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Structure-Activity Relationship, Cytotoxicity and Mode of Action of 2-Ester-substituted 1,5-Benzothiazepines as Potent Antifungal Agents

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Our studies examined the structural features responsible for the antifungal activity of 2-ethoxycarbonyl-1,5-benzothiazepine (**7a**). Three series of 1,5-benzothiazepine derivatives were synthesized and screened for their antifungal activity. The results suggested that the ethoxycarbonyl group at the 2 position and the imine moiety on the seven-membered ring are essential for activity. The most potent of the synthesized analogues (**7a**, **7b**) were further studied by evaluating their cytotoxicity and mode of action (for **7a**). The results showed that compounds **7a** and **7b** were relatively safe for BV_2 cells, but compound **7a** interfered with *Cryptococcus neoformans* cell wall integrity by increasing the chitinase activity. Therefore, compound **7a** was considered safe as an antifungal agent for animal cells.

Keywords 1,5-benzothiazepines, antifungal agents, antifungal mechanism, cytotoxicity, structure-activity relationships

Introduction

1,5-Benzothiazepines exhibit various biological activities, such as blocking Ca^{2+} channels,[1-3] CNS inhibition,[4,5] antiplatelet aggregation, and anti-thrombotic activity;[6] some 1,5-benzothiazepines have become clinically useful drugs.[7] In recent years, a number of 1,5-benzothiazepine derivatives have been evaluated for their antibacterial and antifungal activities,[8-15] and strong efficacy against microorganisms resulted in lead compounds for further investigation. *Cryptococcus neoformans* is an opportunistic fungal pathogen that may cause meningitis in immunocompromised individuals. Meningitis is uniformly fatal if untreated. Up to now, there are few antifungal agents, meanwhile the drug-resistant strains are emerging.. Therefore, researches on drugs against *C. neoformans* are of great significance. During the course of our research on the antimicrobial activity of 1,5-benzothiazepine derivatives, we found that 2-ester-substituted 1,5-benzothiazepine (**7a**) exhibited excellent antifungal activity against *C. neoformans* with a minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) similar to or lower than that of the standard antifungal drug fluconazole.

Based on these findings, we decided to explore the structural features that are important for its antifungal properties. Therefore, using **7a** as the prototypical structure, three series of 1,5-benzothiazepine derivatives were designed, synthesized and evaluated for their antifungal activity against *C. neoformans* (Figure 1). The first series of compounds were benzothiazepines bearing various substituents at the 4 position of the aryl ring (**7a**—**7f**) or various ester groups at the 2 position of the seven-membered ring (**7g**—**7j**) to determine the effects of these substituents on the antifungal activity. The

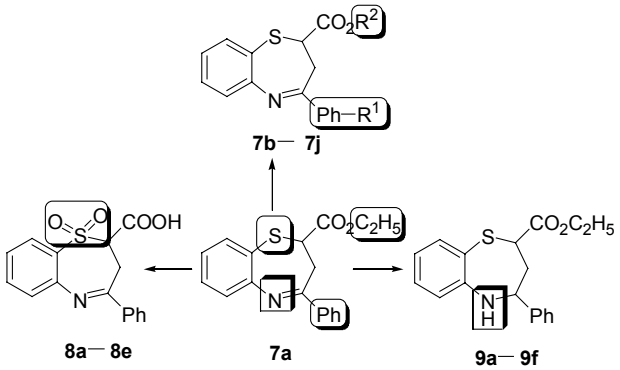

Figure 1 Structures of **7a** and other designed analogues.

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second series of compounds tested were 1,5-benzothiazepines with a sulfone unit (**8a**—**8e**) to evaluate the influence of the oxidation state of the sulfur on activity. The third series of analogues were 2,3,4,5-tetrahedron-1,5-benzothiazepines **9a**—**9f** without a C=N double bond to clarify the role of the imine moiety on the antifungal properties. Furthermore, the most effective antifungal derivatives **7a** and **7b** were further studied for their antifungal activities, cytotoxicities and modes of action (for compound **7a**).

Results and Discussion

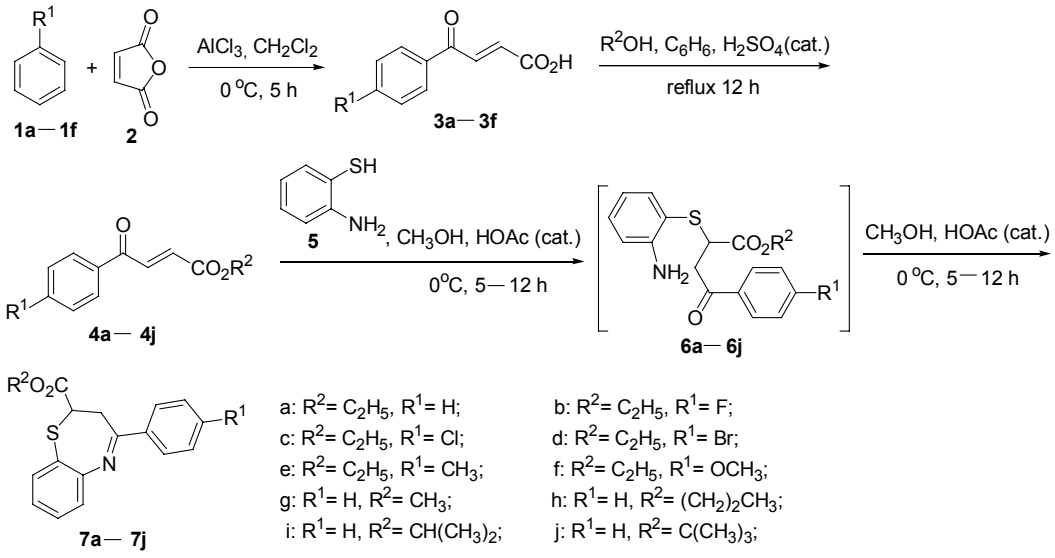
Synthesis of the target compounds

The synthetic route to compounds **7a**—**7j** is depicted in Scheme 1. Compounds **1a**—**1f** reacted with maleic anhydride **2** in the presence of anhydrous aluminum chloride to obtain 4-oxo-4-arylbutenoic acids **3a**—**3f**. The *α*,*β*-unsaturated compounds **4a**—**4j** were prepared by reacting acids **3a**—**3f** with the corresponding alcohols under reflux in a benzene-alcohol solution using concentrated sulfuric acid as a catalyst. However, *tert*-butyl ester analogue **4j** could not be prepared using the above method; therefore, it was obtained by reacting **3a** with *tert*-butyl alcohol at room temperature using anhydrous sodium sulfate as a dehydrating agent. A Michael addition of *o*-aminothiophenol **5** to 4-oxo-4-phenylbut-2-enoate **4a**—**4j** in acetic acid/methanol afforded intermediates **6a**—**6j**, which then underwent an intramolecular cyclization with the loss of water to obtain **7a**—**7j**.

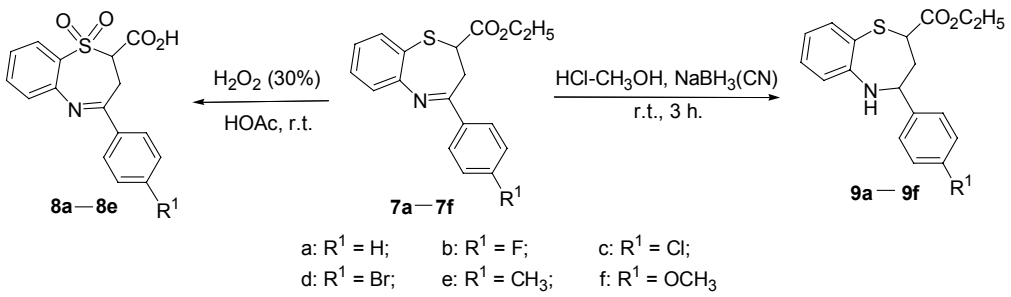
As shown in Scheme 2, compounds **8a**—**8e** were prepared by oxidation of **7a**—**7e** with 30% hydrogen peroxide in the presence of catalytic amounts of acetic acid. Initially, we wanted to synthesize 2-ester-substituted-1,5-benzothiazepines bearing a sulfone functionality, but the reaction did not generate the expected products because the ester group in the 2 position of the seven-membered ring was hydrolyzed to the carboxylic acid during the reaction. This hydrolysis occurred under various reaction conditions (*e.g.*, catalyst, solvent, temperature, and time), which were investigated in trial experiments. Therefore, we could obtain only the 2-carboxyl-substituted analogues **8a**—**8e**.

The third series of analogues **9a**—**9f** were synthesized by reducing the C=N bond of compounds **7a**—**7f**, as illustrated in Scheme 2. The C=N double bond was reduced at pH 4 with sodium cyanoborohydride in an HCl-methanol solution, the bromocresol green was used as a pH indicator. As reported in the literature,[16] so-

Scheme 1 Synthesis of compounds **7a**—**7j**



Scheme 2 Synthesis of compounds **8a**—**8e** and **9a**—**9f**



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dium cyanoborohydride is a pH dependent, selective reducing reagent; however, the ester groups in **7a**—**7f** were inert to this reagent.

All of the synthesized analogues of **7a** were purified by recrystallization with methanol as a solvent or by chromatography on a silica gel column, and their structures were confirmed by elemental analysis, IR, ^{1}H NMR, ^{13}C NMR and MS/HRMS.

In vitro antifungal activities of the synthesized compounds

The synthesized compounds **7a**—**7j** were tested against two strains of *C. neoformans* (one ATCC 34874 and another clinical isolate) using the disk diffusion method with DMSO as a control. As shown in Table 1, compounds **7c**—**7f** with electron-donating or electron-withdrawing groups on the phenyl rings at the position 4 of the seven-membered ring were basically inactive, but the fluorophenyl-substituted 1,5-benzothiazepine (**7b**) exhibited good antifungal activity (average inhibition zone diameter, 19.3 mm). These findings suggested that the electronic effect caused by substituents on the phenyl rings was not important for the bioactivity of the target compounds; however, the appropriate atom (H or F) on the phenyl ring was required. The data in Table 1 also showed that replacement of the 2-ethoxycarbonyl group with other ester groups (2-methoxycarbonyl **7g**, 2-propoxycarbonyl **7h**, 2-isopropoxycarbonyl **7i**, 2-*tert*-butyloxycarbonyl **7j**) caused a marked decrease in antifungal activity. These results demonstrated that the 2-ethoxycarbonyl group was essential for the biological activity of this family of compounds.

Table 1 Zone of growth inhibition for compounds **7a**—**7j** (mm)[a]

Compd	R^1	R^2	*C. neformans* (ATCC)	*C. neoformans* 1# (clinical isolate)
7a	H	CH_2CH_3	23.5±0.5	22.8±0.2
7b	F	CH_2CH_3	18.1±0.2	20.5±0.1
7c	Cl	CH_2CH_3	6.0±0.2	6.0±0.3
7d	Br	CH_2CH_3	6.0±0.2	6.0±0.2
7e	CH_3	CH_2CH_3	6.0±0.3	6.0±0.3
7f	OCH_3	CH_2CH_3	11.7±0.3	6.0±0.3
7g	H	CH_3	6.0±0.2	15.2±0.4
7h	H	$(CH_2)_2CH_3$	12.7±0.3	6.0±0.3
7i	H	$CH(CH_3)_2$	12.9±0.4	6.0±0.4
7j	H	$C(CH_3)_3$	6.0±0.3	6.0±0.2
control[b]	—	—	6.0±0.1	6.0±0.0

[a] All values were measured after 24 h of incubation at 37 ℃ with 200 μg of the tested compound/disk. [b] 1 equiv. volume of solvent (DMSO) was added as a control.

In order to explore the influence of the sulfur oxidation state on the antifungal activity, the sulfur atom on the seven-membered ring was converted to a sulfone, as shown in compounds **8a**—**8e**. The zone data in Table 2 showed that all of the sulfone-bearing analogues were weakly active or inactive against the microorganisms tested. These results indicated that replacement of the sulfur atom and/or ethoxycarbonyl group with a sulfone functionality and/or carboxyl group led to a marked decrease in activity (compare **8a** and **8b** with **7a** and **7b**, respectively). Possible reasons for the decreased bioactivity in **8a**—**8e** include the transformation of the esters into carboxyl groups and/or changes in the sulfur oxidation state, which will be studied further.

To determine the importance of the imine moiety, 2,3,4,5-tetrahydro-1,5-benzothiazepines **9a**—**9f** were prepared by reducing the imine functionalities of **7a**—**7f**. Surprisingly, compounds **9a** and **9b** completely lost their activity compared to **7a** and **7b**, and analogues **9c**—**9f** were also completely inactive (Table 2). These findings suggested that the imine moiety on the seven-membered ring in these benzothiazepines is required for activity.

Table 2 Zone of growth inhibition of compounds **8a**—**8e**, **9a**—**9f** (mm)[a]

Compd	R^1	*C. neoformans* (ATCC)	*C. neoformans* 1# (clinical isolate)
8a	H	9.5±0.1	11.3±0.3
8b	F	10.4±0.3	6.0±0.1
8c	Cl	6.0±0.1	6.0±0.2
8d	Br	10.5±0.2	6.0±0.1
8e	CH_3	12.1±0.4	12.0±0.3
9a	H	6.0±0.2	6.0±0.1
9b	F	6.0±0.3	6.0±0.2
9c	Cl	6.0±0.2	6.0±0.1
9d	Br	6.0±0.1	6.0±0.2
9e	CH_3	6.0±0.2	6.0±0.1
9f	OCH_3	6.0±0.2	6.0±0.1
control[b]	—	6.0±0.1	6.0±0.1

[a] All values were measured after 24 h of incubation at 37 ℃ with 200 μg of the tested compound/disk. [b] An equivalent volume of solvent (DMSO) was added as a control.

Compounds **7a** and **7b**, which had prominent antifungal activities, were subjected to further pharmacological evaluation, including determining the dose dependence of the antifungal activity, MIC, MIC_{80}, and MFC against five strains of *C. neoformans*. The data in Table 3 indicated that compounds **7a** and **7b** showed dose-dependent activity when evaluated in the concentration range of 12.5 to 200.0 μg/disk. At 12.5 μg/disk, the two compounds still exhibited moderate to high activity. The MIC, MIC_{80} and MFC values of **7a**, **7b** are shown in Table 4 and the standard drug fluconazole was used as positive control. The data showed that compounds **7a** (MIC_{80}, 0.5—4.0 μg/mL) and **7b** (MIC_{80}, 0.5—2.0 μg/mL) had MIC_{80} values very similar to that of fluconazole (MIC_{80}, 2.0—4.0 μg/mL). In contrast, the

Table 3 Zone of growth inhibition of compounds **7a** and **7b** at 200.0－12.5 μg/disk (mm) [a]

Fungal strain[b]	7a					7b				
	200.0	100.0	50.0	25.0	12.5	200.0	100.0	50.0	25.0	12.5
1	23.5±0.5	22.4±0.1	21.2±0.3	19.8±0.2	16.9±0.3	18.1±0.2	18.4±0.1	19.8±0.2	17.2±0.1	15.5±0.3
2	22.8±0.2	23.9±0.2	20.8±0.2	21.4±0.4	20.7±0.5	20.5±0.1	19.1±0.2	19.3±0.4	23.2±0.3	20.3±0.2
3	29.1±0.5	25.5±0.4	22.6±0.4	18.7±0.3	15.2±0.2	28.6±0.3	26.2±0.4	21.7±0.3	19.2±0.2	15.1±0.4
4	27.2±0.2	25.0±0.5	20.4±0.4	17.6±0.1	15.3±0.1	27.4±0.2	25.8±0.1	22.7±0.3	19.8±0.1	14.4±0.2
5	25.8±0.3	22.2±0.3	18.6±0.3	15.2±0.3	10.4±0.2	29.3±0.4	28.1±0.2	22.3±0.2	18.1±0.2	13.7±0.3

[a] All values were measured after 24 h of incubation at 37 ℃. An equivalent volume of solvent (DMSO) was added as a control, and the zone of growth inhibition of the control was 6.0±0.1. [b] 1: *C. neoformans* (ATCC), 2－5: *C. neoformans* clinically isolated, 2: *C. neoformans* 1#, 3: *C. neoformans* 2#, 4: *C. neoformans* 3#, and 5: *C. neoformans* 4#.

Table 4 MIC, MIC_{80} and MFC values for compounds **7a**, **7b** and fluconazole (μg/mL)

Fungal strain[a]	7a			7b			Fluconazole		
	MIC	MIC_{80}	MFC	MIC	MIC_{80}	MFC	MIC	MIC_{80}	MFC
1	2.5	2.0	10.0	4.0	2.0	8.0	＞128.0	2.0	＞128.0
2	4.5	4.0	7.5	1.0	0.5	15.0	＞128.0	2.0	＞128.0
3	1.0	0.5	10.0	1.5	0.5	12.5	＞128.0	3.0	＞128.0
4	2.0	1.5	10.0	2.0	0.5	13.0	＞128.0	4.0	＞128.0
5	2.0	0.5	11.5	2.5	0.5	13.0	＞128.0	4.0	＞128.0

[a] 1: *C. neoformans* (ATCC), 2－5: *C. neoformans* clinically isolated, 2: *C. neoformans* 1#, 3: *C. neoformans* 2#, 4: *C. neoformans* 3#, 5: *C. neoformans* 4#. All values were the average of three trials, and the standard deviations were zero.

MIC and MFC values for **7a** (MIC, 1.0－4.5 μg/mL; MFC, 7.5－11.5 μg/mL) and **7b** (MIC, 1.0－4.0 μg/mL; MFC, 8.0－15.0 μg/mL) were much lower than those of fluconazole (MIC, ＞128 μg/mL; MFC, ＞128 μg/mL) for all *C. neoformans* tested. Fluconazole and other triazole antifungals are main choice for the clinical treatment of the systemic fungal infections, but the obvious side effect has been found for them. As a result, new and safe antifungal agents are still needed. The compounds **7a** and **7b**, with good antifungal activity and different structural features from those of fluconazole, will become a new kind of promising antifungal compounds for further optimization.

Cytotoxicities of compounds 7a and 7b

The cytotoxicities of potential agents **7a** and **7b** were evaluated using a BV_2 cell line and a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cytotoxicity of DMSO was used as a control. The relative survival rate of the cells significantly decreased with compound **7a** at concentrations ≥200.0 μg/mL and with compound **7b** at concentrations ≥100.0 μg/mL ($p<0.01$) (Figure 2). This result indicated that compounds **7a** and **7b** were relatively safe for animal cells at concentrations less than or equal to 100.0 and 50.0 μg/mL, respectively, and the safe concentration of fluconazole for animal cells was less than or equal to 200.0 μg/mL.

Mode of action of compound 7a

Compound **7a** was safer than compound **7b** in animal cells. Therefore, the mode of action of compound

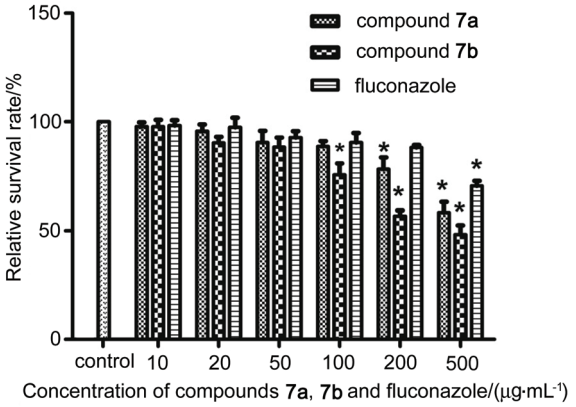

Figure 2 Effects of compounds **7a, 7b** and fluconazole on the survival of cells.

7a was further examined by testing the cell wall integrity and chitinase activity, and performing membrane permeability assays. The fungal strain *C. neoformans* (ATCC) was used.

Cell wall integrity assay One of the defining characteristics of fungi is the structure and composition of their cell walls. Consequently, a cell wall integrity assay was performed to determine whether compound **7a** interfered with cell wall function. When cell wall integrity is compromised, the rapid thermal expansion of the cells most likely results in increased cell death. The effect of compound **7a** on the cell wall function is shown in Figure 3. The number of viable cells in the treated group decreased 10-fold after 40 s of exposure at 65 ℃, indicating increased sensitivity of the treated cells to heat shock. Therefore, compound **7a** interfered with fungal

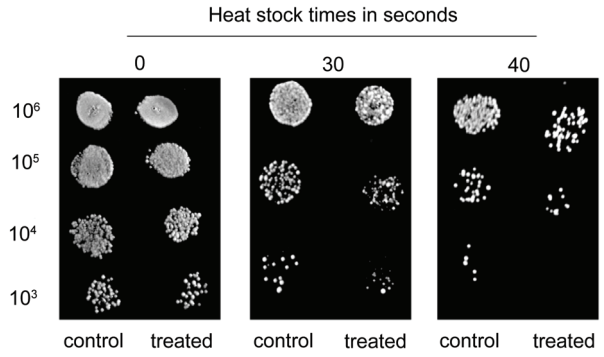

Figure 3 Effect of compound **7a** on cell wall integrity.

cell wall integrity and function.

Chitinase activity assay The chitinase activity of cells was measured after treatment with compound **7a** or 1 equiv. volume of DMSO (control group) for 0, 4, 8, 12, 24, and 32 h. The purpose was to study the influence of compound **7a** on fungal cell wall integrity. Figure 4 showed that chitinase activity significantly increased after treatment with compound **7a** for 12, 24, and 32 h, but did not significantly change in the control group ($p < 0.01$). These results indicated that compound **7a** impaired the cell wall integrity by increasing cell wall chitinase activity.

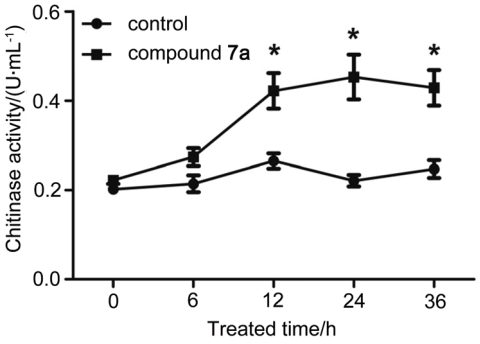

Figure 4 Effect of compound **7a** on cell wall chitinase activity.

Membrane permeability assay Interference of cell membrane function results in membrane permeability changes. Hence, using a conductivity meter, the relative cell membrane permeability rate was measured after treatment with compound **7a** or an equivalent volume of DMSO for 0, 5, 10, 30, 60, 180, and 360 min. The relative permeability rate was found to be similar to that of the control group. With longer treatment times, the relative permeability of the treatment and control groups increased at the same rate (Figure 5). This result indicated that compound **7a** did not affect membrane permeability, and that the reactive site of compound **7a** was not on the cell membrane.

Conclusions

In this study, three series of 1,5-benzothiazepine derivatives were synthesized and their antifungal activities were evaluated to determine the structure-activity relationships with respect to the anti-fungal activity of 2-ester-substituted 1,5-benzothiazepines. The effective antifungal compounds **7a** and **7b** were further studied for their antifungal activity, cytotoxicity and the mechanism of action (for compound **7a**).

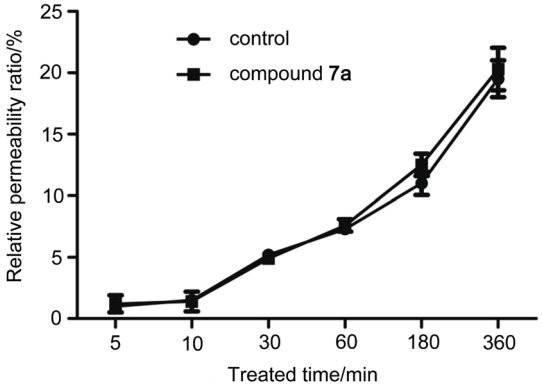

Figure 5 Effect of compound **7a** on membrane permeability.

Preliminary structure-activity relationship studies showed that the ethoxycarbonyl group at the position 2 and the imine moiety on the seven-membered ring in this class of benzothiazepines were critical to their biological activity; the presence of an H or F atom on the phenyl ring at position 4 was also important. The effect of the sulfur oxidation state on the antifungal properties requires further investigation. Of the tested compounds, **7a** and **7b** were the most promising anti-*C. neoformans* compounds, with excellent antifungal properties and low cytotoxicities. Mode of action assays indicated that the reactive site for **7a** as an antifungal agent was the cell wall and not the cell membrane. Compound **7a** may also be a safe antifungal drug for humans and animals.

Experimental

Chemistry

Melting points (℃) were determined using an X-4 digital melting point apparatus. Elemental analysis was performed using a Vario ELIII elemental analyzer without correction. Infrared spectra (in KBr pellets) were recorded using a Vertex 70 FT-IR spectrometer. ^{1}H NMR and ^{13}C NMR spectra were recorded using an AVIII500Q Bruker spectrometer. Chemical shifts (δ) were reported relative to $SiMe_4$ as the internal standard when measured in $CDCl_3$ or DMSO-d_6 solution. Mass spectra were measured using a Xevo mass spectrometer, utilizing electron ionization (EI) at an ionizing energy of 70 eV. High-resolution mass spectra were measured using an apex Ultra mass spectrometer, utilizing electrospray ionization (ESI). All reagents and solvents were dried and distilled according to standard procedures.

General method for preparation of 2,3-dihydro-2-alkoxycarbonyl-4-aryl-1,5-benzothiazepines 7a—7j

A suspension of anhydrous $AlCl_3$ (12.0 g, 90 mmol) and a substituted benzene **1a—1f** (45 mmol) in dry CH_2Cl_2 (150 mL) was cooled to 0 ℃, and maleic anhydride **2** (29.4 g, 30 mmol) in dry CH_2Cl_2 (40 mL) was added. The reaction mixture was stirred in an ice-bath (0 ℃) for 5 h. After completion of the reaction (determined by TLC), a cold aqueous solution of 4 mol/L HCl (100 mL) was added to decompose unreacted $AlCl_3$. The mixture was poured into ice water to precipitate out the crude product, which then was filtered and added to an aqueous, saturated solution of $NaHCO_3$. The resulting mixture was refluxed for 1 h and then filtered to remove the insoluble impurities. Next, the filtrate was acidified with concentrated HCl to crystallize the product, which was filtered and dried to produce compounds **3a—3f**.

To a solution of 4-oxo-4-arylbutenoic acid **3a—3f** (100 mmol) in anhydrous benzene (100 mL), an alcohol (300 mmol) and a catalytic amount of concentrated H_2SO_4 were added. The reaction mixture was refluxed for 12 h with stirring. After cooling, the organic layer was washed with a cold, aqueous, saturated solution of $NaHCO_3$; an aqueous, saturated solution of NaCl; and water. The organic layer was dried and evaporated under reduced pressure, and the crude products **4a—4j** were purified by crystallization from ether.

o-Aminothiophenol **5** (1.25 g, 10 mmol) in dry methanol (10 mL) was added to a solution of **4a—4j** (10 mmol) in dry methanol (20 mL) using acetic acid as a catalyst. The reaction mixture was stirred at 0 ℃ for 5—12 h, then concentrated under reduced pressure to obtain the crude product. The product was either crystallized with methanol or purified by chromatography on a silica gel column with petroleum ether/ethyl acetate (6∶4, *V*/*V*) as the eluent to obtain the corresponding product **7a—7j**.

2-Ethoxycarbonyl-4-phenyl-2,3-dihydro-1,5-benzothiazepine (7a) Yield 2.44 g, 80%; m.p. 99—101 ℃; ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.29 (t, J=7.3 Hz, 3H, -C-CH_3), 4.21 (q, J=7.3 Hz, 2H, -CO-CH_2-), 3.13 (dd, J=13.0, 12.5 Hz, 1H, -C-H), 3.23 (dd, J=5.0, 13.0 Hz, 1H, -C-H), 4.33 (dd, J=5.0, 12.5 Hz, 1H, -S-C-H), 7.11—8.05 (m, 9H, Ph-H); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 14.0, 31.4, 55.9, 61.5, 121.0, 124.8, 125.2, 127.2, 128.7, 130.4, 131.2, 135.4, 137.4, 152.6, 169.2, 170.2; IR (KBr) ν: 1728, 1612 cm^{-1}; MS (70 eV) *m*/*z*: 312.50 $(M+H)^+$. Anal. calcd for $C_{18}H_{17}NO_2S$: C 69.44, H 5.52, N 4.50; found C 69.43, H 5.50, N 4.50.

2-Ethoxycarbonyl-4-(4-fluorophenyl)-2,3-dihydro-1,5-benzothiazepine (7b) Yield 2.79 g, 85%; m.p. 110—111 ℃; ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.29 (t, J=7.0 Hz, 3H, -C-CH_3), 4.21 (q, J=7.0 Hz, 2H, -CO-CH_2-), 3.12 (dd, J=12.0, 13.5 Hz, 1H, -C-H), 3.17 (dd, J=6.0, 13.5 Hz, 1H, -C-H), 4.31 (dd, J=6.0, 12.0 Hz, 1H, -S-C-H), 7.08—8.06 (m, 8H, Ph-H); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 14.0, 31.4, 55.9, 61.6, 115.7, 115.8, 124.9, 125.3, 125.4, 129.5, 129.6, 130.5, 133.4, 163.8, 165.8, 168.0, 170.2; IR (KBr) ν: 1726, 1601 cm^{-1}. HRMS-ESI *m*/*z*: $[M+H]^+$ calcd for $C_{18}H_{16}FNO_2S$: 330.09640, found 330.09234.

2-Ethoxycarbonyl-4-(4-chlorophenyl)-2,3-dihydro-1,5-benzothiazepine (7c) Yield 2.76 g, 80%; m.p. 87—88 ℃; ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.29 (t, J=7.0 Hz, 3H, -C-CH_3), 4.21 (q, J=7.0 Hz, 2H, -CO-CH_2-), 3.11 (dd, J=11.5, 13.5 Hz, 1H, -C-H), 3.15 (dd, J=6.0, 13.5 Hz, 1H, -C-H), 4.31 (dd, J=6.0, 11.5 Hz, 1H, -S-C-H), 7.09—7.91 (m, 8H, Ph-H); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 14.0, 18.4, 31.4, 56.0, 58.4, 61.7, 121.0, 124.9, 125.5, 128.7, 129.0, 130.5, 135.5, 135.8, 137.5, 152.3, 168.1, 170.1; IR (KBr) ν: 1720, 1611 cm^{-1}; MS (70 eV) *m*/*z*: 345.93 $(M+H)^+$. Anal. calcd for $C_{18}H_{16}ClNO_2S$: C 62.57, H 4.65, N 4.40; found C 62.51, H 4.66, N 4.05.

2-Ethoxycarbonyl-4-(4-bromophenyl)-2,3-dihydro-1,5-benzothiazepine (7d) Yield 2.95 g, 76%; m.p. 89—91 ℃; ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.29 (t, J=7.0 Hz, 3H, -C-CH_3), 4.21 (q, J=7.0 Hz, 2H, -CO-CH_2-), 3.12 (dd, J=12.0, 13.0 Hz, 1H, -C-H), 3.16 (dd, J=6.0, 13.0 Hz, 1H, -C-H), 4.31 (dd, J=6.0, 12.0 Hz, 1H, -S-C-H), 7.09—7.99 (m, 8H, Ph-H); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 14.0, 31.3, 56.0, 61.63, 121.0, 124.9, 125.5, 126.0, 128.8, 130.5, 131.9, 135.5, 136.3, 152.3, 168.1, 170.1; IR (KBr) ν: 1720, 1609 cm^{-1}; MS (70 eV) *m*/*z*: 389.82 $(M+H)^+$. Anal. calcd for $C_{18}H_{16}BrNO_2S$: C 55.35, H 4.22, N 3.54; found C 55.39, H 4.13, N 3.59.

2-Ethoxycarbonyl-4-(4-methylphenyl)-2,3-dihydro-1,5-benzothiazepine (7e) Yield 2.92 g, 90%; m.p. 86—88 ℃; ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.29 (t, J=7.0 Hz, 3H, -C-CH_3), 2.43 (s, 3H, Ph-CH_3), 4.21 (q, J=7.0 Hz, 2H, -CO-CH_2-), 3.11 (dd, J=13.0, 12.5 Hz, 1H, -C-H), 3.22 (dd, J=5.0, 13.0 Hz, 1H, -C-H), 4.31 (dd, J=5.0, 12.5 Hz, 1H, -S-C-H), 7.08—7.96 (m, 8H, Ph-H); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 14.0, 20.1, 40.4, 44.6, 57.7, 61.3, 119.5, 120.3, 128.8, 126.6, 128.2, 129.5, 132.8, 137.5, 140.4, 148.9, 170.9; IR (KBr) ν: 1720, 1607 cm^{-1}; MS (70 eV) *m*/*z*: 325.97 $(M+H)^+$. Anal. calcd for $C_{19}H_{19}NO_2S$: C 70.13, H 5.89, N 4.22; found C 70.12, H 5.88, N 4.30.

2-Ethoxycarbonyl-4-(4-methoxyphenyl)-2,3-dihydro-1,5-benzothiazepine (7f) Yield 1.97 g, 58%; m.p. 76—77 ℃; ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.23 (t, J=7.0 Hz, 3H, -C-CH_3), 3.82 (s, 3H, Ph-OCH_3), 4.14 (q, J=7.0 Hz, 2H, -CO-CH_2-), 3.03 (dd, J=13.0, 12.5 Hz, 1H, -C-H), 3.12 (dd, J=5.0, 13.0 Hz, 1H, -C-H), 4.24 (dd, J=5.0, 12.5 Hz, 1H, -S-C-H), 6.91—7.94 (m, 8H, Ph-H); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 14.1, 37.1, 37.2, 55.5, 55.6, 61.7, 113.8, 117.0, 123.9, 127.3, 128.1, 130.5, 135.6, 163.8, 167.2, 170.1, 194.1; IR (KBr) ν: 1720, 1601 cm^{-1}; MS (70 eV) *m*/*z*: 341.85 $(M+H)^+$. Anal. calcd for $C_{19}H_{19}NO_3S$: C 63.07, H 5.87, N 3.60; found C 63.44, H 5.61, N 4.01.

2-Methoxycarbonyl-4-phenyl-2,3-dihydro-1,5-benzothiazepine (7g) Yield 1.19 g, 40%; m.p. 69—70 ℃; ^{1}H NMR (500 MHz, $CDCl_3$) δ: 3.77 (s, 3H, -CO-CH_3), 3.13 (dd, J=13.5, 12.5 Hz, 1H, -C-H), 3.24 (dd,

J=5.5, 13.5 Hz, 1H, -C-H), 4.36 (dd, *J*=5.5, 12.5 Hz, 1H, -S-C-H), 7.09−8.04 (m, 9H, Ph-H); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 37.1, 37.5, 117.0, 120.1, 124.0, 127.4, 128.2, 128.7, 133.5, 135.9, 136.3, 169.0, 195.6; IR (KBr) *v*: 1734, 1612 cm^{-1}; MS (70 eV) *m*/*z*: 298.04 $(M+H)^+$. Anal. calcd for $C_{17}H_{15}NO_2S$: C 63.73, H 4.99, N 4.34; found C 63.86, H 5.08, N 4.71.

2-Propoxycarbonyl-4-phenyl-2,3-dihydro-1,5-benzothiazepine (7h) Yield 1.62 g, 50%; m.p. 55−57 ℃; ^{1}H NMR (500 MHz, $CDCl_3$) δ: 0.97 (t, *J*=7.5 Hz, 3H, -C-CH_3), 1.65−1.72 (m, 2H, -C-CH_2-), 4.07−4.16 (m, 2H, -CO-CH_2-), 3.13 (dd, *J*=13.0, 12.5 Hz, 1H, -C-H), 3.24 (dd, *J*=5.5, 13.0 Hz, 1H, -C-H), 4.35 (dd, *J*=5.5, 12.5 Hz, 1H, -S-C-H), 7.09−8.05 (m, 9H, Ph-H); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 10.3, 21.9, 37.1, 55.9, 67.2, 117.3, 123.8, 127.3, 127.9, 128.2, 128.6, 128.8, 133.4, 135.5, 167.7, 170.3, 195.7; IR (KBr) *v*: 1728, 1611 cm^{-1}; MS (70 eV) *m*/*z*: 325.87 $(M+H)^+$. Anal. calcd for $C_{19}H_{19}NO_2S$: C 69.78, H 5.80, N 4.28; found C 70.12, H 5.88, N 4.30.

2-Isopropoxycarbonyl-4-phenyl-2,3-dihydro-1,5-benzothiazepine (7i) Yield 1.78 g, 55%; m.p. 70−71 ℃; ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.13 (d, *J*=6.5 Hz, 3H, -C-CH_3), 1.20 (d, *J*=6.0 Hz, 3H, -C-CH_3), 4.94−4.99 (m, 1H, -CO-CH-), 3.36 (dd, *J*=5.5, 18.0 Hz, 1H, -C-H), 3.64 (dd, *J*=9.0, 18.0 Hz, 1H, -C-H), 4.12 (dd, *J*=5.5, 9.0 Hz, 1H, -S-C-H), 6.64−7.93 (m, 9H, Ph-H); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 21.4, 21.6, 37.2, 37.5, 56.0, 117.3, 119.6, 123.9, 127.3, 127.9, 128.2, 128.6, 133.4, 136.0, 136.4, 167.8, 195.8; IR (KBr) *v*: 1680, 1614 cm^{-1}; MS (70 eV) *m*/*z*: 325.87 $(M+H)^+$. Anal. calcd for $C_{19}H_{19}NO_2S$: C 69.95, H 5.90, N 4.17; found C 70.12, H 5.88, N 4.30.

2-*tert*-Butyloxycarbonyl-4-phenyl-2,3-dihydro-1,5-benzothiazepine (7j) Yield 2.57 g, 76%; m.p. 101−103 ℃; ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.47 (s, 9H, -C-$(CH_3)_3$), 3.07 (dd, *J*=12.5, 13.5 Hz, 1H, -C-H), 3.17 (dd, *J*=5.5, 13.5 Hz, 1H, -C-H), 4.23 (dd, *J*=5.5, 12.5 Hz, 1H, -S-C-H), 7.07−8.04 (m, 9H, Ph-H); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 27.8, 31.6, 57.1, 81.9, 121.3, 124.7, 125.2, 127.3, 128.7, 130.3, 131.2, 135.5, 137.5, 152.5, 169.3, 169.6; IR (KBr) *v*: 1718, 1607 cm^{-1}; MS (70 eV) *m*/*z*: 340.32 $(M+H)^+$. Anal. calcd for $C_{20}H_{21}NO_2S$: C 70.80, H 6.33, N 4.03; found C 70.77, H 6.24, N 4.13.

General method for preparation of sulfonate-containing 1,5-benzothiazepine derivatives 8a−8e

Aqueous hydrogen peroxide (30%, 3 mL) was added to a solution of 2,3-dihydro-2-ethoxycarbonyl-4-aryl-1,5-benzothiazepine **7a**−**7e** (1 mmol) in acetic acid (20 mL). The reaction mixture was stirred at room temperature until the starting material was consumed (determined by TLC). A small amount of MnO_2 was added to eliminate unreacted H_2O_2. The reaction mixture was then filtered and extracted with diethyl ether (10 mL×3). The combined organic phases were dried with anhydrous $MgSO_4$ and evaporated *in vacuo* to afford the crude residue. This residue was purified by a silica gel column using petroleum ether/ethyl acetate/acetic acid (8∶2∶1, *V*/*V*/*V*) as the eluent.

4-Phenyl-2,3-dihydro-1,5-benzothiazepine-2-carboxylic acid 1,1-dioxide (8a) Yield 0.16 g, 52%; m.p. 208−210 ℃; ^{1}H NMR (500 MHz, DMSO-d_6) δ: 3.61 (dd, *J*=3.5, 18.0 Hz, 1H, -C-H), 3.88 (dd, *J*=8.5, 17.8 Hz, 1H, -C-H), 5.28 (dd, *J*=3.5, 8.5 Hz, 1H, -S-C-H), 11.41 (s, 1H, -CO-OH), 7.25−8.10 (m, 9H, Ph-H); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 29.0, 60.2, 118.7, 123.5, 123.8, 125.3, 128.3, 128.8, 133.7, 135.2, 135.8, 163.5, 194.6; IR (KBr) *v*: 1679, 1579, 1168, 1158 cm^{-1}; MS (70 eV) *m*/*z*: 315.94 $(M+H)^+$. Anal. calcd for $C_{16}H_{13}NO_4S$: C 59.83, H 4.25, N 4.18; found C 60.24, H 4.16, N 4.44.

4-(4-Fluorophenyl)-2,3-dihydro-1,5-benzothiazepine-2-carboxylic acid 1,1-dioxide (8b) Yield 0.18 g, 55%; m.p. 237−238 ℃; ^{1}H NMR (500 MHz, DMSO-d_6) δ: 3.61 (dd, *J*=3.5, 17.5 Hz, 1H, -C-H), 3.87 (dd, *J*=8.5, 18.0 Hz, 1H, -C-H), 5.28 (dd, *J*=3.5, 8.5 Hz, 1H, -S-C-H), 11.41 (s, 1H, -CO-OH), 7.24−8.20 (m, 8H, Ph-H); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 29.0, 60.2, 115.8, 115.9, 118.7, 123.5, 123.8, 125.3, 131.4, 132.6, 132.6, 135.8, 163.5, 164.3, 166.3, 193.3; IR (KBr) *v*: 1693, 1596, 1172, 1160 cm^{-1}. HRMS-ESI *m*/*z*: $[M+Na]^+$ calcd for $C_{16}H_{12}FNO_4S$: 356.03633, found 356.03548.

4-(4-Chlorophenyl)-2,3-dihydro-1,5-benzothiazepine-2-carboxylic acid 1,1-dioxide (8c) Yield 0.16 g, 45%; m.p. 230−232 ℃; ^{1}H NMR (500 MHz, DMSO-d_6) δ: 3.61 (dd, *J*=3.5, 18 Hz, 1H, -C-H), 3.86 (dd, *J*=8.5, 17.8 Hz, 1H, -C-H), 5.28 (dd, *J*=3.5, 8.5 Hz, 1H, -S-C-H), 11.40 (s, 1H, -CO-OH), 7.24−8.12 (m, 8H, Ph-H); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 29.0, 30.7, 60.2, 118.7, 123.5, 123.8, 125.3, 129.0, 130.3, 134.5, 135.2, 135.8, 138.6, 163.5, 193.9; IR (KBr) *v*: 1692, 1592, 1172, 1157 cm^{-1}; MS (70 eV) *m*/*z*: 350.03 $(M+H)^+$. Anal. calcd for $C_{16}H_{12}ClNO_4S$: C 54.90, H 3.51, N 3.84; found C 54.94, H 3.46, N 4.00.

4-(4-Bromophenyl)-2,3-dihydro-1,5-benzothiazepine-2-carboxylic acid 1,1-dioxide (8d) Yield 0.16 g, 40%; m.p. 234−236 ℃; ^{1}H NMR (500 MHz, DMSO-d_6) δ: 3.60 (dd, *J*=3.5, 17.8 Hz, 1H, -C-H), 3.85 (dd, *J*=8.5, 17.8 Hz, 1H, -C-H), 5.26 (dd, *J*=3.5, 8.3 Hz, 1H, -S-C-H), 11.39 (s, 1H, -CO-OH), 7.24−8.03 (m, 8H, Ph-H); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 21.2, 28.8, 60.1, 118.7, 123.5, 123.7, 125.3, 128.5, 129.4, 135.2, 135.8, 144.2, 163.5, 194.0; IR (KBr) *v*: 1720, 1587, 1171, 1154 cm^{-1}; MS (70 eV) *m*/*z*: 393.84 $(M+H)^+$. Anal. calcd for $C_{16}H_{12}BrNO_4S$: C 48.78, H 3.30, N 3.38; found C 48.74, H 3.07, N 3.55.

4-(4-Methylphenyl)-2,3-dihydro-1,5-benzothiazepine-2-carboxylic acid 1,1-dioxide (8e) Yield 0.16 g, 50%; m.p. 232−234 ℃; ^{1}H NMR (500 MHz, DMSO-d_6) δ: 2.41 (s, 3H, Ph-CH_3), 3.56 (dd, *J*=3.5, 17.8 Hz, 1H, -C-H), 3.84 (dd, *J*=8.5, 17.8 Hz, 1H, -C-H), 5.24 (dd, *J*=3.5, 8.3 Hz, 1H, -S-C-H), 11.39 (s, 1H, -CO-OH), 7.25−8.00 (m, 8H, Ph-H); ^{13}C NMR (125 MHz,

DMSO-d_6) δ: 29.0, 30.7, 60.2, 118.7, 123.5, 123.8, 125.3, 127.9, 130.4, 131.9, 135.2, 135.8, 163.5, 194.1; IR (KBr) v: 1720, 1587, 1171, 1154 cm^{-1}; MS (70 eV) m/z: 330.00 $(M+H)^+$. Anal. calcd for $C_{17}H_{15}NO_4S$: C 61.62, H 4.64, N 4.11; found C 61.99, H 4.59, N 4.25.

General method for preparation of 2-ethoxycarbonyl-4-aryl-2,3,4,5-tetrahydro-1,5-benzothiazepines 9a—9f

A trace of bromocresol green in a 2 mol/L HCl-methanol solution was added to a solution of 2,3-dihydro-2-ethoxycarbonyl-4-aryl-1,5-benzothiazepine **7a—7f** (0.11 mmol) in dry methanol (4 mL). When the solution turned yellow, $NaBH_3CN$ (0.7 g, 0.23 mmol) was added. The mixture was then stirred under a N_2 atmosphere at room temperature for 3 h. During the reaction, a HCl-methanol solution was added dropwise to maintain the yellow color. After completion of the reaction (determined by TLC), the mixture was poured into a saturated $NaHCO_3$ solution (5 mL), and the organic layer was separated. The aqueous layer was extracted with three 5 mL portions of ethyl acetate. The combined organic phases were dried with anhydrous $MgSO_4$ and evaporated *in vacuo* to obtain the crude product. The crude mass was purified by crystallizing from methanol or by chromatography using petroleum ether/ethyl acetate (5∶1, *V*/*V*) as the eluent.

2-Ethoxycarbonyl-4-phenyl-2,3,4,5-tetrahydro-1,5-benzothiazepine (9a) Yield 0.32 g, 93%; m.p. 85—86 ℃; ^{1}H NMR (500 MHz, DMSO-d_6) δ: 1.16 (t, J=7.0 Hz, 3H, -C-CH_3), 4.03—4.10 (m, 3H, -CO-CH_2-, -S-C-H), 2.21—2.26 (m, 1H, -C-H), 2.38—2.44 (m, 1H, -C-H), 4.96—4.98 (m, 1H, -N-C-H), 5.38 (d, J=1.5 Hz, 1H, -N-H), 6.70—7.53 (m, 9H, Ph-H); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 14.1, 40.4, 44.5, 58.2, 61.4, 119.7, 120.6, 121.2, 126.8, 127.9, 128.4, 129.0, 133.0, 143.4, 149.0, 171.0; IR (KBr) v: 3393, 1723, 1587 cm^{-1}; MS (70 eV) m/z: 314.28 $(M+H)^+$. Anal. calcd for $C_{18}H_{19}NO_2S$: C 68.48, H 6.13, N 4.44; found C 68.98, H 6.11, N 4.47.

2-Ethoxycarbonyl-4-(4-fluorophenyl)-2,3,4,5-tetrahydro-1,5-benzothiazepine (9b) Yield 0.35 g, 95%; m.p. 92—93 ℃; ^{1}H NMR (500 MHz, DMSO-d_6) δ: 1.16 (t, J=7.0 Hz, 3H, -C-CH_3), 4.08 (q, J=7.0 Hz, 2H, -CO-CH_2-), 3.97 (dd, J=4.5, 10.0 Hz, 1H, -S-C-H), 2.20—2.25 (m, 1H, -C-H), 2.38—2.43 (m, 1H, -C-H), 4.96—4.98 (m, 1H, -N-C-H), 5.43 (d, J=3.0 Hz, 1H, -N-H), 6.71—7.60 (m, 8H, Ph-H); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 14.0, 40.1, 44.0, 57.2, 61.3, 115.6, 115.8, 119.8, 120.5, 121.2, 128.3, 128.4, 128.5, 133.0, 139.2, 148.8, 161.1, 163.1, 170.9; IR (KBr) v: 3402, 1736, 1587 cm^{-1}. HRMS-ESI m/z: $[M+H]^+$ calcd for $C_{18}H_{18}FNO_2S$: 332.11150, found 332.10808.

2-Ethoxycarbonyl-4-(4-chlorophenyl)-2,3,4,5-tetrahydro-1,5-benzothiazepine (9c) Yield 0.34 g, 90%; m.p. 88—90 ℃; ^{1}H NMR (500 MHz, DMSO-d_6) δ: 1.16 (t, J=7.0 Hz, 3H, -C-CH_3), 4.07 (q, J=7.0 Hz, 2H, -CO-CH_2-), 3.90 (dd, J=4.5, 10.0 Hz, 1H, -S-C-H), 2.21—2.26 (m, 1H, -C-H), 2.37—2.43 (m, 1H, -C-H), 4.92—4.93 (m, 1H, -N-C-H), 5.47 (d, J=3.5 Hz, 1H, -N-H), 6.72—7.59 (m, 8H, Ph-H); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 14.1, 39.9, 43.9, 57.6, 61.5, 120.1, 121.0, 121.8, 128.2, 128.8, 129.1, 129.2, 133.3, 133.7, 170.9; IR (KBr) v: 3335, 1722, 1585 cm^{-1}; MS (70 eV) m/z: 347.9 $(M+H)^+$. Anal. calcd for $C_{18}H_{18}ClNO_2S$: C 62.14, H 5.31, N 3.91; found C 62.15, H 5.22, N 4.03.

2-Ethoxycarbonyl-4-(4-bromophenyl)-2,3,4,5-tetrahydro-1,5-benzothiazepine (9d) Yield 0.34 g, 80%; m.p. 87—88 ℃; ^{1}H NMR (500 MHz, DMSO-d_6) δ: 1.16 (t, J=7.0 Hz, 3H, -C-CH_3), 4.08 (q, J=7.0 Hz, 2H, -CO-CH_2-), 3.89 (dd, J=4.5, 10.0 Hz, 1H, -S-C-H), 2.21—2.26 (m, 1H, -C-H), 2.37—2.43 (m, 1H, -C-H), 4.90—4.92 (m, 1H, -N-C-H), 5.48 (d, J=3.5 Hz, 1H, -N-H), 6.72—7.60 (m, 8H, Ph-H); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 14.1, 39.8, 43.8, 57.4, 61.4, 119.9, 120.7, 121.5, 121.7, 128.5, 128.7, 132.0, 133.2, 142.4, 148.7, 170.9; IR (KBr) v: 3328, 1721, 1584 cm^{-1}; MS (70 eV) m/z: 391.86 $(M+H)^+$. Anal. calcd for $C_{18}H_{18}BrNO_2S$: C 54.90, H 4.67, N 3.42; found C 55.11, H 4.62, N 3.57.

2-Ethoxycarbonyl-4-(4-methylphenyl)-2,3,4,5-tetrahydro-1,5-benzothiazepine (9e) Yield 0.34 g, 95%; m.p. 80—81 ℃; ^{1}H NMR (500 MHz, DMSO-d_6) δ: 1.16 (t, J=7.0 Hz, 3H, -C-CH_3), 2.31 (s, 3H, Ph-CH_3), 4.05—4.10 (m, 3H, -CO-CH_2-, -S-C-H), 2.18—2.23 (m, 1H, -C-H), 2.37—2.42 (m, 1H, -C-H), 4.93—4.95 (m, 1H, -N-C-H), 5.32 (d, J=2.5 Hz, 1H, -N-H), 6.69—7.41 (m, 8H, Ph-H); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 14.0, 21.0, 40.4, 44.6, 57.7, 61.3, 119.5, 120.3, 120.8, 126.6, 128.2, 129.5, 132.8, 137.5, 140.4, 148.9, 170.9; IR (KBr) v: 3371, 1724, 1585 cm^{-1}; MS (70 eV) m/z: 327.90 $(M+H)^+$. Anal. calcd for $C_{19}H_{21}NO_2S$: C 69.38, H 6.43, N 4.09; found C 69.69, H 6.46, N 4.28.

2-Ethoxycarbonyl-4-(4-methoxyphenyl)-2,3,4,5-tetrahydro-1,5-benzothiazepine (9f) Yield 0.27 g, 72%; m.p. 86—87 ℃; ^{1}H NMR (500 MHz, DMSO-d_6) δ: 1.16 (t, J=7.0 Hz, 3H, -C-CH_3), 3.76 (s, 3H, Ph-OCH_3), 4.06—4.10 (m, 3H, -CO-CH_2-, -S-C-H), 2.17—2.22 (m, 1H, -C-H), 2.37—2.42 (m, 1H, -C-H), 4.94—4.96 (m, 1H, -N-C-H), 5.29 (d, J=2.5 Hz, 1H, -N-H), 6.68—7.44 (m, 8H, Ph-H); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 14.1, 40.4, 44.6, 55.2, 57.4, 61.3, 114.2, 119.5, 120.3, 120.8, 127.9, 128.3, 132.8, 135.5, 148.9, 159.1, 171.0; IR (KBr) v: 3365, 1728, 1581 cm^{-1}; MS (70 eV) m/z: 343.86 $(M+H)^+$. Anal. calcd for $C_{19}H_{21}NO_3S$: C 66.18, H 6.21, N 3.95; found C 66.45, H 6.16, N 4.08.

Biological evaluations

Cytotoxicity and chitinase activity assays were performed using an Epoch microplate spectrophotometer (BioTek, USA). Membrane permeability was measured using a DDS-307 conductivity meter.

Evaluation of antifungal activity

Inhibition zones were measured by the Espinel-Ingroff disk diffusion method[17] at 12.5—200.0 μg of the tested compound/disk, and the inhibition zones of an

equivalent volume of solvent (DMSO) were measured as a control. The diameter of the disk was 6 mm. Fungi were grown in yeast peptone dextrose (YPD) liquid nutrient medium at 30 ℃, and the cultures were shaken at 200 r/min for 36 h. When the mid-log phase was reached, the fungi concentration was adjusted to $(1-3)\times10^6$ colony forming units (CFU)/mL for the tests.

MIC assays were performed in sterile 96-well plates using the method described by Sarmiento.[18] MIC was recorded as the compound concentration that produced 100% growth reduction. MIC_{80} was recorded as the concentration that produced 80% growth reduction compared to wells with no compound present. The MIC assays were repeated three times.

MFC assays were also conducted in sterile 96-well plates with different compound concentrations inoculated with 200 μL of fungi suspension (2×10^3 CFU/mL). The plates were incubated for 36 h at 30 ℃, and growth was visually observed. Approximately 200 μL of fungi suspension from the wells that did not show growth were plated on nutrient agar. The MFC is the concentration at which fungi failed to grow in the liquid nutrient medium and nutrient agar inoculated with 200 μL of suspension. The MFC assays were repeated three times.

Evaluation of cytotoxicity

The BV_2 cells were cultured at 37 ℃ in 96-well plates at a density of 4×10^4 cells per well with 5% CO_2 in Dulbecco's Modified Eagle Medium (Invitrogen, GIBCO) supplemented with 10% fetal bovine serum (Invitrogen) for 72 h. Compounds **7a**, **7b** and fluconazole were added to each well at final concentrations of 10.0, 20.0, 50.0, 100.0, 200.0, and 500.0 μg/mL. An equivalent volume of DMSO without the compound was added as a control. The cells were incubated for 48 h. Subsequently, 10 μL of aqueous MTT solution (5.0 mg/mL) and the mixture were incubated at 37 ℃ for 4 h. The MTT solution was carefully decanted off, and 100 μL of DMSO was added to each well. The color was measured with an Epoch microplate spectrophotometer at 490 nm with the reference filter set to 620 nm. All MTT assays were repeated three times. Each measurement contained six parallel treatments (wells).

relative survival rate=(treatment A_{490}/negative control A_{620})×100%

Mode of action studies

The fungal strain tested was *C. neoformans* (ATCC), and the concentration of compound **7a** was 100 μg/mL.

Cell wall integrity assay About 2 mL of a 10^6 cells/mL culture of fungus in the mid-log phase was transferred to a culture tube. Compound **7a** or an equivalent volume of solvent (DMSO) without the compound was then added. The cultures were incubated for 12 h with gentle shaking at 30 ℃, and the cell density was adjusted to 10^6 cells/mL. Cell wall disruption was performed by heat-shock treatments in a 65 ℃ water bath for 0, 30, and 40 s. Cell viability was estimated by drop-plate assays using a 10-fold dilution series. About 10 μL of the treated culture was added to YPD nutrient agar and incubated at 30 ℃ for 72 h.

Chitinase activity Fungus cells were grown in YPD liquid nutrient medium at 30 ℃ for 24 h. Compound **7a** or an equivalent volume of DMSO was then added. Cells were harvested at 0, 4, 8, 12, 24, and 32 h. They were washed and ground on ice until at least 95% of cells were disrupted. Chitinase activity was measured in mixed membrane fractions as previously described.[19] One unit of activity was defined as the amount of enzyme mixture that liberated 1 μmol of reducing sugar per minute.

Membrane permeability assay Membrane permeability was measured using a conductivity meter. The cells in mid-log phase were harvested, thrice washed with sterile distilled water, and resuspended in the same buffer to $(1-3)\times10^6$ CFU/mL. Compound **7a** or an equivalent volume of DMSO was then added. The mixture was incubated at 30 ℃, and the conductivity was measured at 0, 5, 10, 30, 60, 180, and 360 min. The mixture was boiled, and the conductivity was measured again. The relative permeability rate was calculated.

Statistical analysis

Values were recorded as the means ±SD. All data were analyzed by the Student's t-test. Differences were considered statistically significant when $p<0.01$.

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