微生物学通报
Microbiology China
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Jun. 20, 2017, 44(6): 1437−1443
http://journals.im.ac.cn/wswxtbcn
DOI: 10.13344/j.microbiol.china.170118

研究报告

Synthesis, characterizations and antimicrobial activities of new thiourea derivatives

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Abstract: [Objective] This study aimed to synthesize novel thiourea derivatives with good antimicrobial activities. **[Methods**] Thiourea derivatives were synthesized through condensation of various primary amines with the *α*-isothiocyanatoacrylic ester (ICE) intermediate; their structures were determined by MS and NMR analysis and their antimicrobial activities were then evaluated. **[Results]** Six novel thiourea derivatives and one new *α*-isothiocyanatoacrylic ester derivative were synthesized through condensation of corresponding primary amines with *α*-isothiocyanatoacrylic ester. The bioactivities of these compounds were tested against several representative pathogenic bacterium and fungus strains. Specifically, the thiourea derivatives showed considerable inhibition activities against *Cryptococcus neoformans*, the pathogen fungus of cryptococcosis. **[Conclusion]** Synthesis of new thiourea derivatives and test their biological activities is a potential way to discover small molecule drug leads.

Keywords: Thiourea derivatives, Isothiocyanate, Antibacterial, *Cryptococcus neoformans*

新结构硫脲类化合物的合成、鉴定及抑菌活性评价

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摘 要:【目的】合成具有抗菌活性的新结构硫脲类化合物。【方法】通过 *α*-异硫氰酸酯中间体与不同伯胺缩合合成硫脲类化合物，利用质谱、核磁分析鉴定结构，并评价其抑菌活性。【结果】合成了六种新结构的硫脲类化合物以及一种 *α*-异硫氰酸酯类衍生物，对几种代表性病原细菌和

Foundation item: National Natural Science Foundation of China (No. 31670032, 31522001); Hundred Talents Program of the Chinese Academy of Sciences
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Received: February 15, 2017; **Accepted:** March 15, 2017; **Published online** (www.cnki.net)**:** March 17, 2017
基金项目：国家自然科学基金项目(No. 31670032，31522001)；中国科学院百人计划经费资助项目
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收稿日期：2017-02-15；接受日期：2017-03-15；优先数字出版日期(www.cnki.net)：2017-03-17

真菌具有抑菌活性。其中，硫脲类化合物对新型隐球菌的抑制效果较为显著。【结论】通过不同结构硫脲类衍生物的合成，可能筛选出具有抑制新型隐球菌等致病菌的前体化合物。

关键词：硫脲衍生物，异硫氰酸酯，抗菌，新型隐球菌

Thiourea has been used as a stimulator to break bud dormancy and increase crop yield[1]. The thiourea group containing compounds have been successfully not only applied in the control of animal pathogenic bacteria, but also reported to possess antifungal, antimalarial, herbicidal, insecticidal, and rodenticidal properties[2-4]. Naturally occurred thiourea compounds are very rare. Zapotidine, a minor constituent of the seeds of the Mexican tree *Casimiroa edulis La Llave et Lej*. (Rutaceae), was isolated in merely 0.001 4 % yield by Kincl et al in 1956[5-6]. Another naturally occurred thiourea derivative anthracylcine FCE 24367 was isolated from the culture broth of *Streptomyces peucetius* and exhibited good antibacterial activities and remarkable cytotoxicity against P388 ascitic leukemia *in vivo*. It should be noted that, the bioactivity of FCE 24367 was weaker than its homologs, in which the thiourea group was replaced by a urea group[7].

Synthesized thiourea derivatives are practically used as components of antithyroid, and anaesthetic drugs, which has inspired medicinal chemists to design and synthesize more thiourea derivatives and test their bioactivities. To date, thiourea derivatives can be readily synthesized by various synthetic routes, among which the most widely approved methods are condensation of primary and secondary amines with isothiocyanate, thiophosgene or their derivatives[8-9]. The synthesized thiourea derivatives mainly belong to three groups of compounds acylthioureas, thiosemicarbazones and alkyl/aryl/heterocyclic-N-substituted thioureas. Acyl thioureas are well known for their prominent pesticidal, fungicidal, antiviral and plant growth regulating activities[10-11]. Thiosemicarbazones display a wide range of bioactivities, and their chemistry and pharmacological applications have been extensively investigated[2]. Among the alkyl/aryl/heterocyclic-N-substituted thioureas, N-alkyl/aryl-4-(3-substituted-3-phenylpropyl) piperazine-thioureas exhibit good spermicidal, anti-trichomonas, and antifungal activities and have no affect on the viability of vaginal epithelium (HeLa cells) and the growth of vaginal flora (*Lactobacillus*), indicating they are suitable for topical vaginal applications[12]. Another work showed that a series of heterocyclic thioureas analogs displayed potent activities against *β*-glucuronidase, an enzyme catalyzing the glucuronosyl-*O*-bonds cleavage at different pathological conditions, such as infection of urinary tract and renal disease, implying the potential of using those heterocyclic thioureas for treatment of the related diseases[3]. Some isoxazolo[4,5-*d*] pyridazine thiourea derivatives were found possessing considerable growth inhibitory activity against the fungal pathogen *Candida albicans*[13]. A SAR study on a variety of 1-(isomericfluorobenzoyl)-3-(isomeric fluorophenyl) thioureas with good antimicrobial activities showed that their antifungal activities were affected by the nature and position of the substituents on their aroyl and aryl rings[11]. N′-[2-(2-thiophene) ethyl]-N′-[2-(5-bromopyridyl)]thiourea was reported as a non-nucleoside inhibitor (NNI) of HIV reverse transcriptase and was capable of inhibiting NNI-resistant HIV[14]; while the other heterocyclic-N-substituted thiourea compound DC27 reduced tyrosine phosphorylation of epidermal growth factor receptor markedly and showed *in vitro* cytotoxicity against human lung carcinoma cells[15]. In recent, an interesting aryl-N-substituted thiourea #326, a potential skeleton for chemical design of future drug therapy for Alzheimer's disease[16], was demonstrated capable of increasing the activity of large-conductance Ca^{2+}-activated K^+ channel, which exerts multiple functions in regulation of microglial immunity[17].

The excellent biological activities of thiourea derivatives promoted us to synthesize more thiourea derivatives through the condensation of various primary amines with the *α*-isothiocyanatoacrylic ester (ICE). In this study, we synthesized six novel thiourea derivatives and one *α*-isothiocyanatoacrylic ester derivative and evaluated their biological activities against common pathogenic bacteria and fungi.

1 Experimental

1.1 Preparation of compounds 1−4

Aniline was added (0.2 g) to a solution of ICE

(0.1 g) in anhydrous ethanol (5 mL). The mixture was refluxed overnight and then cooled to room temperature. After evaporation, the residue was separated by a silica gel column chromatography (petroleum ether/ethyl acetate, 3:1, *V/V*) to afford crude compounds **1**−**4**.

1.2 Isolation of compounds 1−4

After dissolving in a small volume of methanol, compounds **1**−**4** were further purified by semi-preparative HPLC (Zorbax SB-C_{18}, 5 μm, 9.4 mm×250 mm, Agilent Technologies. Santa Clara, CA, USA). And the column was developed with solution A (water with 0.1% trifluoroacetic acid) and acetonitrile at a flow rate of 2 mL/min. The percentage of acetonitrile was changed using the following gradient: 0−3 min, 20%; 3−18 min, 20%−75%; 18−24 min, 75%−100%; 24−26 min, 100%; 26−28 min, 100%−20%; 28−32 min, 20%. The detection wavelengths were 210, 254 and 320 nm. Compounds **1**, **2**, **3** and **4** were eluted at 15.5 min, 16.0 min, 23.5 min and 24.0 min, respectively. After freeze drying in lyophilizer, 7.3 mg of **1**, 2.0 mg of **2,** 10.1 mg of **3** and 4.0 mg of **4** were obtained.

1.3 Preparation of compounds 5−6

Benzylamine (0.1 g) was added to a solution of ICE (0.1 g) in dichloromethane (4 mL). The mixture was stirred at room temperature overnight. After evaporation, the residue was separated by a silica gel column chromatography (petroleum ether/ethyl acetate, 1:1, *V/V*) to afford crude compounds **5**−**6**.

1.4 Isolation of compounds 5−6

After dissolving in a small volume of methanol, compounds **5**−**6** were isolated by semi-preparative HPLC. The column was developed with the same solution A and acetonitrile at a flow rate of 2 mL/min. The percentage of acetonitrile was changed using the following gradient: 0−3 min, 20%; 3−8 min, 20%−75%; 8−18 min, 75%−90%; 18−25 min, 90%−100%; 25−27 min, 100%; 27−28 min, 100%−20%; 28−30 min, 20%. The detection wavelengths were 210, 254 and 320 nm. Compounds **5** and **6** were eluted at 17.5 min and 21.5 min, respectively. Finally, 11.3 mg of **5** and 5.1 mg of **6** were obtained.

1.5 Preparation of compound 7

Propargylamine (0.06 g) was added to a solution of ICE (0.1 g) in dichloromethane (4 mL). The mixture was stirred at room temperature overnight. After evaporation, the residue was separated by a silica column chromatography (petroleum ether/ethyl acetate, 1:1, *V/V*) to afford crude compound **7**.

1.6 Isolation of compounds 7

Compound **7** was refined by semi-preparative HPLC using a same protocol as that for compound **5** and **6**, except the percentage of acetonitrile was changed using the following gradient: 0−3 min, 40%; 3−8 min, 40%−70%; 8−15 min, 70%−80%; 15−17 min, 80%−100%; 17−20 min, 100%; 20−22 min, 100%−40%; 22−25 min, 40%. Compound **7** was eluted at 11.8 min, and 15 mg of **7** was obtained at last.

1.7 Spectroscopic analyses

HPLC analysis was carried out using an Apollo C_{18} column (5 μm, 4.6 mm×250 mm, Alltech. Deerfield, IL, USA) on a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) with solution A (water with 0.1% trifluoroacetic acid) and acetonitrile at a flow rate of 0.8 mL/min. The percentage of acetonitrile was changed using the following gradient: 0−5 min, 20%; 5−25 min, 20%−90%; 25−30 min, 90%−100%; 30−35 min, 100%. The detection wavelengths were 210 nm, 254 nm and 320 nm. LC-MS analysis was performed on an Agilent 1260/6460 Triple-Quadrupole LC/MS system (Santa Clara, CA, USA) with an electrospray ionization source. HR-ESI-MS was performed on an Agilent 1260 HPLC/6520 QTOF-MS instrument (Santa Clara, CA, USA). NMR spectra were recorded at room temperature on a Bruker-500 NMR spectrometer (Billerica, MA, USA).

1.8 Antibacterial assays

The minimum inhibitory concentrations (MICs) were investigated against both gram positive and gram negative bacteria with a micro-broth dilution method using Mueller-Hinton (MH) broth as described according to the Standard of the National Committee for Clinical Laboratory[18-20]. All stock solutions of the tested compounds were prepared to be 40 mmol/L in dimethyl sulfoxide (DMSO), whose concentration in the cultures was critically limited to fewer than 5 percent. The inocula of all tested bacteria were prepared from the broth cultures in log phase growth and diluted to approximately 1×10^6 CFU. To determine the MICs of each compound, we added 20 μL compound stock solution to 160 μL MH broth to make the most concentrated test solution in the first

well, and then extracted 90 μL of the most concentrated test solution into the second well with 90 μL MH broth. The third test well was prepared by dilution of 90 μL of test solution from the second well with 90 μL MH broth also and the following wells were gradually diluted to a proper concentration. Ten microliter of properly diluted bacteria inocula was added into each well at last. MH broth containing bacterial inocula and 5% DMSO without any compound was used as a positive growth control, while MH broth containing only 5% DMSO was adopted as a negative growth control. The MICs were obtained after a growth at 37 °C for 24 h. All experiments were done in triplicate.

1.9 Antifungal assays

MICs for the synthesized compounds against fungi were evaluated with a micro-broth dilution method in 96-well culture plates using Roswell Park Memorial Institute 1640 medium (RPMI-1640) (Mediatech, Inc. China)[21-22]. *C. albicans* SC5314 was cultured on Potato Dextrose Agar for 4−5 days; *C. neoformans* H992 and *C. neoformans* JEC21 were cultured on Yeast Extract Peptone Dextrose solid medium for 2−3 days. The cell suspensions of all tested fungi were prepared to 0.5 McFarland by sterilized water and diluted to approximately 1×10^6 CFU by RPMI-1640. The synthesized compounds at various concentrations were prepared as described in the antibacterial assays, and the positive and negative controls were set in a similar way as those in the bacterial assays. The plates were incubated at 28 °C for 48−72 h to determine the MICs. All experiments were repeated three times.

2 Results

The key intermediate *α*-isothiocyanatoacrylic ester (ICE) used in this study was synthesized according to a reported method[23]. Briefly, ethyl 4-bromocrotonate was reacted with potassium thiocyanate in ethanol to afford ethyl-4-thiocyanatobut-2-enoate, which was distilled at 160 °C to give ICE. ICE was then used to react with commercial available primary amines in different conditions to give a series of thiourea derivatives. Three thiourea containing compounds ethyl-5-methyl-1-phenyl-2-thioxoimidazolidine-4-carb oxylate (**1**), 5-ethylidene-3-phenyl-2-thioxoimidazolidin-4-one (**3**), and N-phenyl-2-(3-phenylthio-ureido)but-2-enamide (**4**), and an ICE derivative ethyl-2-isothiocyanato-3-(phenylamino)butanoate (**2**) were obtained through a reaction of aniline with ICE in anhydrous ethanol by reflux method heating to 80 °C; while the other three thiourea derivatives 3-benzyl-5-ethylidene-2-thioxoim-idazolidin-4-one (**5**), 2-(3-benzyl-thioureido)but-2-enoic acid (**6**), and 2-(3-(prop -2-yn-1-yl)-thioureido)but-2-enoic acid (**7**) were generated by reacting benzylamine and propargylamine with ICE in dichloromethane at room temperature (Figure 1).

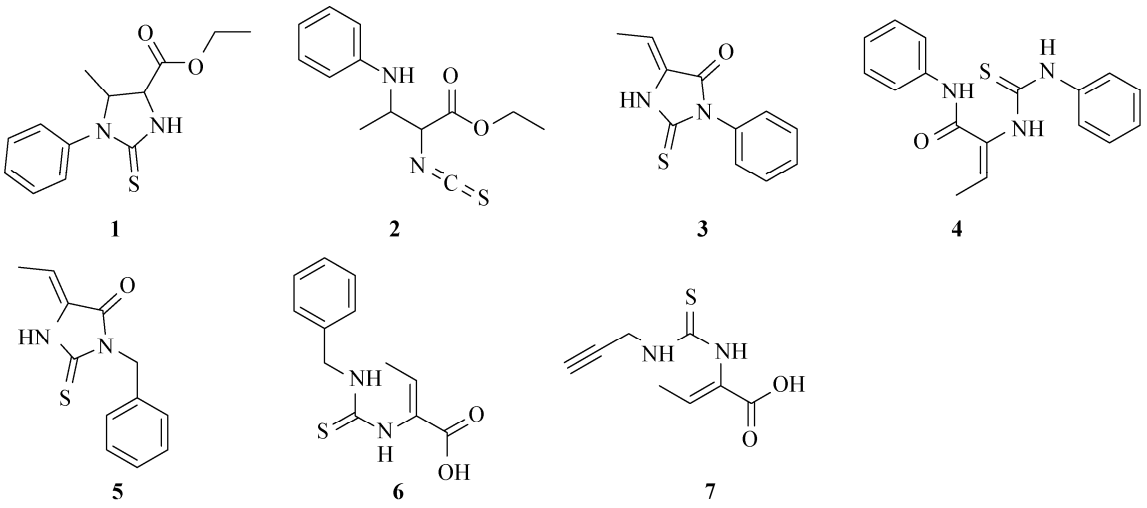

Figure 1 Structures of the seven synthesized compounds in this study
图 1 本研究合成的七种化合物的结构式

Structures of the seven synthesized compounds were determined by HR-MS and NMR analyses (Table 1). Specifically, compounds **1** and **2** have the same chemical formula, but can be distinguished by HMBC analysis. The HMBC correlation from H-5 (δ_H: 4.38) to C-7 (δ_C: 171.8) in compound **1** confirms its thioxoimidazolidine structure; while the absent of this signal in the HMBC spectrum of **2** suggests that it has an intact isothiocyanate moiety (Figure 2). A subsequent circular dichroism analysis showed that both **1** and **2** are racemic substances.

The bioactivities of all synthesized compounds were tested against gram negative pathogenic bacteria *Pseudomonas aeruginosa* ATCC15692 and *Escherichia coli* CGMCC1.1595, gram positive bacteria *Staphylococcus aureus* CGMCC1.89 and *Bacillus subtilis* CGMCC1.1630, and pathogenic fungus strains *Candida albicans* SC5314, *Cryptococcus neoformans* H992, and *Cryptococcus neoformans* JEC21 (Table 2). All of the seven synthesized compounds displayed inhibition activities against both gram positive and gram negative bacteria, and the fungus strain *C. albicans* SC5314 with a MIC range from 49.5 to 264 mg/L. Considerably, compounds **1**, **2**, **5**, and **7** showed good inhibition activities against both of the tested *C. neoformans* strains, and their MICs were determined to be 33, 33, 29 and 24.8 mg/L.

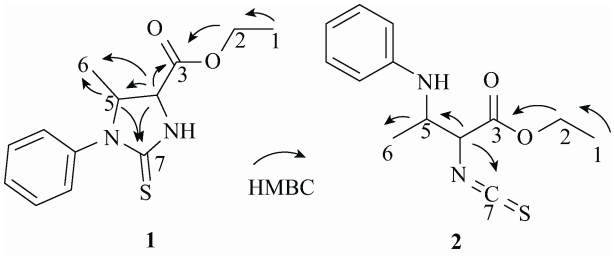

Figure 2 Selected HMBC signals of compounds 1 and 2
图 2 化合物 1 和化合物 2 的 HMBC 谱图

Table 1 Molecular formulas, high resolution mass spectrometry (HR-MS), and NMR spectral data for compounds 1−7 and ICE
表 1 化合物 1–7 和 ICE 的相对分子质量、高分辨质谱以及核磁数据

Compounds	Molecular formula	HR-MS (m/z $[M+H]^+$ calculated/found)	NMR
ICE	$C_7H_{10}NO_2S$	172.042 7/172.043 4	^{1}H-NMR (500 MHz, trichloromethane-d_1): δ6.68 (q, *J*=7.2 Hz, 1H, H-5), 4.28 (q, *J*=7.1 Hz, 2H, H-2), 1.95 (d, *J*=7.2 Hz, 3H, H-6), 1.34 (t, *J*=7.1 Hz, 3H, H-1)
1	$C_{13}H_{16}N_2O_2S$	265.100 5/265.100 8	^{1}H-NMR(500 MHz, methanol-d_4): δ7.42−7.57 (5H, aromatic H), 4.74 (d, *J*=2.5 Hz, 1H, H−4), 4.38 (m, 1H, H-5), 4.32 (q, *J*=7 Hz, 2H, H-2), 1.66 (d, *J*=7 Hz, 3H, H-6), 1.36 (t, *J*=7 Hz, 3H, H-1). ^{13}C-NMR (125 MHz, methanol-d_4): δ171.8 168.6, 136.5, 129.9, 128.3, 123.8, 68.4, 62.4, 46.5, 21.1, 12.9
2	$C_{13}H_{16}N_2O_2S$	265.100 5/265.100 9	^{1}H-NMR (500 MHz, methanol-d_4): δ7.37−7.53 (5H, aromatic H), 4.98 (d, *J*=7.5 Hz, 1H, H-4), 4.55 (m, 1H, H-5), 4.32 (q, *J*=7 Hz, 2H, H-2), 1.47 (d, *J*=7 Hz, 3H, H-6), 1.32 (t, *J*=7 Hz, 3H, H-1). ^{13}C-NMR (125 MHz, methanol-d_4): δ170.9, 166.4, 135.8, 127.4, 122.7, 115.5, 65.4, 61.6, 46.5, 14.1, 12.1
3	$C_{11}H_{10}N_2OS$	219.058 7/219.058 0	^{1}H-NMR (500 MHz，methanol-d_4): δ7.32−7.52 (5H, aromatic H), 6.08 (q, *J*=8 Hz, 1H, H-2), 1.95 (d, *J*=7.5 Hz, 1 H, H-1)
4	$C_{17}H_{17}N_3OS$	312.116 5/312.116 0	^{1}H-NMR (500 MHz, methanol-d_4): δ7.27−7.50 (10H, aromatic H), 5.97 (q, *J*=8 Hz, 1H, H-2), 1.96 (d, *J*=8 Hz, 3H, H-1). ^{13}C-NMR (125 MHz, methanol-d_4): δ178.3, 163.1, 133.4, 130.5, 129.6, 128.5, 128.4, 112.2, 11.3
5	$C_{12}H_{12}N_2OS$	233.074 3/233.074 4	^{1}HNMR (500 MHz, methanol-d_4): δ7.21−7.37 (5H, aromatic H), 5.90 (q, *J*=8 Hz, 1H, H−2), 5.01 (s,1H, H-6), 1.90 (d, *J*=8 Hz,1H, H-1)
6	$C_{12}H_{14}N_2O_2S$	251.084 9/251.084 3	^{1}HNMR (500 MHz, methanol-d_4): δ7.24−7.41 (5H, aromatic H), 5.93 (q, *J*=8 Hz, 1H, H−2), 5.02 (s, 1H, H-6), 1.96 (d, *J*=8 Hz, 3H, H-1)
7	$C_8H_{10}N_2O_2S$	199.053 6/199.053 2	^{1}HNMR (500 MHz, methanol-d_4): δ5.97 (q, *J*=7.5 Hz, 1H, H-2), 4.60 (d, *J*=2.5 Hz, 1H, H-6), 2.71 (t, *J*=2.5 Hz, 1H, H-8), 1.97 (d, *J*=7.5 Hz, 3H, H-1)

Table 2 MIC values (mg/L) for compounds 1−7 against bacterium and fungus strains
表2 化合物1–7的抗细菌和抗真菌的最小抑菌浓度(mg/L)

Compounds	*P. aeruginosa* ATCC15692	*E. coli* CGMCC1.155	*S. aureus* CGMCC 1.89	*B. subtilis* CGMCC1.1630	*C. albicans* SC5314	*C .neoformans* H992	*C .neoformans* JEC21
1	132.0	264.0	132.0	264.0	264.0	33.0	33.0
2	264.0	132.0	264.0	264.0	66.0	33.0	33.0
3	218.0	109.0	109.0	109.0	109.0	54.5	54.5
4	77.8	155.5	77.8.0	311.0	77.8	155.5	38.9
5	232.0	232.0	232.0	232.0	58.0	29.0	29.0
6	250.0	125.0	125.0	125.0	250.0	125.0	31.2
7	49.5	49.5	49.5	49.5	99.0	24.8	24.8

3 Discussion

In this work, we synthesized seven novel compounds, including six thiourea derivatives and one *α*-isothiocyanatoacrylic ester derivative, by three reactions of ICE with primary amines. It was proposed that an initial nucleophilic addition of primary amines to the isothiocyanate of ICE resulted in a key intermediate, which could be converted to compound **1** by an intramolecular aza-Michael addition (Figure 3A, route (a)), or cyclized to give compounds **3** and **5** by an intramolecular nucleophilic substitution of the amino group as showed in Figure 3A, route (b), or reacted at the ester bond through a hydrolysis to give compounds **6** and **7** or through an intermolecular nucleophilic substitution by aniline to generate compound **4** (Figure 3A, route (c)). Interestingly, when reacted at 80 °C with reflux method, aniline could also attack at the olefin double bond of ICE to generate **2** through an intermolecular aza-Michael addition (Figure 3B).

With these compounds in hands, we tested their antibacterial and antifungal activities against several representative pathogenic bacterium and fungus strains. It was showed that all compounds possess inhibition activities against both gram positive and gram negative bacteria and antifungal activities. It is worth noting that compounds **1**, **2**, **3, 5**, and **7** display good activities against the two tested *C. neoformans* strains, however, the MICs of compound **4** and **6** against *C. albicans* SC5314 (77.8 mg/L, 250 mg/L) were higher than *C .neoformans* JEC21 (38.9 mg/L, 31.2 mg/L). As a fast growing disease, cryptococcosis, caused by *C. neoformans* infection, can only be treated with a very limited number of drugs, including fluconazole, amphotericin B, and flucytosine. The data showed here, together with the previous works, which reported thiourea derivatives displaying good activities against *C. neoformans*, encouraged further efforts to screen thiourea containing antibiotics for the treatment of cryptococcosis. Unfortunately, the structural diversities of the seven compounds impeded further

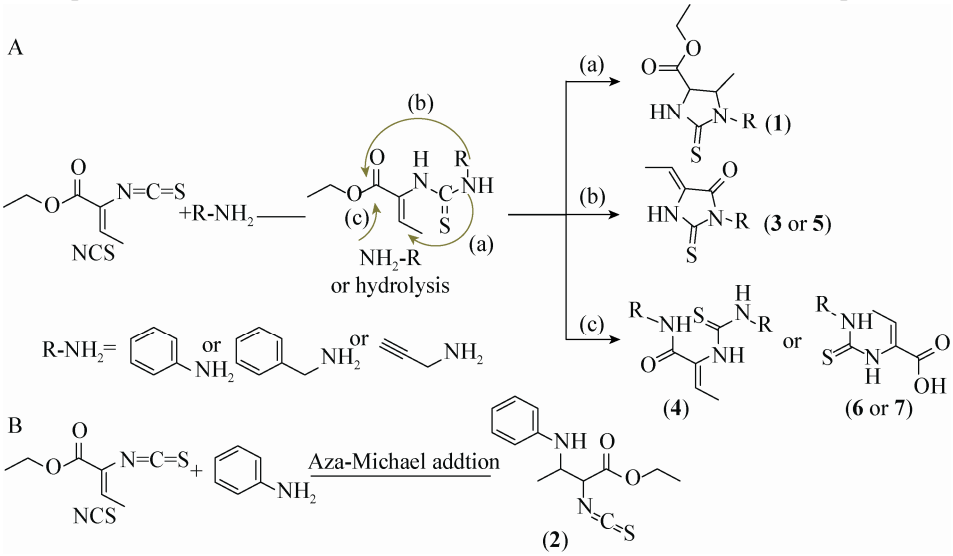

Figure 3 Proposed synthesis routes for compounds 1−7
图3 推测的化合物1−7的合成途径

structure and activity relationship (SAR) analysis. To synthesize a series of structural related thiourea derivatives will be carried out on the bases of this work and a detailed SAR analysis will be an important point in our following studies.

It should be considered that some thioureas are well known toxins and the thiourea derivatives could be toxic to human being. For example, common adverse effects of propylthiouracil, an anti-thyroid drug, are leucopenia, agranulocytosis, and rarely renal disease[24]; thiosemicarbazides could cause convulsions and death of rats within 1 to 3 hours[25]; α-naphthylthiourea and phenylthiourea are pulmonary toxins and induce liver and thyroid tumors in rats[26]. Therefore, it is very important to access the toxicity of a thiourea derivative carefully before it entries any clinical test.

Conclusively, we synthesized six new thiourea derivatives and one α-isothiocyanatoacrylic ester compound in this study and evaluated their antibacterial and antifungal activities. Moreover, the compounds generated in this work, like compounds **2** and **7**, are readily to be used as building blocks to synthesize more thiourea derivatives for drugable small molecules screening.

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