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Synthesis and Characterization of Thiophene-derived Amido Bis-nitrogen Mustard and Its Antimicrobial and Anticancer Activities

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The thiophene-derived amido bis-nitrogen mustard N^2,N^2,N^5,N^5-tetrakis(2-chloroethyl)-3,4-dimethylthiophene-2,5-dicarboxamide was designed and synthesized via five-step reactions from commercially available 2-chloroacetonitrile. This target compound was confirmed by ^{1}H NMR, ^{13}C NMR, MS, IR spectra and elemental analyses, and its structure was further characterized by X-ray single-crystal analysis. The biological activities for the title compound and some intermediates were evaluated *in vitro* for their antibacterial, antifungal and cytotoxic activities. The preliminary results showed that the title compound could inhibit efficiently the growth of the tested microorganisms including drug-resistant bacteria MRSA to some extent. Moreover, the target compound was found to be effective against prostatic carcinoma cell line (PC-3), breast carcinoma cell line (MCF-7), colon carcinoma (LoVo) and lung cancer (A549). Especially, it gave selective antitumor efficacy against prostatic carcinoma cell line (PC-3) at a low dose.

Keywords nitrogen mustard, thiophene, antibacterial, antifungal, cytotoxicity

Introduction

Nitrogen mustard (N-mustard) compounds with special $N(CH_2CH_2Cl)_2$ moiety have been paid considerable attention in medicinal chemistry due to their extensively potential bioactivities in anticancer,[1,2] antibacterial,[3] antifungal[4] and agrochemical field,[5] as well as being important intermediates for the syntheses of azole compounds[6-8] which as predominant antimicrobial drugs have been extensively used in the treatment of various types of microbial infections in current prevalence of pathogens.[9-18] Especially in cancer chemotherapy, they have been playing important roles since the introduction of Mechlorethamine **1** more than sixty years ago. Its higher therapeutic index as clinical drug encouraged much effort toward the research and development of nitrogen mustard compounds including alkyl, aralkyl, aryl, amide and metal supramolecular complex ones. So far more than 70 nitrogen mustards such as aryl N-mustards Chlorambucil **2**, amide-type N-mustard Cyclophosphamide **3**, Estramustine Phosphate **4** and so on (Figure 1) have been employed in clinical treatment of various types of cancer diseases.[19] Especially, Estramustine Phosphate which contains an amido N-mustard moiety has been used for the clinical treatment of advanced prostatic carcinoma with low toxicity.

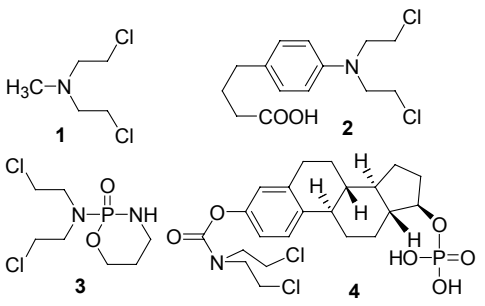

Figure 1 Some clinically used nitrogen mustard agents.

Though N-mustards as anticancer drugs acquired significant therapeutic efficacy against malignant human tumors,[20] they exhibited toxicity to normal tissue, lack of drug-specific affinity to tumor cells and drug resistance in patients with relapsed cancers.[21-23] On the other hand, in recent years a drastic increase in cancer

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diseases has become a serious problem worldwide, which caused the horrendous death of millions of people at a horribly high rate. In spite of the fact that a large number of innovative agents including the metal supramolecular drugs[24-26] are available, these therapeutics are still largely limited for clinical requirement because the survival rate of the malignant tumors is still very low. Therefore, nitrogen mustards have still been attracting great interest due to easy synthesis, lower cost, high inherent chemical reactivity to tumor cells, clinically extensive use with definite mechanism and good therapeutic efficacy. A number of researches were continuously directed toward the development of nitrogen mustards with low toxicity and better curative effects ranging from earlier aliphatic,[27-28] aromatic[29] and phosphamide[30] ones to recent DNA-targeting pyrroloamido alkylating agents.[31-33]

Aliphatic nitrogen mustards possess high chemical reactivity toward tumor cells and thus exhibit good anticancer activities, so a lot of researches focused on this class of compounds including aralkyl N-mustards. Recently, some aliphatic bis-nitrogen mustards which have two sets of N-mustard groups $N(CH_2CH_2Cl)_2$ and four potential covalent sites bonding to DNA per molecule, were designed and synthesized, and were found to display superior therapeutic index to mono-nitrogen mustards (Figure 2). Compounds **5a** and **5b** with two N-mustard groups of equal size and locating in a bisected half had proper log *P* and PSA constants which could result in a high lipid solubility and cell membrane permeability, and thus displayed more effective penetration of the blood-barrier and better cytotoxicity than Mechlorethamine and Cyclophosphamide.[34] Recently, some aralkyl bis-nitrogen mustards were also synthesized,[35-37] and exhibited inhibition potential toward microorganism and tumor cell. In this work, we would like to investigate such bis-nitrogen mustard as target compound and evaluate its biological activity.

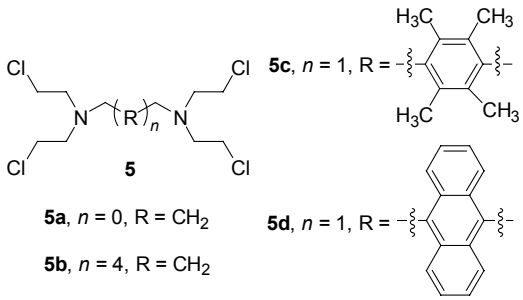


Figure 2 Aliphatic alkyl and aralkyl bis-nitrogen mustards.

Aliphatic nitrogen mustards showed good antitumor activities accompanied with relatively high toxicity, thus much effort has been directed toward the decrease of their toxicities to normal cell. Extensive research provided evidence that the introduction of some conjugated system such as aryl and/or carbonyl moiety into nitrogen mustards could helpfully decrease the toxicity due to the dispersion of N atom electron atmosphere density, which diminished alkylation ability through the attenuation of the nitrogen alkality. Formylmerphalan and Cyclophosphamide are one of the typical representatives of this class of compounds. Their successful clinical use in the treatment of diverse types of human cancers encouraged numerous medicinal chemists to focus on the researches and developments of aromatic and/or amide nitrogen mustards.[38-40] So far, various kinds of aromatic and amido N-mustards as anticancer drugs have been used in clinic, and played important roles during the past decades in the unending struggles against morbidity and mortality caused by cancer. Herein, the conjugation of aryl/carbonyl moiety with N-mustards was employed as an effective strategy for the development of new N-mustards with potentially high efficacy and low toxicity.

Recently, much work showed that incorporation of heterocycle with amido group into aromatic N-mustards could effectively increase anticancer activities. Especially, the pyrroloamido group as typical example was introduced into phenyl N-mustards to be capable of targeting effectively DNA and to show good anticancer activity in murine cancer.[41,42] Thiophene ring, as the most prominent bioisostere of pyrrole in medicinal chemistry, having been receiving great interest, was often employed as an important building block and extensively introduced into target compounds in modern drug design, and could significantly enhance biological activities.[43] Especially, some of those derivatives containing amido group possessed good inhibition potency to tumor cell.[44] Several thiophene compounds such as Teniposide and Raltitrexed have shown excellent antitumor efficacy which were being used effectively in clinical therapy. In view of this, we would like to incorporate thiophene ring, instead of the pyrrolyl moiety, in combination with amido group into nitrogen mustards to prepare the first thiophene-derived amido bis-nitrogen mustard **12**, and investigate its anticancer activity.

Although nitrogen mustards as anticancer drugs have been widely used in clinic and played important roles in chemotherapy of various cancer diseases, as antimicrobial agents they have also been exploited in recent years along with the drastic emergence of multiple-drug-resistant organisms which evoked urgent need for new antimicrobial drugs. It was found that some nitrogen mustards with chloroethyl amine moiety showed broad-spectrum antibacterial and antifungal activities with significantly inhibitory efficiency.[6-8,38,45] Some N-mustard derivatives including bis(2-bromoethyl)-amine and their azole-substituted compounds such as triazole, imidazole, benzimidazole ones and so on exhibited good biological activities. For example, compound **6** (Figure 3) with bromoethyl amine moiety displayed excellent activities toward the tested bacterial strains including MRSA with MIC values ranging from 0.25 to 4 μg/mL, which were equivalent or even better

potency in comparison with the clinical drug Norfloxacin. More importantly, this compound also exhibited significant antifungal activities against *Candida albicans* ATCC 76615 with MIC values of 0.25—2 μg/mL, which were comparable to Fluconazole (MIC = 0.25 μg/mL). These results showed that nitrogen mustards possessed large probability as new type of potential antimicrobial agents. Reasonably, we are interested in investigating the antimicrobial activity of the prepared thiophene amido bis-nitrogen mustard **12** containing four chloroethyl amine moieties.

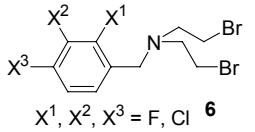


Figure 3 Analogs of nitrogen mustards.

The target thiophene-derived bis-nitrogen mustard **12** was conveniently synthesized through etherization of chloroacetonitrile, cyclization by base catalysis, hydrolysis of nitrile, halogenation of dicarboxylic acid and amidation reaction. The synthetic route is outlined in Scheme 1. According to the achievements of above literatures, such target molecule with bis-nitrogen mustard containing four bioactive chloroethylamino groups might give stronger antitumor and antimicrobial activities. The conjugated system of thiophene ring with the amido groups should be helpful for dispersing the N atom electron density, and thereby make the title compound **12** low toxic to normal cells and high cytotoxic to tumor cells. Naturally, we evaluated its anticancer efficacy against four tumor cells including prostatic carcinoma cell line (PC-3), breast carcinoma cell line (MCF-7), colon carcinoma (LoVo) and lung cancer (A549), and screened its antibacterial and antifungal activities *in vitro*. Furthermore, our previously obtained results showed that antimicrobial activity strongly depended on the water solubility[9-18] and it was found that imidazole hydrochloride and triazolium as well as imidazolium possessed good water solubility and resulted in an increase of antimicrobial efficiency. Therefore, we prepared the hydrochloride **13** and nitrate **14** from compound **12** and investigated their biological activities.

Experimental

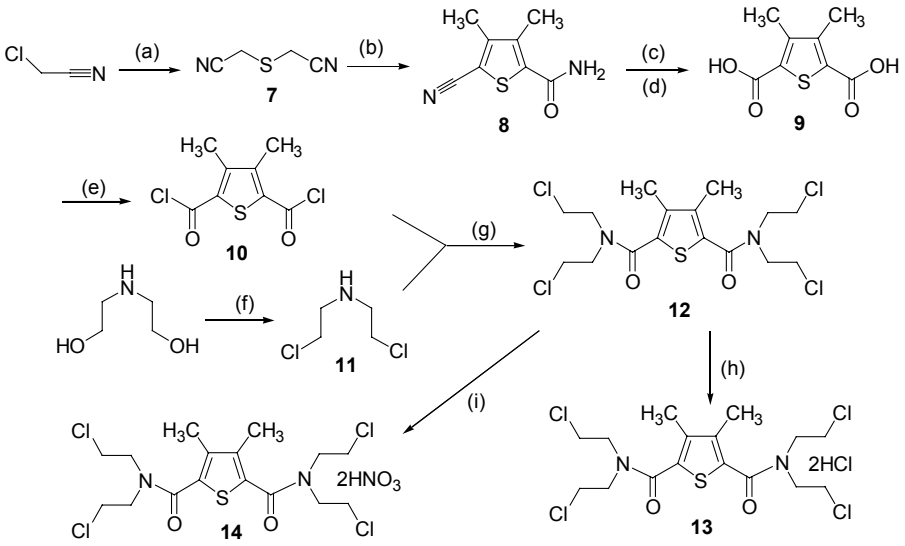
Reagents and measurements

Melting points were determined using an X-6 melting point apparatus and are uncorrected. Infrared (IR) spectra were recorded on a Bio-Rad FTS-85 (Bio-Rad, Cambridge, MA, USA) using KBr disks in the 4000—400 cm^{-1} region. NMR spectra were recorded using TMS as an internal reference standard on a Bruker AV 300 spectrometer. The mass spectra were recorded on LCMS-010A. Elemental analyses were carried out on a Carlo Erba model EA 1106 elemental analyzer. TLC analyses were done using silica gel plates; Column chromatography was performed on silica gel (300—400 mesh) column. All chemicals and solvents were of AR grade and, when necessary, were purified by standard methods.

Synthesis of bis(cyanomethyl)sulfide (7)

A mixture of sodium sulfide nonahydrate (24.0 g, 0.1 mol), benzyltrimethylammonium chloride (1.0 g) and chloroacetonitile (15.1 g, 0.2 mol) in the solvent of water/dichloromethane (100 mL, 1/1, *V*/*V*) was stirred at

Scheme 1 Synthetic route of thiophene-derived amido bis-nitrogen mustards



Reagents and conditions: (a) Na_2S, CH_2Cl_2, H_2O, benzyltrimethylammonium chloride, 0—5 ℃; (b) butane-2,3-dione, $NaOCH_3$, 0—5 ℃; (c) NaOH aqueous solution (8 mol/L), 100 °C, (d) hydrochloric acid (4 mol/L); (e) $SOCl_2$; (f) $SOCl_2$; (g) Et_3N, CH_2Cl_2, 0—5 ℃; (h) hydrochloric acid (4 mol/L), methanol; (i) nitric acid (4 mol/L), ethyl ether

0—5 ℃ under N_2 protection. After 30 min the reactants were stirred for 3 h at room temperature, and then the organic layer was separated and dried over anhydrous sodium sulfate. Evaporation of the solvent and recrystallization from methanol (5 mL) gave desired bis(cyanomethyl)sulfide **7** as colorless crystal (8.0 g). Yield 71.4%; m.p. 45—47 ℃ [lit.[46] 45—47 ℃].

Synthesis of 5-cyano-3,4-dimeth-ylthiophene-2-carboxamide (8)

To a solution of bis(cyanomethyl)sulfide **7** (11.2 g, 0.1 mol) and butane-2,3-dione (17.2 g, 0.2 mol) in methanol (50 mL) was added quickly 1 mol/L sodium methoxide solution in methanol (200 mL, 0.2 mol). The mixture was stirred for 15 min at 0—5 ℃, then water (20 mL) was added to quench the reaction, and methanol was removed by evaporation under vacuum to give a brown solid. The crude product was washed with water to afford the desired compound **8** as white solid (16.9 g). Yield 94.2%; m.p. 192—194 ℃ [lit.[46] 192—193 ℃].

Synthesis of 3,4-dimethyl thiophene-2,5-dicarboxylic acid (9)

Compound **8** (18.0 g, 0.1 mol) in aqueous sodium hydroxide (100 mL, 8 mol/L) was refluxed for 48 h, then the reaction mixture was cooled and the pH value was adjusted to 3—4 with 0.1 mol/L hydrochloric acid. The resulting precipitate was collected, washed with water and dried in oven at 120 ℃ to afford product **9** as white solid (19.6 g). Yield 98.0%; m.p. 325—326 ℃ [CAS NO. 19799-13-4: 327—328 ℃]; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.43 (s, 2H, COOH), 2.26 (s, 6H, CH_3); IR (KBr) v: 2927, 1747 (C=O), 1419, 1375, 1174 cm^{-1}; MS m/z: 200 $[M]^+$, 155 $[M-45]^+$.

Synthesis of 3,4-dimethyl thiophene-2,5-dicarbonyl dichloride (10)

Compound **3** (2.0 g, 10.0 mol) in thionyl chloride (10 mL) was refluxed for 6 h and the surplus thionyl chloride was distilled off. Recrystallization of the crude product from petroleum ether (60—90 ℃) gave acyl chloride **10** as white solid (1.5 g). Yield 64.7%; m.p. 78—79 ℃; IR (KBr) v: 2959, 2853 (CH_3), 1754 (C=O), 1203, 1171, 917 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 2.42 (s, 6H, CH_3); MS m/z: 237 $[M]^+$.

Synthesis of bis(2-chloro ethyl)amine hydrochloride (11)

To a stirring solution of diethanolamine (21.0 g, 0.2 mol) in chloroform (20 mL) was added thionyl chloride (25 mL) dropwise in ice-bath. The reactants were stirred for 1 h at room temperature and then refluxed overnight. The excess thionyl chloride was distilled off to afford crude product. The further recrystallization of the crude product from acetone gave bis(2-chloroethyl)amine hydrochloride **11** as colorless crystal (23.2 g). Yield 81.7%; m.p. 216—218 ℃ [lit.[46] 216—217 ℃].

Synthesis of N^2,N^2,N^5,N^5-tetrakis(2-chloroethyl)-3,4-dimethylthiophene-2,5-dicarboxamide (12)

To a suspension of thiophene-2,5-dicarbonyl dichloride **10** (1.2 g, 5.0 mmol) and bis(2-chloroethyl)amine (1.8 g, 10.0 mmol) in dry dichloromethane (20 mL) was added dropwise slowly triethylamine (5 mL) under stirring at 0—5 ℃. The resultant mixture was warmed gradually to room temperature, stirred for 30 h, and then treated with ethyl acetate (50 mL). After filtration, the filtrate was evaporated under reduced pressure and the resulting residue was purified by silica gel column chromatography using ethyl acetate/petroleum ether (1/1, *V*/*V*) as eluent to afford the target compound **12** as colorless crystal (1.2 g). Yield 53.6%; m.p. 104—105; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 3.86—3.82 (t, J=6 Hz, 8H, CH_2Cl), 3.71 (bs, 8H, $CONCH_2$), 2.17 (s, 6H, CH_3); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 161.70 (C=O), 146.76 (thienyl 2-C), 129.90 (thienyl 3-C), 48.60 (NCH_2), 45.80 (CH_2Cl); IR (KBr) v: 2977, 2918, 2863 (CH_3, CH_2), 1628 (C=O), 1216 (C—N), 1152 cm^{-1}; MS m/z: 449 $[M+H]^+$. Anal. calcd for $C_{16}H_{22}Cl_4N_2O_2S$: C 42.87, H 4.95, N 6.25; found C 42.90, H 4.91, N 6.28.

Synthesis of N^2,N^2,N^5,N^5-tetrakis(2-chloroethyl)-3,4-dimethylthiophene-2,5-dicarboxamide hydrochloride (13)

To a stirring solution of compound **12** (90 mg, 0.2 mmol) in methanol (2 mL) was added hydrochloric acid (4 mol/L, 2 mL) dropwise at room temperature. The reactants were stirred for 24 h at 40 ℃. After the reaction came to the end (monitored by TLC, eluent, ethyl acetate/petroleum ether, 1/2, *V*/*V*), the excess hydrochloric acid was distilled off under reduced pressure and the residue was recrystallized from acetone to afford compound **13** (91 mg) as colorless crystal. Yield 86.4%; m.p. 213—216 ℃; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 4.59 (bs, 8H, CH_2Cl), 4.01—3.97 (t, J=6 Hz, 8H, $CONCH_2$), 2.53 (s, 6H, CH_3); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 165.39 (C=O), 146.27 (thienyl 2-C), 130.96 (thienyl 3-C), 48.53 (NCH_2), 45.90 (CH_2Cl); IR (KBr) v: 2957, 2853 (CH_3, CH_2), 1723 (C=O), 1250 (C—N), 1154 cm^{-1}; MS m/z: 449 $[M+H-2HCl]^+$. Anal. calcd for $C_{16}H_{24}Cl_6N_2O_2S$: C 36.87, H 4.64, N 5.38; found C 36.74, H 4.68, N 5.35.

Synthesis of N^2,N^2,N^5,N^5-tetrakis(2-chloroethyl)-3,4-dimethylthiophene-2,5-dicarboxamide nitrate (14)

To a stirring solution of compound **12** (90 mg, 0.2 mmol) in ethyl ether (5 mL) and chloroform (5 mL) was added nitric acid (4 mol/L, 1 mL) dropwise at room temperature. The reactant mixture was stirred for 24 h at 30 ℃. After the reaction came to the end (monitored by TLC, eluent, ethyl acetate/petroleum ether, 1/2, *V*/*V*), the excess nitric acid was distilled off under reduced pressure and the residue was recrystallized from acetone to afford compound **14** (79 mg) as colorless crystal. Yield 69.3%; m.p. 216—218 ℃; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 4.55 (bs, 8H, CH_2Cl), 3.80 (bs, 8H, $CONCH_2$), 2.52 (s, 6H, CH_3); ^{13}C NMR (75 MHz,

DMSO-d_6) δ: 165.42 (C=O), 146.27 (thienyl 2-C), 130.95 (thienyl 3-C), 48.59 (NCH_2), 45.84 (CH_2Cl); IR (KBr) v: 2964, 2877 (CH_3, CH_2), 1713 (C=O), 1255 (C—N), 1157 cm^{-1}; MS m/z: 449 $[M+H-2HNO_3]^+$. Anal. calcd for $C_{16}H_{24}Cl_4N_4O_8S$: C 33.46, H 4.21, N 9.76; found C 33.52, H 4.18, N 9.72.

X-ray crystallography

A single crystal of the title compound with dimensions of 0.25 mm×0.22 mm×0.20 mm was chosen for X-ray diffraction study. The data were collected on Bruker APEXII diffractometer equipped with graphite-monochromated Mo Kα (λ=0.71073 Å) radiation by using a ω-2θ scan mode in the range of $1.9°<\theta<26.0°$ at 293(2) K. A total of 13412 reflections were collected with 4008 independent ones (R_{int}=0.018), of which 3342 observed reflections with $I>2\sigma(I)$ were used in the structure solution and refinement. The final R=0.042 and wR=0.126 ($w=1/[\sigma^2(F_o^2)+(0.0694P)^2+1.0128P]$, where $P=(F_o^2+2F_c^2)/3$, S=1.04, $(\Delta/\sigma)_{max}<0.001$, $\Delta\rho_{max}$=0.88 and $\Delta\rho_{min}$=−0.63 e/Å^{-3}). Cell refinement and data reduction were carried out using SAINT software (Bruker AXS Inc., Madison, Wisconsin, USA). The structure of the target compound was solved by directed methods using the program SHELXS97 and refined on F^2 by full-matrix least-squares techniques (SHELXL97).[48] The hydrogen atoms were placed by calculation and refined using riding model. Details of crystal data including selected bond lengths and bond angles were given in Table 4, hydrogen bonds were given in Table 3, the ORTEP drawing of the molecule was shown in Figure 5 and the molecular packing was shown in Figure 6.

Antibacterial and antifungal test

The *in vitro* minimal inhibitory concentrations (MICs) of tested compounds were determined by the micro-broth dilution method in 96-well microtest plates[49,50] according to the method recommended by the National Committee for Clinical Laboratory Standards (NCCLS). Chloramphenicol and Fluconazole were used as standard drugs. Initially the tested compounds **7**, **8**, **9**, **12**, **13** and **14** were dissolved in DMSO to prepare the stock solution, and then the test compounds and reference drugs were prepared in Mueller-Hinton broth (Guangdong Huaikai Microbial Sci. & Tech Co., Ltd, Guangzhou, Guangdong, China) by twofold serial dilution to give the required concentrations.

The prepared compounds were evaluated for their antibacterial activity against *S. aureus*, MRSA, *B. subtilis*, *M. luteus* as Gram-positive bacteria, *E. coli*, *P. vulgaris*, *S. typhi*, *P. aeruginosa* as Gram-negative bacteria. The bacterial suspension was adjusted with sterile saline to a concentration of 1×10^5 CFU/mL. 100 μL of each bacterial culture with equal volume of each concentration of tested compounds dilutions were added to each well. These dilutions were inoculated at 37 ℃ for 24 h. To ensure that the solvent had no effect on bacterial growth, a control test was performed with test medium supplemented with DMSO at the same dilutions as used in the experiment. The growth was monitored visually and spectrophotometrically. The lowest concentration (highest dilution) required to arrest the growth of bacteria was regarded as MIC. The MICs were summarized in Table 5.

The compounds were evaluated for their antifungal activities against *C. albicans* and *S. cerevisiae*. A spore suspension in sterile distilled water was prepared from 1 day old culture of the fungi growing on Sabouraud agar (SA) media. The final spore concentration was $1—5\times10^3$ spore•mL^{-1}. From the stock solutions of the tested compounds and reference antifungal drug Fluconazole, dilutions in sterile RPMI 1640 medium (Neuronbc Laboraton Technology Co., Ltd, Beijing, China) were made resulting in eleven wanted concentrations of each tested compounds. 100 μL of each fungus culture with equal volume of each concentration of tested compounds dilutions were added to each well. These dilutions were inoculated and incubated at 35 ℃ for 24 h. The growth was monitored visually and spectrophotometrically. The lowest concentration (highest dilution) required to arrest the growth of fungi was regarded as MIC. The MICs were summarized in Table 5.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetra zolium bromide (MTT) assay for cytotoxicity

The human prostatic carcinoma cell line (PC-3), breast carcinoma cell line (MCF-7), colon carcinoma (LoVo) and lung cancer (A549) were purchased from the Chongqing Medical University, China. Ham's F12 Nutrient Mixture, enriched with 10% heat inactivated foetal bovine serum (FBS) and 1% of penicillin-streptomycin was used for cell cultivation and to perform the tests. The cytotoxicity was investigated using the MTT assay. Stock solution (100 mmol/L) of test compound **12** was prepared in DMSO and stored at −20 ℃ prior to dilution into each tested concentration for the biological assay. Cell suspensions were diluted to 10^5 cells/mL, suitably prepared and distributed in plates of culture with 96 wells (100 μL in each well), 100 μL of each tested concentration was added to each well. The plate was incubated at 37 ℃ for 48 h. Then, 25 μL of MTT solution (5 mg/mL) was added to each well, and the mixture was incubated at 37 ℃ for another 4 h. At the end of this period, the culture medium with the MTT excess was aspirated and after that, 100 μL of DMSO was added to each well to dissolve the formazan crystals. The optical density (OD) of the wells was measured at 490 nm. Results were evaluated by comparing the absorbance of the wells containing compound treated cells with the absorbance of wells containing 0.1% DMSO alone (solvent control). Conventionally, cell growth inhibition can be defined as

$$\text{Cell growth inhibition} = (OD_{\text{cell negative control}} - OD_{\text{compound control}})/(OD_{\text{cell negative control}} - OD_{\text{blank}}) \times 100\%$$

All assays were performed in triplicate and mean ±

SD value was used to estimate cell growth inhibition rate. The cell growth inhibition rates were summarized in Table 6 and Figure 7.

Results and Discussion

Synthesis

The target thiophene bis-nitrogen mustard **12** was prepared starting from commercial chloroacetonitrile. Chloroacetonitrile easily reacted with sodium sulfide in the mixture solvent of dichloromethane and water using benzyltrimethylammonium chloride as catalyst to produce bis-cyanomethyl sulfide **7** as white solid in good yield via a slightly improved procedure that is to perform this reaction under N_2 protection which could effectively avoid the oxidation of thioether **7**. The yield for compound **7** in our experiment was up to 72% which is much higher than the literature yield 54%.[46] The cyclization of thioether **7** with butane-2,3-dione in the presence of sodium methoxide formed the intermediate 5-cyano-3,4-dimethylthiophene-2-carboxamide **8**, which was prepared according to a known procedure with a little improvement. The difference between the literature[47] and this improved method is to add some water into the cyclization reaction mixture in the organic phase before evaporating the solvent, this could efficiently avoid the formation of byproducts at a higher temperature, and produce pure product as white solid without further recrystallization. Compound **8** was hydrolyzed by aqueous solution of sodium hydrate, acidified by hydrochloric acid, washed with water and methanol consecutively, and then dried in oven to yield 3,4-dimethylthiophene-2,5-dicarboxylic acid **9** as powder. For the hydrolysis of compound **8**, a suitable concentration of the sodium hydrate should be 20%—30%, neither higher nor lower concentration was favorable for the formation of compound **9**. The treatment of compound **9** with fresh thionyl chloride conveniently and efficiently produced the 3,4-dimethylthiophene-2,5-dicarbonyl dichloride **10** with high purity after recrystallization from petroleum ether. The thiophene acyl chloride **10** was unstable in moist air because it is very sensitive to water due to hydrolysis of acyl chloride, so it is better to be used immediately for the next step reaction. The amidation of thiophene dicarbonyl dichloride **10** with bis(2-chloroethyl)amine hydrochloride **11** suspended in methylene chloride using triethylamine as catalyst at 0 ℃ readily produced the target compound **12** in moderate yield after chromatographic purification on silica gel. It is better to control the reactant ratio for compound **10** and bis(2-chloroethyl)amine at 1∶2 because the excess bis(2-chloroethyl)amine would react with itself in the alkalinity condition. Additionally, this reaction should be performed under N_2 protection because of the sensitivity of reactants **10** and **11** to moisture which resulted in low yield of title compound.

Analysis of spectra

The synthesized compounds were characterized by MS, IR and NMR spectra. The spectral data were consistent with the assigned structures and listed in the experimental section. The MS spectra of the compounds showed $[M+H-2HCl]^+$, $[M+H-2HNO_3]^+$, $[M+H]^+$ or $[M]^+$ peaks, in agreement with their corresponding molecular formulas.

In IR spectra, intermediate **9** gave broad absorption bands at 3000—2500 cm^{-1} which suggested the presence of carboxyl group. Another strong absorption at 1747 cm^{-1} should be attributed to the stretching vibration of C=O group. The disappearance of the characteristic COOH absorption in compound **10** suggested that the carboxyl group underwent chlorination with thionyl chloride. Moreover, the C=O group in compound **10** appeared with strong absorption at higher frequency in comparison with carboxyl one owing to the electrophilic inductive effect of chlorine moiety. However, the vibration frequency of C=O group in target compound **12** was greatly shifted to lower wave number due to the presence of strong electron-donating effect of $N(CH_2CH_2Cl)_2$ moiety which resulted in the increase of carbonyl electron atmosphere density. Moreover, it was found that the carbonyl vibration frequency in hydrochloride **13** and nitrate **14** was shifted to a higher wave number in contrast to compound **12**, which was mainly responsible for inductive effects of the positive charge on N atom in amide moiety. The characteristic absorption of C=O group was given in Table 1.

Table 1 Some typical spectral data for compounds **9**—**10** and **12**—**14**

Compd.	IR/cm^{-1}	^{1}H NMR δ			^{13}C NMR δ
	C=O	Thiophene-CH_3	CH_2Cl	NCH_2	C=O
9	1747	2.26	—	—	—
10	1754	2.42	—	—	—
12	1628	2.17	3.84	3.71	161.70
13	1723	2.53	4.59	3.99	165.39
14	1713	2.52	4.55	3.90	165.22

The ^{13}C NMR spectra showed peaks at δ 161.70—165.39 which were assigned to the C=O groups in compounds **12**—**14**. An obviously downfield shift (δ 3.52—3.69) for the carbon signal of amide groups in compounds **13** and **14** was observed in contrast to compound **12** as a result of the strong electron withdrawing character of positively charged amide group, as seen in Table 1.

The CH_3 protons in compounds **9**—**10** and **12**—**14** all gave downfield ^{1}H NMR signals as singlet varying from δ 2.17 to 2.53 due to the formation of large conjugation system between thienyl ring and C=O moiety which resulted in the enhancement of deshielding for methyl protons. The chemical shifts of CH_3 groups increased in the order **12**<**9**<**10**<**13**≈**14** with the increase of electron-withdrawing inductive effects of carbonyl moieties in the order $CONR^1R^2$<COOH<COCl

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<(CONR^{1}R^2).

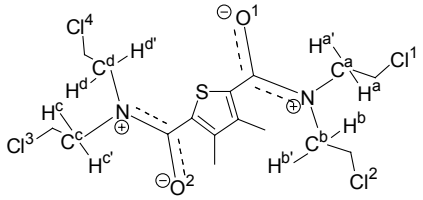

Figure 4 The molecular structure of compound **12**.

The methylene protons in the hydrochloride **13** and nitrate **14** displayed downfield shifts in the range of δ 0.20—0.77 in comparison with the corresponding compound **12** due to the positive charge on the amido group which could obviously affect on the chemical shifts of the neighboring groups. Surprisingly, the methylene protons of compounds **12**—**14** gave abnormal splits instead of normal triplet at the integration ratio of 1∶2∶1. This fact would be partially attributed to the concentrations of tested compounds. As seen in Figure 4, the C—N bond in amide group possessed partially double bond property and thus was fixed. The chemical environment of C^a atom was not equal to C^b atom. Therefore, the electrophilic inductive effects of O and Cl atoms to the H^a and H^b atoms were different, resulting from the different distances between them. In order to further clarify the steric effect of the target compound, the minimize energy calculation of compound **12** was carried out and it showed that O atom has different distances with the four protons. For O^1—H^a and O^2—H^c the distance was 2.421—2.734 Å, and for O^1—H^b and O^2—H^d the distance was 3.769—3.941 Å. But the C^l—H distance was in a wide range because the CH_2Cl group could revolve freely, for this reason and in order to confirm the structure, the target compound was crystallized to afford single crystal and further characterized by X-ray diffraction analysis. The calculative and actual bond lengths were listed in Table 2.

Table 2 The calculative length and actual length of atom O on the four methylene groups

Bond	Calculative length/Å	Actual length/Å
O(1)—H(a)	2.734	2.516
O(1)—H(b)	3.874	3.769
O(2)—H(c)	2.421	2.533
O(2)—H(d)	3.941	3.800

Single-crystal structure of target compound 12

A crystal of compound **12** suitable for X-ray analysis was grown from a mixture solution of ethyl acetate and petroleum ether by slow evaporation at room temperature. An ORTEP drawing of **12** was given in Figure 5 while Figure 6 depicted the unit cell packing. The structure belongs to monoclinic system with space group $P2_1/c$: a=7.9238 (4) Å, b=21.1712 (11) Å, c=12.6186 (7) Å, β=99.2380 (10)° and Z=4. The structural arrangement of compound **12** shows that the two amide groups adopted *trans*-conformation arrangement compared with the central thienyl ring so the four terminal 2-chloroethyl arms are oriented in the different orientation. As indicated in Figure 6, in the solid state, these molecules are bonded together with Cl···H—C hydrogen bonds into an H-bonding-driven three-dimensional network, corresponding O(7)···H(3 A), O(7)···O(3), and O(7)···H(3A)—O(3) data are 2.33 Å, 3.19 Å and 145.1°, respectively. The H-bonding data showed that the O and Cl atoms could form week H-bonding effects among the molecules which could play important roles in the chemical and biological activity.

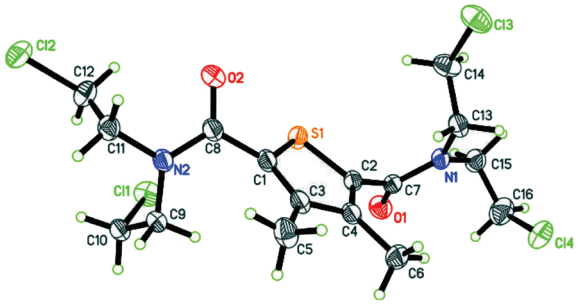

Figure 5 ORTEP of compound **12** at 50% probability.

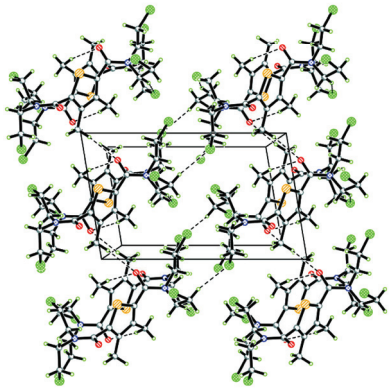

Figure 6 Packing of compound **12** along the *a* axis. The dashed lines represent the intermolecular hydrogen bonds.

Table 3 Hydrogen-bond geometry [Å and (°)]

D—H···A	D—H	H···A	D···A	D—H···A
C(14)—H(14)B···O^{i}	0.97	2.45	3.257(3)	141
C(14)—H(14)B···Cl^{ii}	0.97	2.80	3.632(3)	145
C(6)—H(6)B···O1^{iii}	0.96	2.54	3.474(3)	166
C(5)—H(5)B···O1^{iv}	0.96	2.54	3.477(3)	165

Symmetry codes: (i) x, $-y+1/2$; $z-1/2$; (ii) $x-1$, y, $z-1$; (iii) $-x+1$, $-y$, $-z+1$; (iv) $x+1$, y, z.

Pharmacology

Compounds **7**—**9** and **12**—**14** were evaluated for their antibacterial activities against Gram-positive and negative bacteria, including *S. aureus*, MRSA, *B. subtilis*, *M. luteus*, *E. coli*, *P. vulgaris*, *P. aeruginosa*, *S. typh* as well as antifungal activities against *C. albicans*, *S. cerevisiae* by two fold serial dilution method. The

activities of the synthesized compounds were compared with standard drugs Chloramphenicol and Fluconazole. The antimicrobial activities of the tested compounds were reported as MICs (mmol/L), and were shown in Table 5.

Antibacterial and antifungal activities The antimicrobial assay revealed that the target compound **12** could inhibit effectively the growth of tested bacteria and fungi including the drug-resistant strain MRSA, whereas the intermediates **7** and **9** were not sensitive to all the tested microorganisms even at higher concentrations. Surprisingly, compound **8** with amido and cyano groups showed better antibacterial and antifungal efficiency than the other two intermediates and gave a lower MIC value of 1.75 mmol/L against the tested bacteria strains that is 2—3 folds active than compounds **7** and **9**. It was noteworthy that the biological activities decreased in the order of the target compounds **12**> hydrochloride **13** and nitrate **14**>amdio thiophene **8**> carboxyl thiophene **9**. Thioether **7** with no thiophene ring was the most inactive one. These facts suggested that the amido residue, heterocyclic thienyl ring and chloroethylamino moiety exert the synergistic effect on biological activity.

Compared with the referenced drugs chloramphenicol and fluconazole in clinic, Table 5 showed that the target compound **12** exhibited poor to moderate inhibitory activities *in vitro* against most of the tested Gram-positive and Gram-negative bacteria as well as fungi. Notably, Proteus vulgaris was the most sensitive to compound **12** with lower MIC value of 0.07 mmol/L which was comparable to the clinical drug Chloramphenicol (MIC 0.012 mmol/L) to some extent.

Unexpectedly, although compounds **13** and **14** had a better water solubility than compound **12**, they were less active against the tested microorganisms especially the Gram-negative one. This result was different from our previous work.[9-18] Probably, this was due to the fact that penetration of hydrochloride and nitrate into the cell was more difficult for microorganisms which have a more lipophilic cell wall.[51]

Cytotoxicity The cytotoxicity of the title compound was determined *in vitro* on a panel against four human cancer cell lines derived from different cancer types including human prostatic carcinoma cell line (PC-3), colon carcinoma (LoVo), lung cancer (A549) and breast carcinoma cell line (MCF-7), using Fluorouracil as standard drugs. The results were shown in Table 6 and Figure 7.

As shown in Figure 7, the preliminary cytotoxicities showed that the target compound **12** demonstrated poor to moderate antiproliferative effect on all tested cell lines. Figure 7 clearly demonstrated that human prostatic carcinoma cell line (PC-3) was more sensitive to the title compound **12** than other three tested tumor cells: lung cancer (A549), colon carcinoma (LoVo) and breast carcinoma cell line (MCF-7). Among all tested cells, compound **12** toward human prostatic carcinoma cell line (PC-3) was the most active, while the lowest inhibitory efficiency was against breast carcinoma cell

Table 4 Selected bond lengths (Å) and bond angles (°) for the target compound **12**

Bond	Length	Bond	Length	Bond	Length
C(1)—S(1)	1.717(2)	C(15)—N(1)	1.469(3)	C(2)—C(7)	1.494(3)
C(2)—S(1)	1.724(2)	C(10)—Cl(2)	1.784(3)	C(3)—C(4)	1.440(3)
C(7)—O(1)	1.231(3)	C(12)—Cl(1)	1.766(4)	C(3)—C(5)	1.497(3)
C(8)—O(2)	1.227(3)	C(14)—Cl(4)	1.786(3)	C(4)—C(6)	1.504(3)
C(7)—N(1)	1.350(3)	C(16)—Cl(3)	1.777(3)	C(9)—C(10)	1.505(4)
C(8)—N(2)	1.350(3)	C(1)—C(3)	1.366(3)	C(11)—C(12)	1.490(4)
C(9)—N(2)	1.465(3)	C(1)—C(8)	1.502(3)	C(13)—C(14)	1.498(3)
C(11)—N(2)	1.468(3)	C(2)—C(4)	1.365(3)	C(15)—C(16)	1.514(4)
C(13)—N(1)	1.460(3)				
Bond	Angel	Bond	Angel	Bond	Angel
O(1)-C(7)-N(1)	121.0(2)	C(7)-N(1)-C(13)	124.28(19)	C(4)-C(2)-S(1)	112.36(17)
O(1)-C(7)-C(2)	119.5(2)	C(7)-N(1)-C(15)	117.58(19)	C(7)-C(2)-S(1)	116.12(17)
N(1)-C(7)-C(2)	119.6(2)	C(13)-N(1)-C(15)	117.75(17)	C(1)-C(3)-C(4)	111.6(2)
O(2)-C(8)-N(2)	122.0(2)	C(8)-N(2)-C(9)	123.80(19)	C(1)-C(3)-C(5)	124.5(2)
O(2)-C(8-C(1)	120.3(2)	C(8)-N(2)-C(11)	118.4(2)	C(4)-C(3)-C(5)	123.9(2)
N(2)-C(8)-C(1)	117.7(2)	C(9)-N(2)-C(11)	117.63(19)	C(2)-C(4)-C(3)	112.1(2)
N(2)-C(9)-C(10)	110.34(19)	C(1)-S(1)-C(2)	91.11(11)	C(2)-C(4)-C(6)	125.2(2)
C(9)-C(10)-Cl(2)	110.00(19)	C(3)-C(1)-C(8)	127.6(2)	C(3)-C(4)-C(6)	122.7(2)
N(2)-C(11)-C(12)	113.7(2)	C(3)-C(1)-S(1)	112.81(17)	C(11)-C(12)-Cl(1)	112.3(3)
N(1)-C(15)-C(16)	112.64(19)	C(8)-C(1)-S(1)	119.44(16)	N(1)-C(13)-C(14)	114.0(2)
C(15)-C(16)-Cl(3)	110.22(18)	C(4)-C(2)-C(7)	131.1(2)	C(13)-C(14)-Cl(4)	110.80(15)

Table 5 Antibacterial and antifungal activities *in vitro* of thiophene derivatives expressed as MIC (mmol/L)[a,b]

Compd.	Gram-positive bacteria				Gram-negtive bacteria				Fungi	
	S. aureus	MRSA	*B. subtilis*	*M. luteus*	*E. coli*	*P. vulgaris*	*S. typhi*	*P. aeruginosa*	*C. albicans*	*S. cerevisiae*
7	>4.5[c]	>4.5	>4.5	>4.5	>4.5	>4.5	>4.5	>4.5	>4.5	>4.5
8	1.75	1.75	>1.75	1.75	1.75	1.75	>1.75	1.75	>1.75	>1.75
9	>2.56	>2.56	2.56	2.56	2.56	2.56	>2.56	2.56	>2.56	>2.56
12	0.28	0.14	0.56	0.14	0.07	0.56	0.14	0.28	0.14	0.14
13	0.49	>0.99	0.12	>0.99	>0.99	0.99	>0.99	>0.99	>0.99	>0.99
14	0.44	0.89	0.44	0.89	>0.89	0.89	>0.89	0.89	>0.89	0.89
Chloramphenicol	0.012	0.096	0.096	0.024	0.012	0.012	0.024	0.048	—	—
Fluconazole	—	—	—	—	—	—	—	—	0.0033	0.013

[a] Minimum inhibitory concentrations were determined by micro broth dilution method. [b] *S. aureus*, *Staphylococcus aureus* (ATCC 25923); MRSA, *Staphylococcus aureus* (N315); *B. subtilis*, *Bacillus subtilis* (ATCC 6633); *M. luteus*, *Micrococcus luteus* (ATCC 4698); *E. coli*, *Escherichia coli* (ATCC 25922); *P. vulgaris*, *Proteus vulgaris* (ATCC 6896); *S. typhi*, *Salmonella typhi* (ATCC 9484); *P. aeruginosa*, *Pseudomonas aeruginosa*; *C. albicans*, *Candida albicans* (ATCC 76615); *S. cerevisiae*, *Saccharomyces cerevisiae* (ATCC 9763).

Table 6 *In vitro* cytotoxic activities of compound **12** compared with Fluorouacil

Compd.	Percentage growth inhibition (%) at 25 μmol/L in different cell lines			
	PC-3	LoVo	A549	MCF-7
12	33.61*, #	11.07#	15.03*, #	6.09#
5-Fluorouracil	50.17*	51.63*	34.44*	47.67*

$p < 0.01$ means statistically significantly difference between treatment and control as evaluated by Student's t-test. * $p < 0.01$, compared with the cell negative group (negative control); # $p < 0.01$, compared with the cell group treated with fluorouacil (positive control).

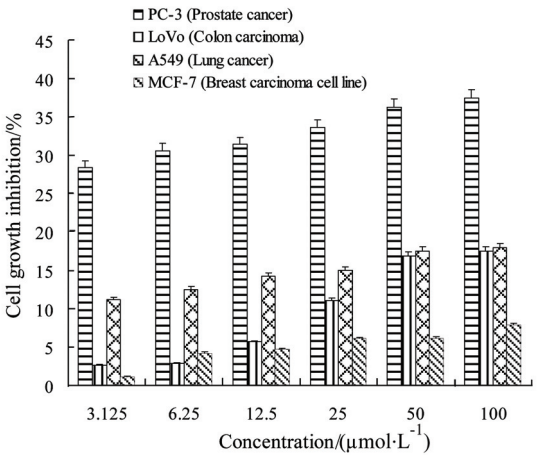

Figure 7 Cell growth inhibition of compound **12**.

line (MCF-7). Compound **12** could selectively inhibit the cell growth of PC-3 at micromolar concentration, however, there was no concentration-dependent response pattern. As noticed in Figure 7, for higher tested concentrations, 25 μmol/L (11.2 μg/mL), 50 μmol/L (22.4 μg/mL) and 100 μmol/L (44.8 μg/mL), the average growth inhibition rates of PC-3 cell line were 35.2%, 36.2% and 38.6%, respectively. Meanwhile, for lower tested concentrations, 3.12 μmol/L (1.4 μg/mL), 6.25 μmol/L (2.8 μg/mL) and 12.5 μmol/L (5.6 μg/mL), the average growth inhibition activities against the tumor cells were 31.9%, 31.2% and 32.3% respectively. It is clearly observed that these tumor cell growth inhibition rates were slightly changed for each tested concentration and compound **12** did not show obviously dose-dependent antiproliferative property. However, as shown in Table 6, the other three tested tumor cells, colon carcinoma (LoVo), lung cancer (A549) and breast carcinoma cell line (MCF-7) were not sensitive to the target mustard in comparison to the clinically used Fluorouracil and the cell growth inhibition percentages were 11.07%, 11.14% and 3.92% to each cell line at 25 μmol/L. The cell growth inhibition rate did not change obviously along with different dose used as shown in Figure 7.

In conclusion, the thiophene-derived amido bis-nitrogen mustard exhibited poor to moderate cytotoxic activities against all the tested tumor cell lines. In particular, PC-3 cell line was the most sensitive to the target mustard among the four tested tumor cells. Compound **12** exerted a higher cytotoxic activity against PC-3 tumor cell at a lower dose which indicated that compound **12** with a thiophene core and two amide nitrogen mustard moieties could be a new and potential scaffold for the development of novel anticancer agents.

Furthermore, whether or not the target mustard **12** show dose-dependent response pattern, this compound might act as DNA groove binder due to its special structure making the cell DNA a dominant target for antiproliferative activity as other literatures described. It would be worth for pharmacological scientists to confirm by other experiments.[52]

Conclusions

The thiophene-derived amido bis-nitrogen mustard

12 was designed and synthesized via five-step reactions starting from commercially available 2-chloroacetonitrile, and characterized by NMR, MS, IR spectra and elemental analyses. Its single crystal was successfully cultivated from a mixture solvent of ethyl acetate and petroleum ether, and further analyzed by X-ray crystal diffraction on Bruker APEXII diffractometer. Antimicrobial assay *in vitro* showed that the target compound exhibited broad inhibitory efficacy and better bioactivities than its intermediates. *N*,*N*-Bis(2-chloroethyl)amine, amide and nitrile moieties are helpful for the title mustard to increase its antimicrobial activity. The antitumor evaluation for the target nitrogen mustard **12** exhibited selective inhibitory activities against PC-3 tumor cell line, which made the thiophene bis-amido nitrogen mustard a potential and promising antitumor scaffold for further modification and optimization.

Acknowledgements

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