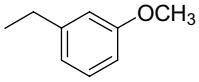
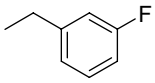
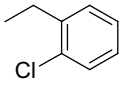
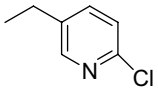
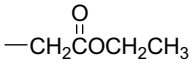
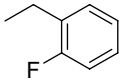
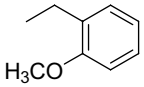
[a] Reaction was conducted at 4 °C for 2 h, and then at room temperature for 5 h.

Table 3. Oxidation of thiadiazole sulfides to the corresponding product

R	Product	Yield (%) (mp °C)
	**9a**	43.7 (106–108)
	**9b**	44.9 (124–126)
	**9c**	42.1 (168–170)
	**9d**	50.4 (123–125)
	**9e**	50.4 (116–118)
	**9f**	50.4 (133–134)
$-CH_2CH_2OCH_2CH_3$	**9g**	51.5 (77–79)

As could be observed from Tables 3 and 4 that the alkyl sulfide is, however, more easily oxidized to the corresponding sulfoxide as compared to the aralkyl sulfides. The yield was low with *p*-fluorobenzyl sulfide due to easy over oxidation into sulfone. In order to avoid side product and over oxidation of the sulfide to sulfone, we preferred to settle for a relatively lower yield of the product. It should be noted, however, that due to their differences in polarity, it is easy to remove sulfone from sulfoxide. Thus, a wide range of aromatic, aliphatic, and

Table 4. Oxidation of oxadiazole sulfides to the corresponding products

R	Product	Yield (%) (mp °C)
	**10a**	42.0 (126–128)
	**10b**	47.30 (103–105)
	**10c**	51.0 (121–123)
	**10d**	46.0 (128–130)
	**10e**	48.5 (147–149)
	**9f**	45.3 (99–101)
	**10g**	42.4 (154–156)
	**10h**	39.8 (134–136)

heterocyclic sulfides with 1,3,4-oxadiazole or 1,3,4-thiadiazole moiety were converted by this procedure into their corresponding sulfoxides in moderate yields.

2.2. Antifungal activity and structure–activity relationship

Three fungi, for example, *Gibberella zeae*, *Fusarium oxysporium*, and *Cytospora mandshurica* were employed in the fungicidal bioassay using mycelial growth rate method.[16] The results of preliminary bioassays were compared with that of a commercial agricultural fungicide, Hymexazol. As indicated in Table 5, most of the synthesized compounds showed certain antifungal activities against the tested fungi. The data provided in Table 5 indicate that introduction of 1,3,4-oxadiazole in sulfoxide might improve their antifungal activities. The interesting thing is the conversion of the 1,3,4-oxadiazole in sulfoxides **10a**–**10h** into 1,3,4-thiadiazole in sulfoxides **9a**–**9g** weakened their antifungal activities. Among these compounds containing 1,3,4-oxadiazole moiety, **10a** (R = benzyl) is much more active against *G. zeae*, *F. oxysporum*, and *C. mandshurica* than the other ones **10b**–**10h** (R = 3-methoxyben-

Table 5. Inhibition[a] effect of sulfoxides derivatives at 50 μg/mL against phytopathogenic fungi

Compound	Inhibition rate[a] (%)		
	G. zeae	*F. oxysporum*	*C. mandshurica*
Hymexazol[b]	53.68 ± 1.03	52.11 ± 0.97	52.02 ± 1.12
9a	26.88 ± 1.33	15.54 ± 0.97	15.99 ± 1.00
9b	34.83 ± 0.89	4.58 ± 1.17[c]	8.42 ± 0.85
9c	5.45 ± 1.09	3.31 ± 0.85	6.63 ± 1.14
9d	8.29 ± 0.67	8.65 ± 1.02	10.97 ± 0.87
9e	14.93 ± 0.76	11.70 ± 0.87	11.99 ± 1.13
9f	6.40 ± 0.72	2.54 ± 0.77	3.83 ± 0.99
9g	16.82 ± 1.17	8.14 ± 0.87	16.07 ± 0.75
10a	53.26 ± 2.21	52.28 ± 1.46	56.09 ± 1.43
10b	37.69 ± 1.46	30.50 ± 1.94	25.71 ± 0.94
10c	7.29 ± 1.17	−0.88 ± 1.27	−0.31 ± 0.79
10d	7.79 ± 1.21	7.04 ± 0.86	−0.33 ± 0.88
10e	31.91 ± 1.44	14.95 ± 1.04	11.91 ± 1.26
10f	18.09 ± 1.37	1.47 ± 1.08	6.90 ± 1.16
10g	11.31 ± 1.24	4.40 ± 0.63	2.82 ± 1.03
10h	29.90 ± 1.50	32.26 ± 1.07	18.18 ± 1.02

[a] Average of three replicates.
[b] The commercial agricultural fungicide, Hymexazol was used for the comparison of activity.
[c] The values were estimated statistically by SPSS 11.5 software using a personal computer.

zyl, 3-fluorobenzyl, 2-chlorobenzyl, (2-chloropyridine-5-yl)methyl, 2-ethoxycarbonylmethyl, 2-fluoro-benzyl, 2-methoxybenzyl). Compound **10a** at 50 μg/mL inhibited the growth of *G. zeae*, *F. oxysporum*, and *C. mandshurica* at 53.26%, 52.28%, and 56.09%, respectively, which is close to that of Hymexozole (53.68% against *G. zeae*, 52.11% against *F. oxysporum*, 59.02 % against *C. mandshuric* at 50 μg/mL).

Further bioassays disclosed that compound **10a** showed remarkable inhibitory effect on nine kinds of plant pathogenic fungi. The EC_{50} of compound **10a** on *G. zeae*, *F. oxysporum*, *C. mandshurica*, *Rhizoctonia solani*, *Thanatephorus cucumeris*, *Phytophthora infestans*, *S. sclerotiorum*, *Botrytis cinerea*, *Colletotrichum gloeosporioides*, were 28.84 μg/mL, 58.48 μg/mL, 63.97 μg/mL, 44.67 μg/mL, 23.44 μg/mL, 33.11 μg/mL, 21.63 μg/mL, 19.91 μg/mL, 39.45 μg/mL, respectively (Table 6). Compound **10a** had more potent antifungal activities against most of the tested fungi, and showed a broad-spectrum bioactivity.

2.2.1. The morphology changes of hyphal. As shown in Figure 1, we can find that the hyphal of the control was slippy, vimineous, and branched normally. The endosome of the cell distributed evenly. But after treatment of *S. sclerotiorum* with compound **10a** at the concentration of 100 μg/mL for 24 h, most of its hyphal became distorted, malformed, and got entangled. In addition, the cell of the hypha swelled and some of its endosome condensed, leaked out, and formed blanks (Fig. 1).

2.2.2. Effect of compound 10a on sporule germination of *S. sclerotiorum*. From Figure 2 we can observe that when *S. sclerotiorum* was treated with **10a** and a commercial agricultural fungicide—Hymexazol, the sporule germination ratio (%) showed declining tendency with the increase of the concentration. When the concentration was lower than 40 μg/mL, the sporule germination ratio with **10a** treatment was found to be higher than that treated by Hymexazol. On the other hand, when the concentration was higher than 40 μg/mL, the sporule germination ratio with **10a** treatment was lower than that of the Hymexazol. At 80 μg/mL concentration, the ratio was 12.6% and showed a more rapid decreasing tendency as compared to that observed with Hymexazol treatment. When the concentration reached 100 μg/mL, the sporule germination ratio of the treated was 5.4%, 39.3% lower than that obtained with Hymexazol treatment (Fig. 2).

Table 6. Toxicity of **10a** on nine kinds of fungi

Compound	Fungi	Toxic regression equation[a]	EC_{50} (μg/mL)[a]	*r*
10a	*G. zeae*	$Y = 1.81x + 2.36$	28.84 ± 5.6	0.991
	F. oxysporum	$Y = 1.59x + 2.19$	58.48 ± 7.1	0.989
	C. mandshurica	$Y = 0.98x + 3.23$	63.97 ± 4.9	0.996
	R. solani	$Y = 1.65x + 2.28$	44.67 ± 5.9	0.966
	T. cucumeris	$Y = 2.43x + 1.67$	23.44 ± 7.7	0.983
	P. infestans	$Y = 1.43x + 2.82$	33.11 ± 3.5	0.965
	B. cinerea	$Y = 2.75x + 1.33$	21.63 ± 4.3	0.978
	S. sclerotiorum	$Y = 1.67x + 2.83$	19.91 ± 3.3	0.991
	C. gloeosporioides	$Y = 1.61x + 2.43$	39.45 ± 5.1	0.987
Hymexazol[b]	*G. zeae*	$Y = 1.92x + 2.27$	26.4 ± 7.1	0.969
	F. oxysporum	$Y = 1.05x + 3.46$	29.1 ± 7.6	0.994
	C. mandshurica	$Y = 0.87x + 3.84$	21.4 ± 9.3	0.961
	R. solani	$Y = 2.73x + 1.33$	52.1 ± 6.1	0.995
	T. cucumeris	$Y = 0.95x + 3.48$	40.4 ± 9.7	0.965
	P. infestans	$Y = 1.60x + 2.76$	25.1 ± 9.3	0.941
	S. sclerotiorum	$Y = 3.48x + 2.57$	5.12 ± 4.5	0.931
	B. cinerea	$Y = 3.55x + 2.63$	4.63 ± 3.9	0.926
	C. gloeosporioides	$Y = 0.91x + 3.91$	15.8 ± 8.2[c]	0.983

[a] Average of three replicates.
[b] The standard compound was used for the comparison of activity.
[c] The values were estimated statistically by SPSS 11.5 software using a personal computer.

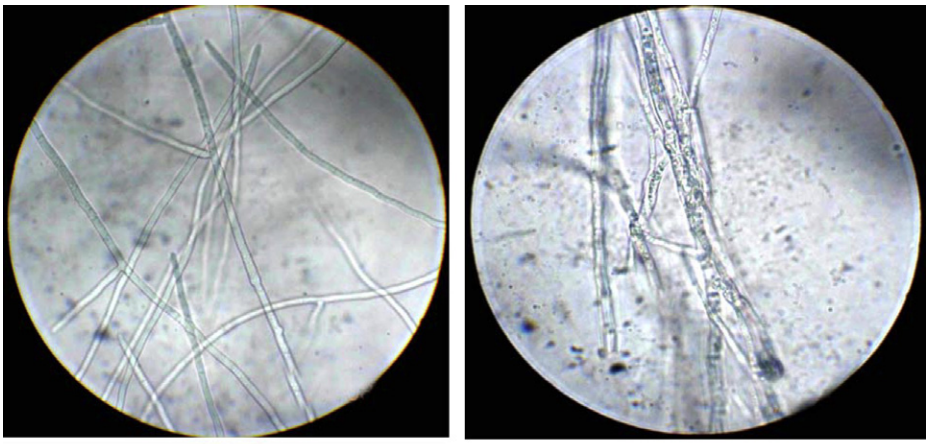


Figure 1. Microphotograph of the hyphal morphology of *S. sclerotiorum* treated with compound **10a** (800×).

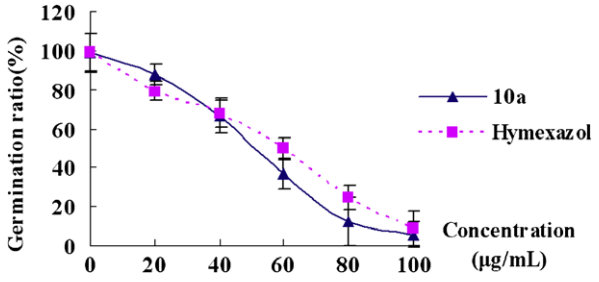


Figure 2. The effect of compound **10a** on sporule germination of *S. sclerotiorum* (solid lines) is compared with Hymexazol (dashed lines).

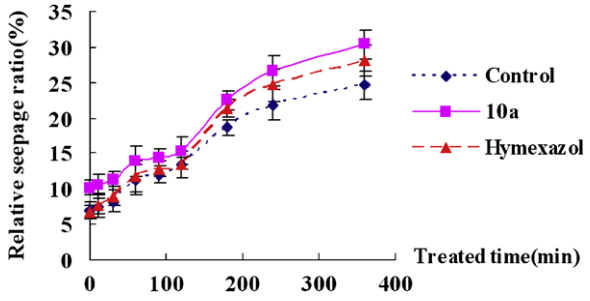


Figure 3. The effect of compound **10a** on membrane permeability of *S. sclerotiorum* is compared with the Control (dashed blue lines) and Hymexazol (dashed red lines).

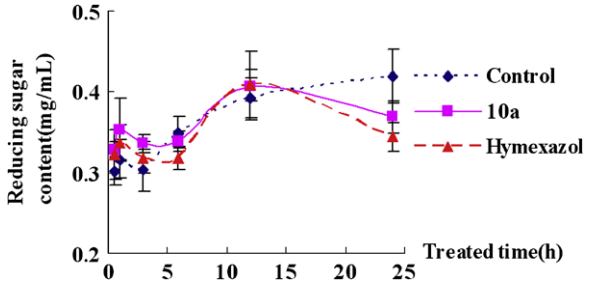


Figure 4. Changes of mycelial reducing sugar content, **10a** (solid lines), Control (dashed blue lines), and Hymexazol (dashed red lines).

2.2.3. Membrane permeability of *S. sclerotiorum*. After *S. sclerotiorum* was treated with compound **10a** (100 μg/mL), the relative permeability rate of the cell membrane was always higher than that of the control and the Hymexazol treatment. After being treated with **10a** for 240 min, the relative permeability rate was found to be 26.7% while the rate under identical conditions with control and Hymexazol treated were 21.9% and 24.6%, respectively. With longer treatment time, the relative permeability of the control rose gradually, but the one treated with compound **10a** and Hymexazol did not rise at the same rate. The results showed that when *S. sclerotiorum* was incubated with compound **10a**, the cell membrane was destroyed quickly and the relative permeability of *S. sclerotiorum* treated with Hymexazol was a little higher than that of the control. The results may be attributed to the fact that compound **10a** attacks the cell membrane of *S. sclerotiorum* (Fig. 3).

2.2.4. Changes of mycelial reducing sugar content. In the beginning up to 3 h, mycelial reducing sugar content of *S. sclerotiorum* for the treated one was higher than the control, being 0.30 mg/mL for the control against 0.34 mg/mL for the treated which was 13.3% higher than that of the control. After 3 h, although the content of control continued to rise, but the one treated with **10a** reached its peak value after 12 h and then started to fall. At the end of 24 h, the mycelial reducing sugar content of the **10a** treated was 0.37 mg/mL, which was 11.9% lower than that of the control and 8.8% higher than that with the Hymexazol treated one (Fig. 4).

2.2.5. Changes of mycelial chitosan content. From Figure 5, we can find that when *S. sclerotiorum* was treated with compound **10a**, the mycelial chitosan content, namely the content of D-GlcNAc in the mycelial cell, started to fall at the beginning before showing a rising tendency that continued till 12 h, and then began to decline again. On the other hand, the one treated with control showed an increasing tendency throughout and always maintained a lead compared to the one treated with **10a** ex-

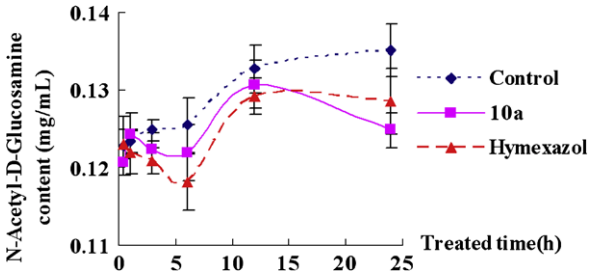


Figure 5. Changes of mycelial D-GlcNAc content, **10a** (solid lines), Control (dashed blue lines), and Hymexazol (dashed red lines).

cept at the end of 1 h. The mycelial chitosan content was 0.12 mg/mL after being cultivated with compound **10a** at the end of 6 h, which was 7.7% lower than that of the control (the control was 0.13 mg/mL). After treatment with compound **10a** after 24 h, the D-GlcNAc content was 0.12 mg/mL, which was 14.3% and 7.7% lower than that of the control and the Hymexazol, respectively (the control was 0.14 mg/mL, the Hymexazol was 0.13 mg/mL) (Fig. 5).

2.2.6. Changes of mycelial chitinase activity. When treated with compound **10a**, the mycelial chitinase activity of *S. sclerotiorum* showed a fluctuating tendency. After being cultured for 12 h, the mycelial chitinase activity of *S. sclerotiorum* began to fall. Both control and the treated one showed similar behavioral pattern up to 6 h and reached their respective lowest values at the end of this period, the control began to rise constantly thereafter. After being inoculated for 24 h, the D-GlcNAc content that was catalyzed by chitinase without treatment was found to be 0.129 mg/mL. The corresponding values for the one treated with compound **10a** and Hymexazol were 0.125 mg/mL and 0.124 mg/mL, respectively (Fig. 6).

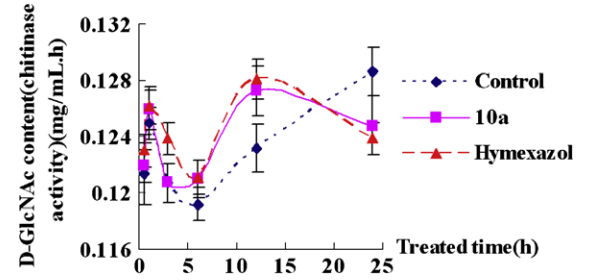


Figure 6. Changes of mycelial chitinase activity, **10a** (solid lines), Control (dashed blue lines), and Hymexazol (dashed red lines).

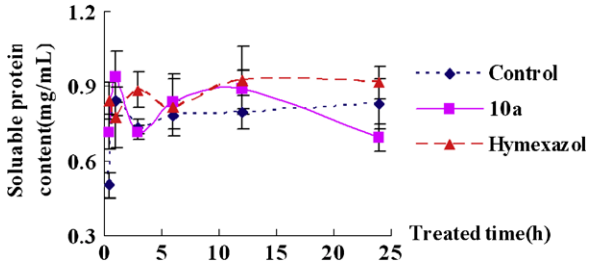


Figure 7. Changes of mycelial soluble protein content, **10a** (solid lines), Control (dashed blue lines), and Hymexazol (dashed red lines).

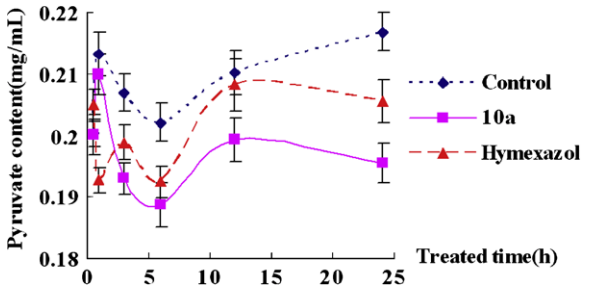


Figure 8. Changes of mycelial pyruvate content, **10a** (solid lines), Control (dashed blue lines), and Hymexazol (dashed red lines).

2.2.7. Changes of mycelial soluble protein content. As we can see from Figure 7, when *S. sclerotiorum* was treated with compound **10a** for 0.5 h, the mycelial soluble protein content was 0.72 mg/mL, 43% higher than that of the control (0.50 mg/mL). After 6 h of inoculation, the mycelial soluble protein content for the control was always higher but similar as compared to the one treated with Hymexazol. Although the compound treated one showed an irregular pattern up to 12 h, the mycelial soluble protein content revealed a gradual but uniform decreasing tendency thereafter. At the end of 24 h, the mycelial soluble protein content of the treated one was 0.69 mg/mL, for the control the value was 0.83 mg/mL, and the one with commercial fungicide Hymexazol it was 0.91 mg/mL. So the results seem to indicate that compound **10a** can inhibit the synthesis of protein while the acting site of Hymexazol may not be necessarily the soluble protein (Fig. 7).

2.2.8. Changes of mycelial pyruvate content. As has been shown in Figure 8, the mycelial pyruvate content of *S. sclerotiorum* treated with compound **10a** was lower than that of the control throughout the experiment. With Hymexazol treated one, the changes were irregular up to 12 h before showing a uniform decreasing pattern. After being cultivated for 24 h, the mycelial pyruvate content of *S. sclerotiorum* treated with **10a** was found to be 0.19 mg/mL, which was 13.6% lower than the control and 9.52% lower than with the Hymexazol treatment, respectively. These data indicate that compound **10a** can inhibit the activity of lactate dehydrogenase (LDH) thereby inducing the decrease of the pyruvate content (Fig. 8).

3. Conclusion

In summary, the present method of the formation of sulfoxide derivatives by *m*CPBA oxidation in dichloromethane offers several advantages such as fast reaction rate and moderate yield. It was also found that title compound **10a** displayed good antifungal activity and had a wide spectrum of bioactivity. Using the mycelial growth rate method in the laboratory, the mechanisms of action of **10a** against *S. sclerotiorum in vitro* were studied. The results showed that **10a** had high inhibitory effect on the growth of most of the fungi with the EC_{50} values ranging from 13.9 to 63.9 μg/mL. After *S. sclerotiorum* was treated with compound **10a** at 100 μg/mL, the permeability

of the cell membrane rose and most of its hyphal became distorted, malformed, and got entangled. The cell of the hypha swelled and its endosome condensed, malformed, leaked out, and formed blanks. From these results, it can be concluded that the cell membrane and the morphology of *S. sclerotiorum* may be destroyed and changed by compound **10a**. After treatment with compound **10a** at 100 μg/mL for 12 h, the soluble protein content began to fall and perhaps the activity of proteolytic enzymes started to decrease. But these were not obvious when *S. sclerotiorum* was treated with Hymexazol, perhaps due to the fact that the main site of attack by Hymexazol was not the same. In addition, the mycelial reducing sugar and D-GlcNAc content also showed decreasing trends indicating that the production of energy probably could be inhibited by compound **10a**. At the same time, the mycelial pyruvate content and chitinase activity showed decreasing tendency that may be attributed to suppression of lactate dehydrogenase and chitinase activities by compound **10a**. The fact that only 5.4% of its spore bourgeoned, could possibly explain the results of high inhibition rate (%) while using compound **10a** against nine kinds of fungi (Table 6). Although, some preliminary studies were conducted, the precise way in which compound **10a** efficiently inhibits the growth of the hypha still remains to be ascertained. So, further investigation requires to be done in the future research.

4. Experimental

4.1. Analysis and instruments

The melting points of the products were determined on a XT-4 binocular microscope (Beijing Tech Instrument Co., China) and were not corrected. The IR spectra were recorded on a Bruker VECTOR22 spectrometer in KBr disks. ^{1}H and ^{13}C NMR (solvent $CDCl_3$) spectra were recorded on a JEOL-ECX 500 NMR spectrometer at room temperature using TMS as an internal standard. Elemental analysis was performed on an Elementar Vario-III CHN analyzer. The reagents were all of analytical grade or chemically pure. Analytical TLC was performed on silica gel GF254. Column chromatographic purification was carried out using silica gel. 3,4,5-Trimethoxyphenylhydrides **3** and 5-(3,4,5-trimethoxyphenyl)-1,3,4-thiadiazole-2-thiol **4** were prepared according to the literature method as described.[12] The optical density (OD) was determined on WFZUV-2100 UV–Vis Spectrophotometer. The conductivity was done by DDS-11A Conductivity Meter. The microphotograph of the hyphal morphology was taken from a OLYMOPUS Microscope.

4.1.1. Preparation of 5-(3,4,5-trimethoxyphenyl)-1,3,4-oxadiazole-2-thiol (5). A mixture of 0.56 g (10 mmol) of potassium hydroxide, 2.26 g (10 mmol) of compound **3**, and 1.14 g (15 mmol) of carbon disulfide in 50 mL of absolute ethanol was refluxed for 8 h. After the solvent was evaporated in vacuum, the residue was dissolved in ice-cold water and acidified with dilute hydrochloric acid. The precipitate was filtered off, washed with water, dried, and recrystallized from absolute ethanol to give compound **5**. The structure was confirmed by ^{1}H NMR, ^{13}C NMR, IR, and elemental analysis (see Supporting Information).

4.1.2. Preparation of 2-substituted methylthio-5-(3,4,5-trimethoxyphenyl)-1,3,4-thiadiazole (7a–7g). To a 50 mL, three-necked, round-bottomed flask equipped with a magnetic stirrer were added 1.5 mmol of **4**, 20 mL of distilled water, and 2 mL (3%, w/w) of NaOH solution. The mixture was stirred at room temperature for 10 min. Then 1.5 mmol of halide **6** and 17.2 mg (0.15 mmol) of indium were added. The resulting mixture was stirred at room temperature for 4 h, and then filtered. The white solid resulted was washed with 5% Na_2CO_3 solution and distilled water, dried under vacuum, and recrystallized from ethanol to give compound **7** (for **7g**, after the completion of the reaction, extracted with CH_2Cl_2 and dried over $MgSO_4$. The solvent was removed and the crude product was purified by chromatography (petroleum/ether, 2/1)) (see Supporting Information).

4.1.3. Preparation of 2-substituted methylthio-5-(3,4,5-trimethoxyphenyl)-1,3,4-oxadiazole (8a–8h). A 50 mL round-bottomed flask equipped with a magnetic stirrer was charged with **5** (1.5 mmol) and 2 mL (3%, w/w) of sodium hydroxide solution. The mixture was dissolved in 20 mL of distilled water. The flask was stirred at room temperature for 10 min, and then halide **6** (1.5 mmol) and indium tribromide (0.15 mmol) were added to the reaction mixture and stirred at room temperature for 4 h. The mixture was filtered and the white solid obtained was washed with 5% Na_2CO_3 solution and distilled water, dried under vacuum, and recrystallized from ethanol to give compound **8** (see Supporting Information).

4.1.4. Preparation of 2-substituted sulfinyl-5-(3,4,5-trimethoxyphenyl)-1,3,4-thiadiazole (oxadiazole) (9a–9g, 10a–10h). To a three-necked round-bottomed flask equipped with a magnetic stirrer and containing Sulfide (1.0 mmol) and dichloromethane (10 mL) was added dropwise 1.6 mmol *m*CPBA dissolved in 10 mL dichloromethane over a period of 15 min. The reaction mixture was first stirred at 0 °C for 2 h, and then stirring was continued for another 5 h at room temperature. The mixture was washed with 0.25 mol/L Na_2HPO_4 solution (3× 20 mL), dried over $MgSO_4$. The solvent was removed and the crude product was purified by column chromatography (petroleum/ether, 1:1) (see Supporting Information).

4.2. Antifungal assay

The antifungal activity of all synthesized compounds were tested against three pathogenic fungi, namely *G. zeae*, *F. oxysporum*, *C. mandshurica* by the poison plate technique.[16]

Compounds were dissolved in 1 mL acetone before mixing with 90 mL potato dextrose agar (PDA). The final concentration of the compounds in the medium was tested at 50 μg/mL. All kinds of fungi were incubated in PDA at 27 ± 1 °C for 4 days to get new mycelium

for antifungal assay. Then mycelia dishes of approximately 4 mm diameter were cut from culture medium and one of them was picked up with a sterilized inoculation needle and inoculated in the center of PDA plate aseptically. The inoculated plates were incubated at 27 ± 1 °C for 5 days. Acetone in sterile distilled water served as control, while Hymexazole severed as positive control. For each treatment, three replicates were conducted. The radial growth of the fungal colonies was measured and the data were statistically analyzed. The inhibiting effects of the test compounds in vitro on these fungi were calculated by the formula: $I(\%) = [(C - T)/(C - 0.4)] \times 100$, where C represents the diameter of fungi growth on untreated PDA, and T represents the diameter of fungi on treated PDA while I means inhibition rate.

Compound **10a** was tested against nine pathogenic fungi namely *G. zeae*, *F. oxysporum*, *C. mandshurica*, *P. infestans*, *R. solani*, *T. cucumeris*, *C. gloeosporioides*, *B. cinerea*, *S. sclerotiorum* at different concentrations of 100, 50, 25, 12.5, and 6.25 μg/mL.The EC_{50} values were estimated statistically by Probit analysis with the help of Probit package of SPSS 11.5 software using a personal computer. The average EC_{50} (μg/mL) was taken (effective dose for 50% inhibition μg mL^{-1}) from at least three separate analyses for inhibition of growth using the Basic LD_{50} program version 1.1.[16]

4.2.1. The hyphal morphology observation of *S. sclerotiorum*. Compound **10a** was added in sterilized Czapek media (0.2% $NaNO_3$, 0.131% $K_2HPO_4{\cdot}3H_2O$, 0.05% KCl, 0.05% $MgSO_4{\cdot}7H_2O$, 0.00183% $FeSO_4{\cdot}7H_2O$, 3% sucrose, pH 6.8)[17] which had incubated *S. sclerotiorum* for a few days, and the final concentration was 100 μg/mL. Then incubated together at 27 °C. After 24 h, observed under microscope. Acetone (1.0 mL) served as the control.

4.2.2. Effect of compound 10a on sporule germination of *S. sclerotiorum*.[18] Compound **10a** was mixed with PDA medium at the concentrations of 0 (Control), 20, 40, 60, 80, and 100 μg/mL, for each treatment, three replicates were conducted. Poison media plates were prepared using 9 cm Petri dishes, 0.5 mL sporule in suspension was spread at poison media plates. This was followed by incubation at 27 °C and then after 24 h, observed at every 2 h. When the germination of the control bourgeoned over 90%, investigated the germination of every concentration, and observed three eyeshots per Petri dish. The germination rate was calculated using the following formula:

$$\text{Germination rate } (\%GR) = 100 \times G/S$$

where GR represents the germination rate, G represents the number of germinated sporule and S represents the number of all observed sporule.

4.2.3. Preparation of the crude extract of mycelium (*S. sclerotiorum*).[19] The six mycelial discs (4 mm diameter) taken from a starting colony growing on PDA were placed in an Erlenmeyer flask containing 90 mL of sterilized Czapek. Then these were incubated in a whirly shaker (140 rpm, 27 °C). After 15 days, compound **10a** was dripped into the culture media and the tested concentration was 100 μg/mL. Then mycelium was filtered, collected, and washed orderly at 0.5, 1, 3, 6, 12, and 24 h. After soaking the water with filter paper, the dried mycelium was weighed and then preserved at −20 °C.

The dry mycelium (1.0 g) in cold mortar was mixed with Tris–HCl buffer (2.5 mL, 0.05 mol/L, pH 7.5), triturated into paste quickly, and then centrifuged at 4 °C, 15,000*g* for 30 min. The clean upper layer was preserved at −20 °C. Every treatment had three repetitions.

4.2.4. Effect of 10a on the relative permeability rate of cell membrane.[20] Collected the mycelial of *S. sclerotiorum* which incubated in a few days and washed with double distilled water. 1 g of the mycelial was placed in 15 mL centrifuge tubes containing compound **10a**, at 100 μg/mL concentration. Then measured the conductivity at 0 (J_0), 5, 10, 30, 60, 180, 360 min (J_1), gradually. After boiling which was followed by cooling, the conductivity (J_2) was measured. In the end, calculated the permeability by the formula: $P\% = \frac{J1-J0}{J2-J0} \times 100\%$.

4.2.5. Detection of mycelial reducing sugar content.[21] The 200 μL of upper clear layer of mycelial extract was mixed with 3,5-dinitrosalicylic acid (DNS, 400 μL), and then the mixture was boiled for 5 min. It was then quickly cooled to room temperature and diluted to a volume of 2.5 mL using distilled water. Absorbance value of the mixture measured at 540 nm was converted into the value of glucose content (mg) by the standard curve of glucose. Tris–HCl buffer served as the control.

4.2.6. Detection of mycelial chitosan content.[22] The upper clear layer (200 μL) of mycelial extract was mixed with $K_2B_4O_7$ solution (100 μL), and then the mixture was boiled for 3 min. After quickly cooling to room temperature, the mixture was added into 3 mL of 1% 3,2′-dimethyl-4-aminobiphenyl (DMAB). Subsequently, it was incubated for 20 min at 36 °C, and then cooled down to room temperature again. The absorbance value of the mixture was measured immediately at 544 nm. Tris–HCl buffer served as the control.

4.2.7. Detection of mycelial chitinase activity.[23,24] The mixture of the upper clear layer (400 μL) with chitin colloid (200 μL) was dripped into clear centrifugal tube. The tube was incubated for 1 h (37 °C), and then boiled for 5 min. After being centrifuged at 5000 rpm for 10 min, the mixture (400 μL) mixed with $K_2B_4O_7$ solution (200 μL, 0.8 M) was boiled, and then cooled down quickly. This was followed by the addition of 3 mL of 1% DMAB. The mixture was kept at 36 °C for 20 min, and then cooled down to room temperature. The absorbance value of the mixture was measured immediately at 544 nm. The control was boiled before incubating.

4.2.8. Detection of mycelial soluble protein content.[21] The Coomassie brilliant blue G-250 dye-binding technique was adopted to observe the changes of protein content. The upper clear layer of mycelial extract (100 μL) was mixed with coomassie brilliant blue G-250 solution (3 mL), and then the mixture was kept still for 5 min.

The absorbance value of the mixture was measured at 595 nm. Tris–HCl buffer served as the control.

4.2.9. Detection of mycelial pyruvate content.[25] The upper clear layer (100 μL) of mycelial extract was mixed with 2,4-dinitrophenylhydrazine (500 μL, 2.5 mM), and then the mixture was swayed evenly and incubated for 10 min (37 °C), this was followed by the addition of NaOH (5000 μL, 0.4 M), then swayed uniformly. The absorbance value of the mixture measured at 520 nm was converted into the value of pyruvate content (mg) by the standard curve of pyruvate. Tris–HCl buffer served as the control.

4.3. Statistical analysis

All the statistical analyses were done under SPSS 11.5 using personal computer. Each experiment had three replicates and all experiments were run three times with similar results. Measurements from all the replicates were combined and treatment effects analyzed.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.02.006.

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