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Full Length Research Paper

The preliminary study of inhibitory mechanism of sulfone compounds xa202 and xt208 against *Gibberella zeae*

Linhong Jin, Jiang He, Ming He, Weiming Xu and Song Yang*

State Key Laboratory Breeding Base of Green Pesticide and Agricultural Bioengineering, Key Laboratory of Green Pesticide and Agricultural Bioengineering, Ministry of Education, Guizhou University, Huaxi District, Guiyang, 550025, Guizhou Province, P. R. China.

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A series of novel methyl sulfone derivatives containing 1,3,4-oxadiazole (thiadiazole) moiety were bioassayed *in vitro* against *Gibberella zeae* using mycelial growth rate method. The bioassay results showed that compounds xa202 and xt208 displayed excellent antifungal activity against *G. zeae* with EC_{50} values of 3.49 µg/ml and 3.73 µg/ml, respectively. The mechanism for compounds xa202 and xt208 against *G. zeae* were studied. After treating with compounds xa202 and xt208 at 50 µg/ml for 24 h, the permeability of fungi cell membranes, the conductivity and the concentration of calcium ions (Ca^{2+}) in mycelial solution showed increasing tendency, while the chitinase content experienced a slight decline initially and then increased rapidly. Compounds xa202 and xt208 were also found to have more potent inhibitory activities against ergosterol biosynthesis than a commercial fungicide, carbendazol. The inhibitory rates for xa202 and xt208 were 32.04% and 55.34%, respectively.

Key words: *Gibberella zeae*, mechanism of action, conductivity, calcium ions and ergosterol biosynthesis.

INTRODUCTION

G. zeae, the perfect stage of *Fusarium graminearum* is one of the most devastating pathogens of all classes of wheat and other small grain worldwide that not only reduces crop yield greatly (Bai and Shaner, 1994; McMullen et al., 1997; Su et al., 2007) but also contaminates grain with deoxynivalenol and other trichothecene toxins that can be harmful to human beings and animals that consume infected grain (Vesonder et al., 1976; Forsyth et al., 1977; Li et al., 2008). Head blight disease of wheat and barley was prevalent in regions facing the Pacific Ocean, especially in eastern China (Wang et al., 1982). In recent years, because of continued moist weather during the crop growing season and the failure of chemical control in some areas due to benzimidazole fungicides resistance (especially carbendazim) in the pathogen population (Chen et al., 2009), Fusarium head blight (FHB) have been endemic in the wheat-producing areas of Anhui province of China, which have caused an estimated 20 to 50% of reduction and even completely failure of harvests in many wheat-producing areas. Therefore, the design of new compounds to deal with *G. zeae* has become one of the most important areas of antibacterial research today.

In the present work, we studied herein the effect of nineteen novel methyl sulfone derivatives containing 1, 3, 4-oxadiazole (or thiadiazole) moiety in the design of new antifungal compounds. The bioassay results showed that all compounds displayed excellent antifungal activity against *G. zeae* with EC_{50} values ranging from 3.49 to 19.63 µg/mL (Table 1). The compound 2-(methylsulfonyl)- 5-phenyl-1,3,4-oxadiazole (xa208) and 2-(methylsulfonyl)-5-(4-chlorophenyl)-1,3,4-oxa-diazole (xt202) displayed high antifungal activity.

In order to investigate the mechanism of antifungal activity of compounds xa202 and xt208, herein we studied their effect on chitinase activity, permeability, and ergosterol content and the impact on calcium ion channel against *G. zeae*. To the best of our knowledge, this is the

*Corresponding author. E-mail: gdjhzx@gmail.com.

first report of how sulfone derivatives affect the ergosterol content and calcium ion channel of *G. zeae*.

MATERIALS AND METHODS

Pathogenic fungus and compounds

Gibberella zeae come from the Culture Collection Center of Nankai University and activated in the key Laboratory of Green Pesticide and and Agricultural Bioengineering, Ministry of Education, Guizhou University. The tested compounds were synthesized at the Key Laboratory of Green Pesticide and Agricultural Bioengineering, Ministry of Education, Guizhou University. The 99.4% carbendazim was purchased from National Pesticide Quality Control Center-Shenyang. The potato dextrose agar (PDA) comes from the key Laboratory of Green Pesticide and Agricultural Bioengineering, Ministry of Education, Guizhou University.

Experiment of inhibitory activity

Nineteen compounds (at 25.0, 12.5, 6.25, 3.13, and 1.56 µg/ml respectively) and carbendazim (at 5.0, 0.5, 0.1, 0.05, 0.01 µg/ml respectively) were tested against *Gibberella zeae* using mycelial growth rate method (Huang, 2000; Boué et al., 2005). Blank control was set in the experiments and three replicates were conducted for each treatment. The inhibitory effects of the test compounds *in vitro* against *G. zeae* were calculated by the formula: I (%) = [(*C*-*T*)/(C-0.4)]*100, where *C* represents the diameter of fungi growth on untreated PDA, and *T* stands for the diameter of fungi on treated PDA while I denotes the inhibition rate. After calculating toxicity regression with SPSS and drawing the toxicity regression equations, the corresponding correlative coefficient and the EC_{50} values were obtained.

Mycelium culture

Gibberella zeae was incubated in PDA in the conical flask containing 90 ml Czapek's medium at 27±1°C, pH 6.5, 120 r/min for three weeks to get new mycelium for antifungal assay (Fang, 1998). 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. After three weeks compounds, xa202 and xt208 (at 50 µg/ml) were added to the conical flask, while acetone served as blank control and carbendazim served as positive control. For each treatment, three replicates were conducted. And then the mycelium was collected separately at 0.5, 1.0, 3.0, 6.0, 12.0, and 24.0 h by filtering, and washed with distilled water to remove the undissolvable substances. The 0.5 g dry mycelium collected at different treatment time was respectively added into the freezing mortar, then mixed with 3.0 ml buffer solution (Tris-HCl: 0.05 mol/L, pH 7.54) and a little quartz sand. Under the freezing condition the mixture was quickly ground into pasty. The slurry was packed into the 1.5 ml centrifuge tube and then centrifuged at 4°C, 15000 g for 30 min. Finally the supernatant was collected and packed into the 1.5 ml centrifuge tube and kept in the refrigerator at -20°C.

Assay of chitinases activity

Reported methods (Gu and Hu, 1994; Rodriguex-Kabana, 1983; Liu et al., 2008) was used for the chitinases activity assay. The supernatant was added into the 1.5 ml centrifuge tube and then mixed with 0.2 ml colloidal chitin. The mixture was kept at 37°C for one h and then boiled for five min, centrifuged at 4°C, 5000 r/min for ten min. than 0.3 ml supernatant of the centrifugate was transferred to an 1.5 ml centrifuge tube and mixed with 0.1 ml potassium borate solution. The mixturewas kept in boiling water for three min and then immediately cooled to room temperature, added another 3 ml 1% dimethylamine borane solution was than added into the mixture and the resulted solution kept at 36°C for 20 min, then immediately cooled to room temperature, and finally each sample was measured with the 722 spectrophotometer at 544 nm wavelength and triplicate measurements were carried out for Each sample was repeated three times.

Content determination of calcium ion content

Standard calcium ions solutions were prepared by mixing $CaCl_2$ with ultrapure water at different concentrations of 0.00, 1.00, 5.00, 10.00, 20.00, 25.00 µg/ml respectively. To a 25 ml volumetric flask was added 0.5 ml solution of supernatant stored in the -20°C refrigerator, then ultrapure water was added to a total volume of 25 ml. Three replicates were conducted for each treatment. Analytical Methods (Liu, 2008): calcium ion content was tested using a light source of calcium hollow-cathode lamp at 422.7 nm wavelength, with lamp current set at 6.0 mA and slit-width being 0.2 nm, the air flow rate was 460 L/h and the acetyle flow rate was 1.8 L/min.

Effect of compounds xa202, xt208 and carbendazim on the relative permeability rate of cell membrane (Liu et al., 2008)

After incubated for two weeks, *Gibberella zeae* mycelium was collected and washed with ultrapure water. Then 1.0 g mycelium was placed in 100 ml beakers containing carbendazim, xa202 and xt208 at 50.00 µg/ml concentration, wIth acetone served as blank control and carbendazim served as postive control. The conducti-bility of each sample was respectively determined by DDS-11A conductivity meter at 0 (J_0)10, 30, 60, 90, 120, 180 and 240 min, and the value were recorded as J_1. Then the mixture was boiled and then cooled to room temperature and the conductivity (J_2) was measured again. Three replicates were conducted for each experiment. The permeability was then calculated by the formula:

$$\text{the relative conductivity of certain times (\%)} = \frac{J_1 - J_0}{\times 100\%\ J_2 - J_0}$$

Change of ergosterol content

After incubated for two weeks, *Gibberella zeae* mycelium was collected and washed with ultrapure water. Then 0.05 g mycelium was added to 50 ml mortar and grounded into fine powder. Reported protocol was then followed to extract ergosterol (Hang Ꝑ et al., 2006; Pappas and Fisher, 1979). The chromate-graphy conditions were: Luna C18 column (250 mm×4.6 mm, 5 µm), 100% methanol as the eluent, flow rate 1.00 ml/min, detection wavelength 282 nm, column temperature 25°C, column pressure of 81 bar, and an injection volume of 20 µl.

RESULTS AND DISCUSSION

Antifungal activity

The *in vitro* antifungal activities of all the compounds were tested

Table 1. Inhibitory effect of nineteen compounds against *G. zeae*.

Entry	Regression equation	Correlative coefficient	EC_{50} (μg/mL)
carbendazim	y=1.544x + 7.090	0.973	0.044±0.027
xa2010	y=3.662x + 1.305	0.975	10.23 ± 1.10
xa201	y=5.140x - 1.565	0.943	18.92 ± 3.16
xa202	y= 3.517x + 3.093	0.935	3.49± 0.14
xa204	y=2.676x + 2.900	0.970	6.10± 0.52
xa205	y=1.604x + 2.911	0.988	20.00 ± 0.59
xa206	y=5.067x - 2.990	0.965	37.76 ± 8.31
xp209	y=7.724x - 4.155	0.923	15.31 ± 0.62
xp210	y=2.822x + 2.072	0.925	19.63± 26.18
xp211	y=3.030x + 3.162	0.835	4.05± 0.89
xp212	y=6.083x - 1.743	0.920	12.82 ± 1.04
xp219	y=2.614x + 3.122	0.982	5.22± 0.66
xt208	y=3.469 x + 3.017	0.929	3.73± 0.25
xt215	y=2.311 x + 2.928	0.908	7.89± 0.75
xx218	y=2.866x + 1.745	0.927	13.68 ± 1.44
xx226	y=2.597x + 1.822	0.891	16.75 ± 5.12
xx223	y=2.736x + 2.137	0.945	11.12 ± 1.30
xx219	y=3.383x + 1.498	0.941	10.84 ± 1.84
xx217	y=2.923x + 2.098	0. 956	9.84± 1.84
xh215-1	y=2.311x + 2.443	0.876	12.76 ± 2.52

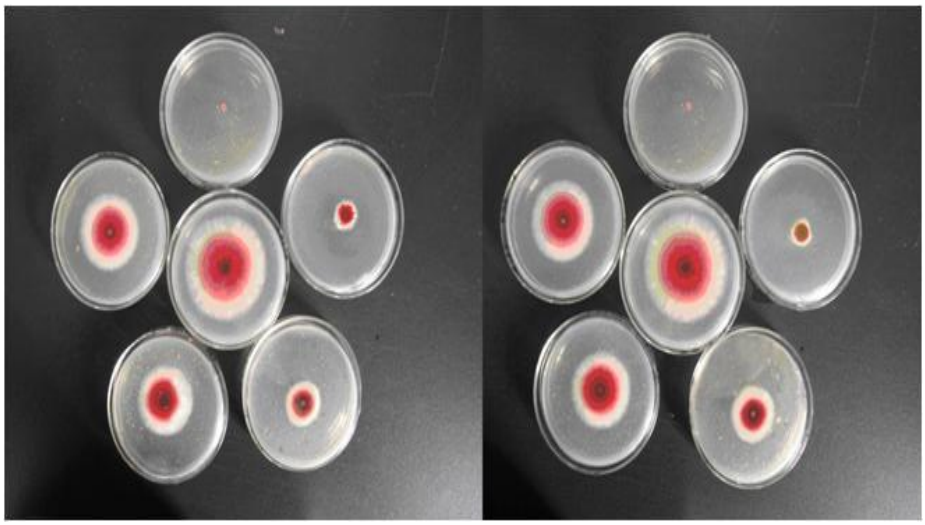

Figure 1. Inhibitory activity of xa202 and xt208 against mycelial growth of *G. zeae*. The left-hand chart and the right-hand chart represent respectively the inhibitory picture of xa202 and xt208 (concentration are 25.0, 12.5, 6.25, 3.13, 1.56 μg/ml). The concentration in the clockwise direction at the chart arranged from large to small order.

against *G. zeae* using mycelial growth rate method (Boué et al., 2005). The toxicity regression equations, correlative coefficients and EC_{50} values of nineteen novel methyl sulfone derivatives containing 1, 3, 4-oxadiazole (thiadiazole) moiety were shown in Table 1. The bioassay results showed that all the compounds exhibited promising antifungal activities against *G. zeae* with EC_{50} values ranging from 3.49 to 19.63 μg/ml. and Compounds xa202 and xt208 were found to have excellent antifungal activities, the EC_{50} values of 3.49 and 3.73 μg/ml, respectively.

Change of chitinases activity

The mycelium of *G. zeae* was treated with compounds xa202 and xt208. The D-GlcNAc content (chitinase activity) was respectively assayed at 0.5, 1.0, 3.0, 6.0, 12.0 and 24.0 h. The results are presented in Figure 2. It is evident that the chitinases activity of *G. zeae* showed a fluctuating tendency. After being cultured for 24 h, the D-GlcNAc content (compound xa202） was very close to that of the blank control but lower than carbendazim treatment during the first two h, and then increased

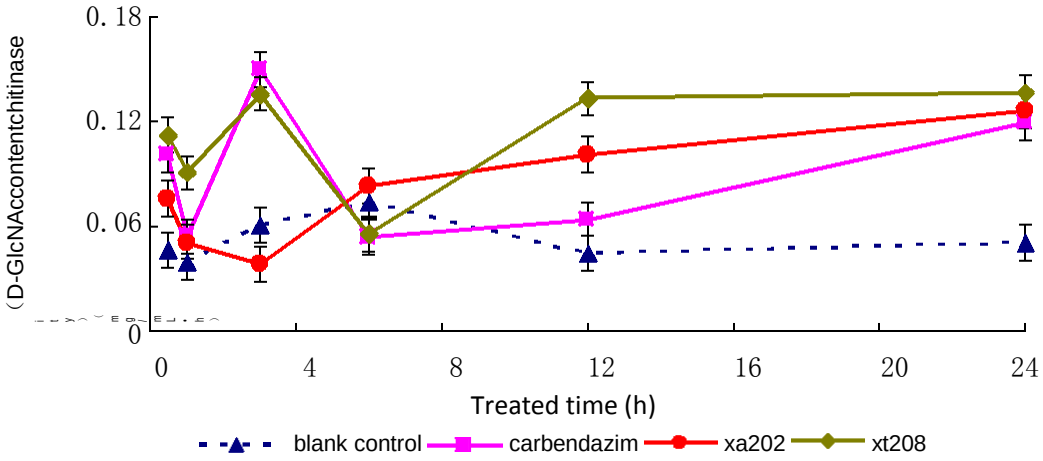

Figure 2. Change of the mycelial chitinase activity in the mycelium of *G. zeae*.

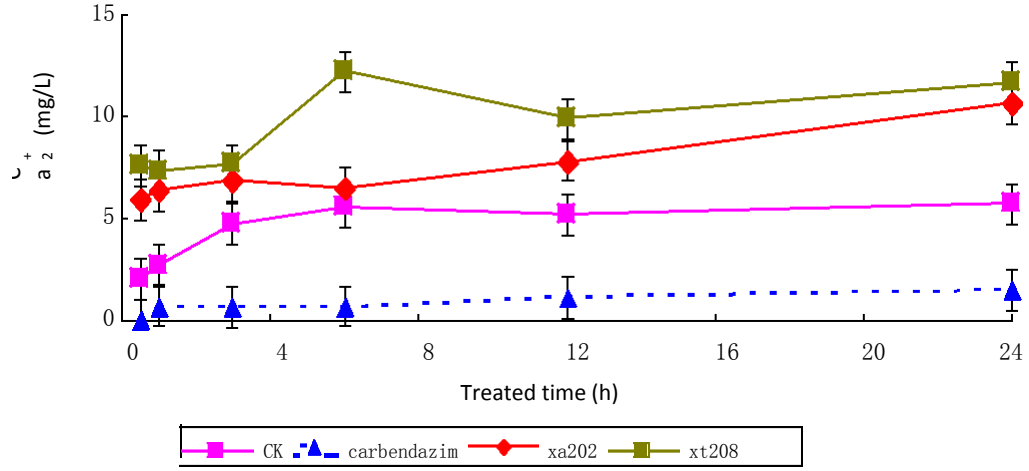

Figure 3. Content change of the Ca^{+2} in the mycelium of *Gibberella zeae*.

rapidly during the next 20 h compared to the two controls. As for compound xt208, the D-GlcNAc content reached the highest point after being cultured for 3 h, and during 24 h, the D-GlcNAc content was higher than that of the blank control but close to that of the carbendazim control.

Content change of calcium ion

G. zeae was treated with compounds xa202 and xt208, while acetone served as blank control and carbendazim served as positive control. The content of calcium ion was assayed in 24 h (Figure 3). Conclusively, the calcium ion content increased gradually for compounds xa202, xt208 and controls. After being treated with compound xt208 for 6 h, the calcium ion content reached the peak at 13 mg/L and decreased steadily during the next 18 h, while the maximum content calcium ion of compound xa202 was only 10 mg/L after being treated for 24 h. However, as for the two compound, the calcium ion content was higher than that of the controls during 24 h. probably due to the effect of two compounds on the calcium channel of *Gibberella zeae* cell.

Membrane permeability

G. zeae was treated with compounds xa202 and xt208. The changes of the relative conductivities are shown in Figure 4. After being treated for 4 h, the relative permeability rate of compound xt208 treatment was in consistent with carbendazim control, while the relative permeability rate of compound xa202 treatment was higher than that of xt208 and carbendazim treatment. All of the results may be ascribed to the fact that the two compounds could attack the cell membrane of *G. zeae* and caused the protoplasm leakage in cell, and thus the

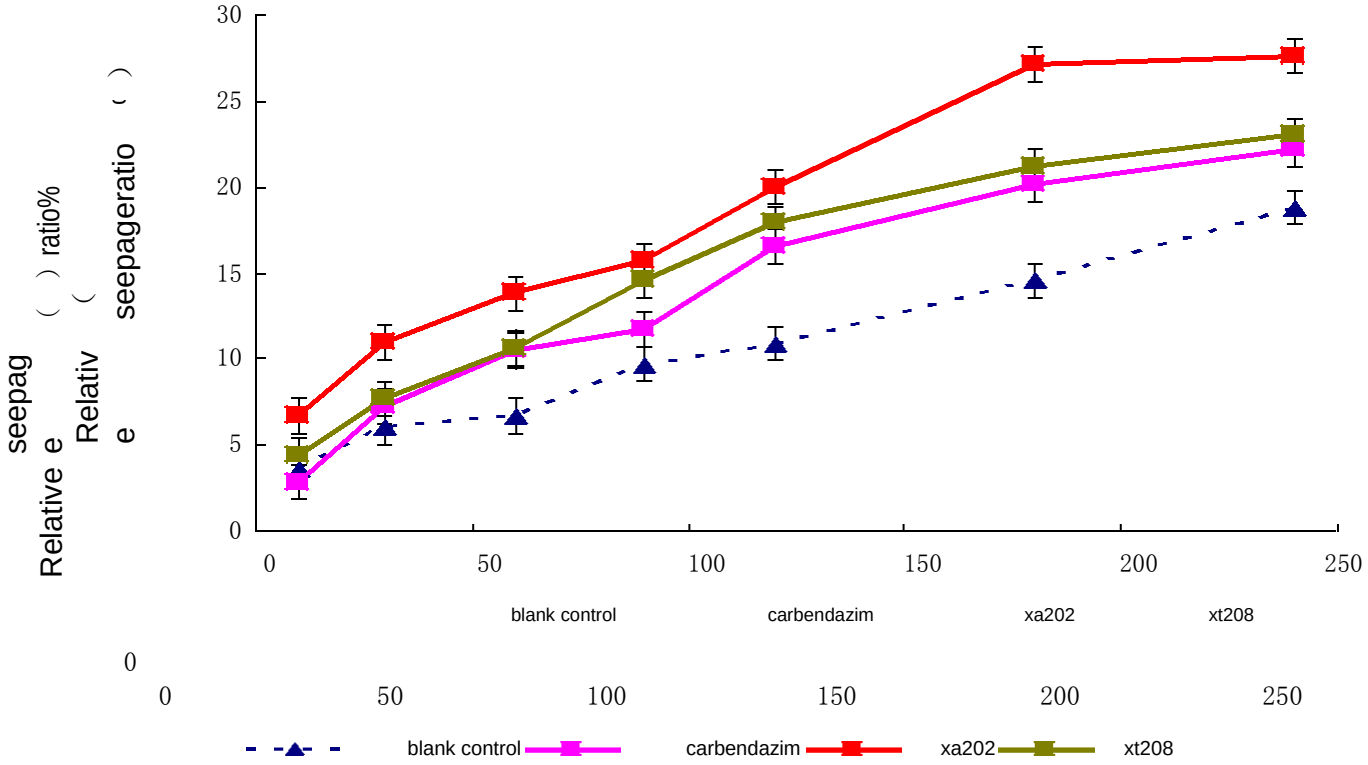

Figure 4. Effect of compounds xa202 and xt208 on membrane permeability of *G. zeae*.

Table 2. The content of ergosterol in the *G. zeae* mycelium.

Compound numbers	Concentration (μg/ml)	Concentration of Ergosterol in the treated-samples (μg/ml)	Content of ergosterol in mycelium (mg/g)	Inhibition on ergosterol in the treated samples (%)
blank control	---	5.13 ± 1.030	1.03 ± 0.089	---
carbendazim	12.5	4.19 ± 0.612	0.84 ± 0.034	18.45
xa202	12.5	3.48 ± 0.122	0.70 ± 0.037	32.04
xt208	12.5	2.29 ± 0.166	0.46 ± 0.011	55.34

conductivity increased.

Change of ergosterol content

The changes of ergosterol content are shown in Table 2. Compound xt208 was found to have good ergosterol biosynthesis inhibitory activity, with the inhibitory rate amount of 55.34%, which was higher than that of carbendazim control (32.04%). However, compound xa202 had a negligible inhibitory rate towards ergosterol biosynthesis. Further study was carried out to investigate whether compounds xt208 and xa202 were ergosterol biosynthesis inhibitors or not. Figure 5 showed that the retention time of ergosterol was 14 min and the absorption peak was fully separated from other impurity peaks.

Conclusion

The novel methyl sulfone derivatives containing 1, 3, 4-oxadiazole (thiadiazole) moiety compounds were tested against *G. zeae* using mycelial growth rate method. The bioassay results showed that compounds xa202 and xt208 displayed excellent antifungal activities against *G. zeae* and the mechanism for compounds xa202 and xt208 against *G. zeae* were studied. The results showed that compounds xa202 and xt208 could affect the calcium channel and the membrane permeability of *G. zeae* cell. They could also affect the ergosterol synthesis of fungal cell, Previously we have reported a few simple and common biochemical changes of the mycelium of *G. zeae* when treated with xa202 and xt208. Although some new points has been clarified in the present study, the exact molecular mechanism of the fungicidal activities of

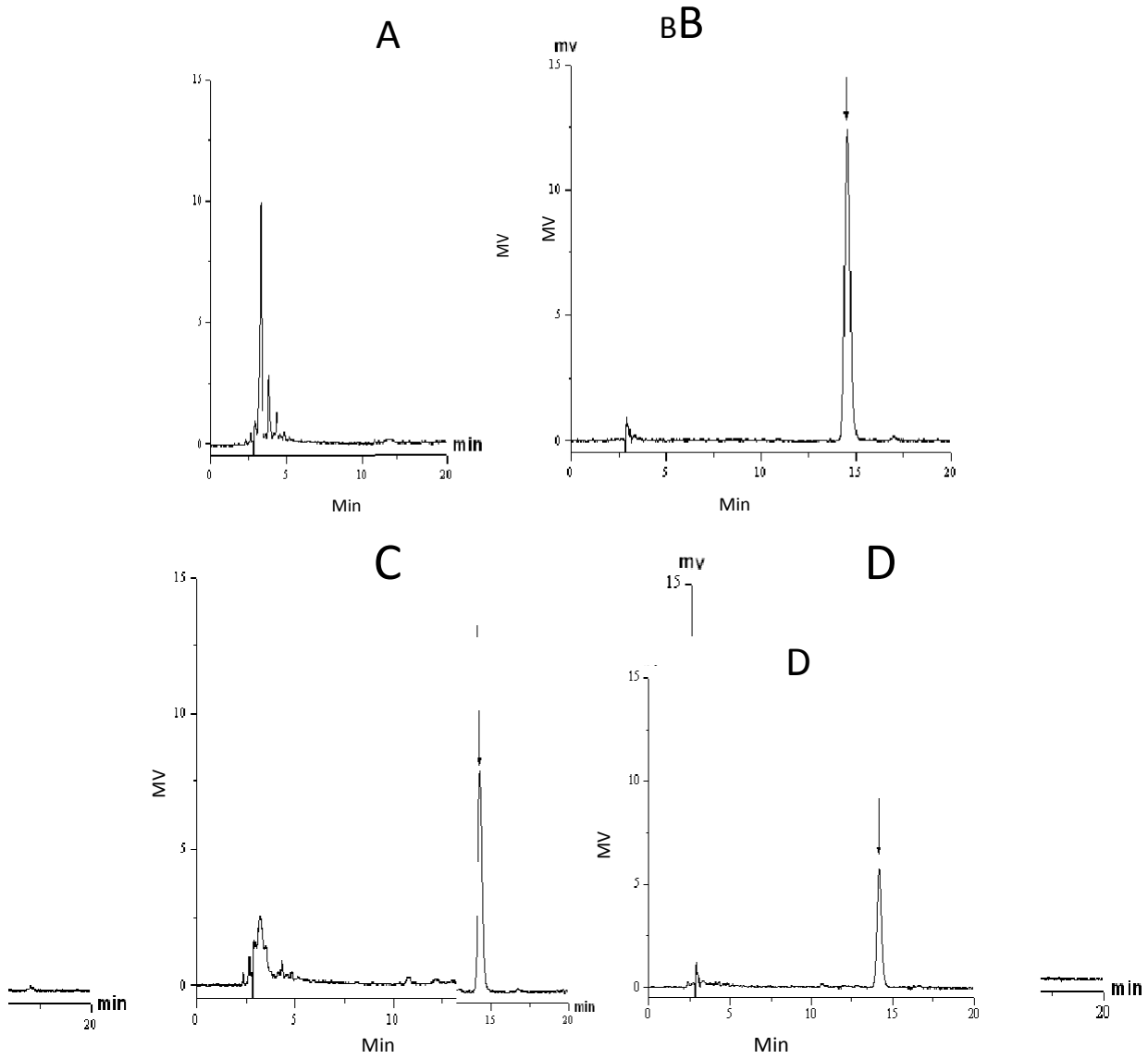

Figure 5. Chromatogram of ergosterol in mycelium with the different conditions. A: blank control; B: carbendazim; C: xa202; D: xt208.

compounds xa202 and xt208 is still under way. And considered the complication of the biochemical action inside the fungus cell, intense investigations need to be conducted in the future to unravel these questions.

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