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Synthesis and *in vitro* antiprotozoal activities of water-soluble, inexpensive phenothiazinium chlorides

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ABSTRACT

Tropical diseases are serious infectious diseases caused by protozoan parasites in tropical and subtropical regions. Novel, effective, safe, and inexpensive drugs are required to treat the parasites and contribute to the global goal of eradication. A series of phenothiazinium chlorides were synthesized and evaluated for *in vitro* activities against *Plasmodium falciparum*, *Trypanosoma cruzi*, *Trypanosoma brucei rhodesiense*, and *Leishmania donovani*. Notably, 3,7-bis(piperidinyl)phenothiazinium chloride showed IC_{50} of 0.097 μmol L^{-1} against *T. cruzi* and 3,7-bis(benzyl(methyl)amino)phenothiazinium chloride exhibited IC_{50} of 0.081 μmol L^{-1} against *L. donovani*, although the cytotoxicities of these compounds against L-6 cells were observed at low concentration.

1. Introduction

Malaria, Chagas disease, Leishmaniasis, and African sleeping sickness are the main tropical diseases infected by parasitic protozoa. Even inhabitants of temperate zones are exposed to the danger of infection owing to global warming.

Malaria, which is caused by the *Plasmodium falciparum* parasites, exists in more than 100 countries. In 2008, there were an estimated 243 million cases of malaria worldwide, and malaria accounted for an estimated 863 thousand deaths [1]. In many parts of the world, parasites have developed resistance to a number of anti-malarial medicines [2]. Chagas disease, which is caused by *Trypanosoma cruzi*, is a very serious public health problem in several countries, with 18 million people known to be infected with the parasite and an additional 100 million being at risk of infection. The lack of interest shown by pharmaceutical companies for developing anti-*T. cruzi* drugs makes Chagas one of the major "neglected" diseases of the world [3]. Benznidazole and nifurtimox are used as medicines for Chagas disease, but these drugs were developed for veterinary use more than 30 years ago, and have low efficacy and high acute toxicity [4,5]. Phosphinopeptides [6], tipifarnib analogues [3] were recently evaluated for anti-*T. cruzi* activity; however, there is no effective treatment for the prevalent chronic form of Chagas disease [7]. Over 10 million patients are infected by leishmaniasis [8]. Pentostam, amphotericin B, and miltefosine are used to treat leishmaniasis, but these are either highly toxic or very expensive compounds. African trypanosomiasis, caused by *Trypanosoma brucei rhodesiense*, has reappeared in several areas over the past 30 years. Most medicines for this disease, such as melarsoprol, have been used for 60 years and have substantial side effects [9,10]. Generally, the presently used drugs possess severe side effects and do not provide complete eradication of the disease. In addition, drug resistance has become a major problem in the treatment of tropical diseases. Therefore, new promising compounds are urgently needed [11].

Developing countries have many patients infected by parasitic protozoa. Thus, the identification of effective, low-toxicity, and inexpensive antiprotozoal candidates is a challenge for synthetic chemists, biochemists and medicinal chemists. Our previous work is based on the π-delocalized lipophilic cation (DLC) hypothesis [12]. Quaternary ammonium salts of rhodacyanine and

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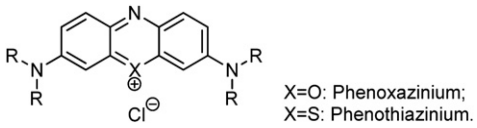

Fig. 1. Structures of phenoxazinium and phenothiazinium.

phenoxazinium derivatives were prepared. Rhodacyanines, which are easily prepared by several steps in high yields, show good *in vitro* activity against *P. falciparum* and *L. donovani* [13–17]. Phenoxaziniums, which are water-soluble, inexpensive and high purity dyes, exhibit potent antiprotozoal activities, especially against *P. falciparum* and *T. cruzi* [18–21].

Methylene blue having a phenothiazinium skeleton is still explored as a potential candidate for anti-malarial medicine [22,23]. On the basis of the structure similarity of phenoxazinium and phenothiazinium (Fig. 1), we have synthesized several new derivatives of methylene blue and evaluated their biological activities. Here, we report our findings, which provide important information to medicinal chemists working in related areas.

2. Experimental

2.1. Chemistry

2.1.1. General

Melting points were measured on an X-4 microscope electro thermal apparatus (Taike, China) and the results are uncorrected. Infrared spectra (IR) were recorded on a Nicolet 5200 FT-IR instrument using solid samples dispersed in KBr pellets. UV–vis spectra were recorded on a Perkin–Elmer λ-17 spectrometer using a 1 cm square quartz cell. ^{1}H NMR and ^{13}C NMR spectra were collected on Varian-300 or 400 NMR spectrometer, tetramethylsilane was used as the internal reference for the analyses and CD_3OD as solvent; TMS was used as an internal standard for ^{1}H NMR and solvent peak was used as an internal standard for ^{13}C NMR. High resolution mass spectra were recorded on a Finnigan MAT 95 mass spectrometer (ESI^+). Elementary analyses were conducted on a Carlo Erba-MOD1106 elementary analysis apparatus. Phenothiazinium tetraiodide was obtained by reported method [24].

2.1.2. Synthesis

2.1.2.1. General procedure. Phenothiazinium tetraiodide (1.45 g, 2.0 mmol) was slowly added to a solution of the requisite dialkylamine (12 mmol) in methanol (50 mL) during a time of 30 min. The resulting solution was allowed to stir at room temperature for 8 h. The mixture was concentrated to approximate 20 mL and precipitated by addition of diethyl ether (100 mL). The crude product was collected by filtration, and purified by column chromatography ($CHCl_3$:CH_3OH = 10:1, silica gel (200–300 mesh)). The solvent was removed by evaporator under reduced pressure, then the residue obtained was dissolved in 1:1 CH_3OH:$CHCl_3$ and passed through an IRA-400 (Cl) resin exchange column to obtain phenothiazinium chloride. The product was finally further purified by column chromatography ($CHCl_3$:CH_3OH = 10:1, silica gel (300–400 mesh)).

*2.1.2.2. 3,7-Di(pyrrolidinyl)phenothiazinium chloride (**1a**).* Purple powder, yield: 15%, mp: >250 °C; UV–vis (MeOH), λ_{max}(nm) (l g ε (L mol^{-1} cm^{-1})): 660 (5.1); IR (KBr pellet cm^{-1}) υ_{max}: 2936, 2867 (alkyl-CH), 1590, 1523, 1398 (phenothiazinium skeleton), 1146 (C–N); ^{1}H NMR(300 MHz; CD_3OD; Me_4Si) δ_H: 7.91 (2H, d, J = 9.4, 2 × C*H*), 7.32 (2H, d, J = 9.4, 2 × C*H*), 7.21 (2H, s, 2 × C*H*), 3.72 (8H, br, 2 × N(CH_2)$_2$), 2.16 (8H, br, 2 × (CH_2)$_2$); ^{13}C NMR (101 MHz; CD_3OD) δ_C: 153.0(*C*), 139.5(*C*H), 136.7(*C*), 135.5(*C*), 120.6(*C*H), 108.0 (*C*H), 50.9(NCH_2), 50.6(NCH_2), 26.2(CH_2), 26.1(CH_2); m/z(ESI^+) 336.1545 ([M − Cl^-]$^+$, $C_{20}H_{22}N_3S^+$ requires 336.1534); Calcd. for $C_{20}H_{22}ClN_3S\cdot 2.5H_2O$: C, 57.61; H, 6.53; N, 10.08; Found: C, 57.84; H, 6.58; N, 9.71.

*2.1.2.3. 3,7-Di(piperidinyl)phenothiazinium chloride (**1b**).* Purple powder, yield: 17%, mp: >250 °C; UV–vis (MeOH), λ_{max}(nm) (l g ε (L mol^{-1} cm^{-1})): 667 (4.3); IR (KBr pellet cm^{-1}) υ_{max}: 2929, 2853 (alkyl-CH), 1595, 1522, 1398 (phenothiazinium skeleton) and 1146 (C–N); ^{1}H NMR(300 MHz; CD_3OD; Me_4Si) δ_H: 7.93 (2H, d, J = 9.6, 2 × CH), 7.60 (2H, d, J = 9.7, 2 × CH), 7.51 (2H, d, J = 2.6, 2 × CH), 3.88 (8H, br, 2 × N(CH_2)$_2$), 1.82 (12H, br, 2 × (CH_2)$_3$); ^{13}C NMR (101 MHz; CD_3OD) δ_C: 154.5(*C*), 139.7(*C*H), 137.0(*C*), 136.2(*C*), 120.1 (*C*H), 107.8(*C*H), 50.6(NCH_2), 27.7(CH_2), 22.2(CH_2); m/z(ESI^+) 364.1854([M − Cl^-]$^+$, $C_{22}H_{26}N_3S^+$ requires 364.1847); Calcd. for $C_{22}H_{26}ClN_3S\cdot 2.5H_2O$: C, 59.38; H, 7.02; N, 9.44; Found: C, 59.25; H, 7.26; N, 9.43.

*2.1.2.4. 3,7-Bis(methyl(phenyl)amino)phenothiazinium chloride (**1c**).* Purple powder, yield: 15%, mp: 108 °C; UV–vis (MeOH), λ_{max}(nm) (l g ε (L mol^{-1} cm^{-1})): 652 (4.1); IR (KBr pellet cm^{-1}) υ_{max}: 1602, 1484, 1385 (phenothiazinium skeleton), 1125(C–N); ^{1}H NMR (300 MHz; CD_3OD; Me_4Si) δ_H: 7.97 (2H, d, J = 8.6, 2 × CH), 7.61–7.27 (14H, m, Ar–H), 3.69 (6H, br); ^{13}C NMR (101 MHz; CD_3OD) δ_C: 155.8 (*C*), 145.6(*C*), 139.5(*C*H), 138.1(*C*), 136.8(*C*), 131.9(*C*H), 130.1(*C*H), 127.6(*C*H), 121.9(*C*H), 108.4(*C*H), 42.6(CH_3); m/z(ESI^+) 408.1560 ([M − Cl^-]$^+$, $C_{26}H_{22}N_3S^+$ requires 408.1534); Calcd. for $C_{26}H_{22}ClN_3S\cdot 2.5H_2O$: C, 63.86; H, 5.56; N, 8.59; Found: C, 63.72; H, 5.67; N, 8.49.

*2.1.2.5. 3,7-Bis(benzyl(methyl)amino)phenothiazinium chloride (**1d**).* Purple powder, yield: 17%, mp: 118 °C; UV–vis (MeOH), λ_{max}(nm) (l g ε (L mol^{-1} cm^{-1})): 655 (4.6); IR (KBr pellet cm^{-1}) υ_{max}: 1595, 1489, 1392 (phenothiazinium skeleton), 1144(C–N); ^{1}H NMR (400 MHz; CD_3OD) δ_H: 7.98 (2H, d, J = 9.6, 2 × CH), 7.58 (2H, d, J = 9.7, 2 × CH), 7.45 (2H, d, J = 2.5, 2 × CH), 7.42–7.28 (10H, m, 2 × C_6H_5), 5.03 (4H, s, 2 × CH_2), 3.45 (6H, s, 2 × CH_3); ^{13}C NMR (101 MHz; CD_3OD) δ_C: 155.8(*C*), 139.8(*C*), 137.3(*C*H), 136.9(*C*), 136.4 (*C*), 130.3(*C*H), 129.2(*C*H), 127.9(*C*H), 120.5(*C*H), 107.7(*C*H), 57.5 (CH_2), 40.7(CH_3); m/z(ESI^+) 436.1864 ([M − Cl^-]$^+$, $C_{28}H_{26}N_3S^+$ requires 436.1842); Calcd. for $C_{28}H_{26}ClN_3S\cdot 2.5H_2O$: C, 65.04; H, 6.04; N, 8.13; Found: C, 64.95; H, 6.21; N, 8.12.

*2.1.2.6. 3,7-Bis(dibenzylamino)phenothiazinium chloride (**1e**).* Purple powder, yield: 16%, mp: 110 °C; UV–vis (MeOH), λ_{max}(nm) (l g ε (L mol^{-1} cm^{-1})): 654 (5.0); IR (KBr pellet cm^{-1}) υ_{max}: 1595, 1487, 1393 (phenothiazinium skeleton), 1140(C–N); ^{1}H NMR(400 MHz; CD_3OD) δ_H: 7.98 (2H, d, J = 9.4, 2 × CH), 7.56 (2H, d, J = 9.7, 2 × CH), 7.47–7.36 (22H, m, Ar–H), 5.09 (4H, br, 2 × CH_2), 5.09 (4H, br, 2 × CH_2); ^{13}C NMR (101 MHz; CD_3OD; Me_4Si) δ_C: 156.1(*C*), 140.1(*C*), 137.8(*C*H), 136.7(*C*), 136.6(*C*), 130.3(*C*H), 129.2(*C*H), 127.9(*C*H), 121.2 (*C*H), 108.0(*C*H), 56.3(CH_2); m/z(ESI^+) 588.2515 ([M − Cl^-]$^+$, $C_{40}H_{34}N_3S^+$ requires 588.2473); Calcd. for $C_{40}H_{34}ClN_3S\cdot 2.5H_2O$: C, 71.78; H, 5.87; N, 6.28; Found: C, 71.65; H, 5.92; N, 6.25.

2.2. Antiprotozoal activities

The tests were performed at pH 7.4 as microplate assays using *T. b. rhodesiense* (STIB900), *T. cruzi* (Tulahuen C4), *L. donovani* (MHOM-ET-67/L82), and *P. falciparum* K_1 (resistant to chloroquine and pyrimethamine), and the cytotoxicity was assessed with rat skeletal myoblasts (L-6 cells) as described previously [20]. The following substances were used as reference standards: melarsoprol (*T. b. rhodesiense*), benznidazole (*T. cruzi*), miltefosine

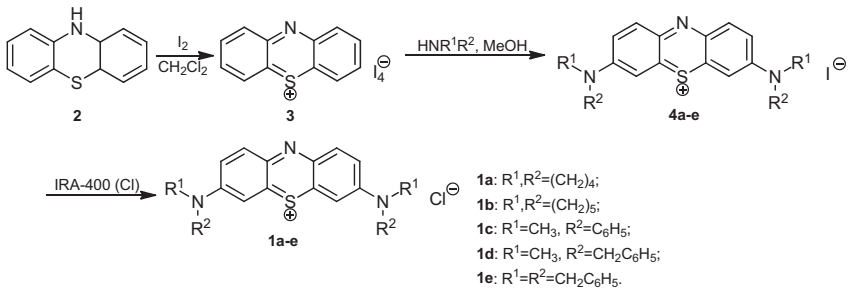

Fig. 2. Synthesis of phenothiazinium chlorides (**1a**–**e**).

(*L. donovani*), chloroquine (*P. falciparum*), and podophyllotoxin (cytotoxicity assay).

3. Results and discussion

3.1. Chemistry

Several synthetic routes have been reported for the preparation of phenothiazinium dyes [25], and only a few were suitable for our experiment since they had multi-step reactions and gave mixed products, while the phenothiazinium dyes are notoriously difficult to separate and purify [26]. As phenothiazinium compounds had been obtained for biological evaluation by Brown et al. [27], it was decided therefore to use the same synthetic route [28,29], which allows the reaction to progress to completion under ambient temperature and pressure in air. Thus, symmetrically disubstituted phenothiaziniums were obtained (Fig. 2).

The compounds (**1a**–**e**) exhibit absorption maxima in the range 652–667 nm in methanol solution, and the characteristic absorption peaks of phenothiazinium skeleton are located at ca. 1590–1398 cm^{-1} in their IR spectra. It was difficult to obtain good NMR spectra of the phenothiazinium iodides (**4a**–**e**), indeed several papers reporting such compounds did not report their NMR spectra [24,26]. The nitrate salt of phenothiazinium, which can be prepared by the reported method, might be suitable for characterization [30]; however, traces of silver ions might affect the result of the biological assay results. So, IRA-400 (Cl) exchange resin was used to obtain the phenothiazinium chlorides. After the purification by column chromatography, the NMR spectra of phenothiazinium chlorides were easily obtained in deuterated methanol solution. The NMR spectra of **1a** are shown in Fig. 3. The chemical shift of tertiary carbons is located at 105.4, 118.0, 136.9 ppm, and the chemical shift of quaternary carbon atom is located at 132.8, 134.0, 150.3 ppm. The peaks of secondary carbon were shown by four peaks. Both ^{1}H NMR and ^{13}C NMR are clearly demonstrated that phenothiazinium chlorides are symmetric structures in methanol solution.

In order to obtain specific structures of phenothiazinium salts, pyrrolidine, piperidine, *N*-methylaniline, *N*-methylbenzylamine and dibenzylamine were selected for the amines in the reaction.

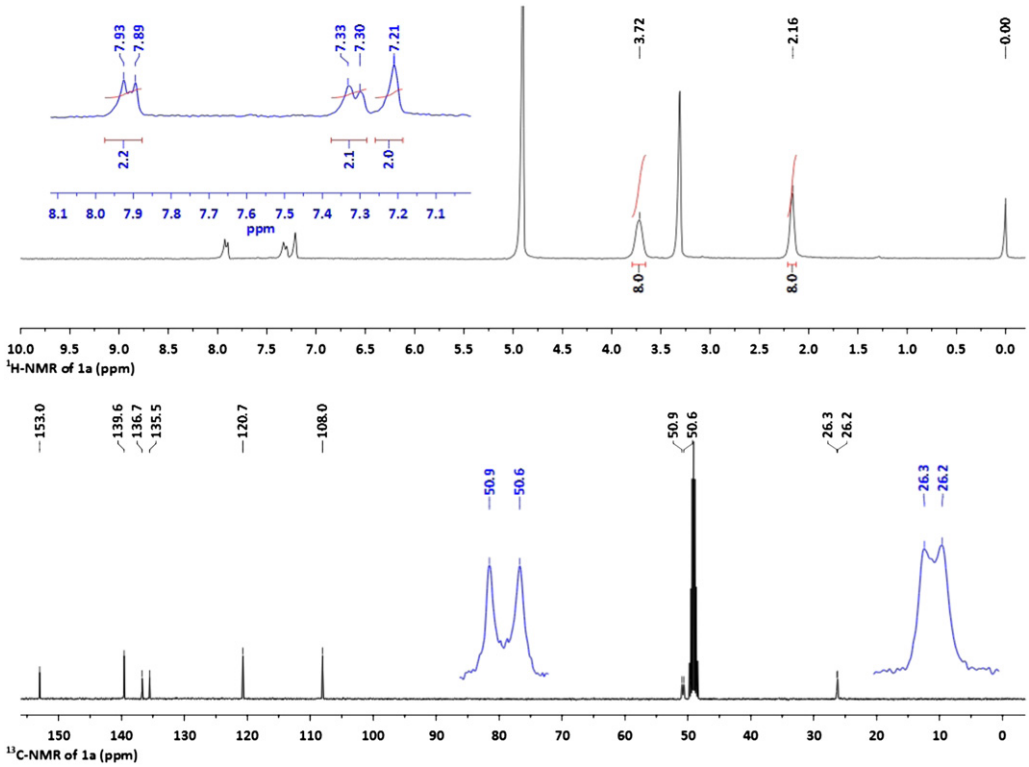

Fig. 3. NMR spectra of **1a**.

Table 1
Antiprotozoal and cytotoxic activities (IC_{50} values, μmol L^{-1}) of title compounds (**1a**–**e**)[a].

Compd.	*P. falc.*K_1		*T. cruzi*		*T.b. rhod.*		*L. don.*, axenic		Cytotox. L6
	IC_{50}	SI[b]	IC_{50}	SI[b]	IC_{50}	SI[b]	IC_{50}	SI[b]	IC_{50}
1a	0.005	18.40	ND[c]	ND[c]	0.089	1.06	0.150	0.62	0.094
1b	0.010	53.12	0.097	5.31	0.103	5.05	1.055	0.49	0.517
1c	2.500	3.49	7.905	1.10	0.396	22.04	0.748	11.68	8.739
1d	0.084	32.30	0.603	4.53	0.566	4.82	0.081	33.77	2.731
1e	0.188	21.02	0.713	5.55	0.266	14.88	0.207	19.14	3.957
Chloroquine[d]	0.148								
Benznidazole[d]			0.886						
Melarsoprol[d]					0.006				
Miltefosine[d]							0.280		
Podophyllotoxin[d]									0.02

[a] Values indicate the inhibitory concentration of a compound or standard in μM, which is necessary to achieve 50% growth inhibition (IC_{50}). Data shown are values from two replicate experiments.
[b] Selectivity index = IC_{50} value for L6/IC_{50} value for *P. falciparum*, *T. b. rhodesiense*, *T. cruzi*, or *L. donovani*.
[c] Not determined due to insufficient activity.
[d] Standard samples.

Diphenylamine was also used for the reaction, but no product can be obtained owing to steric hindrance and weak nucleophilicity.

3.2. Biological evaluations

3.2.1. Antiprotozoal activities

Antiprotozoal and cytotoxic activities of the phenothiazinium chlorides (**1a**–**e**) are shown in Table 1. All compounds show insufficient activities against *T. b. rhodesiense*. 3,7-Di(pyrrolidinyl) phenothiazinium chloride (**1a**) shows median inhibitory concentration (IC_{50}) of 0.005 μmol L^{-1} against *P. falciparum* K_1 strain, and it is moderately active compared with rhodacyanine [13–17] and phenoxazinium [18–21] derivatives.

Notably, 3,7-di(piperidinyl)phenothiazinium chloride (**1b**) shows IC_{50} of 0.097 μmol L^{-1} against *T. cruzi*, which reveals that it might be a lead compound for the Chagas disease, since there are no good candidates for the treatment of this type disease at present. 3,7-Bis(benzyl(methyl)amino)phenothiazinium chloride (**1d**) exhibits IC_{50} of 0.081 μmol L^{-1} against *L. donovani*.

3.2.2. Cytotoxicity

The IC_{50} value to the normal L6 cell, 3,7-di(pyrrolidinyl)phenothiazinium chloride (**1a**, IC_{50} = 0.094 μmol L^{-1}) with five-membered ring shows more than five times toxicity comparing with the 3,7-di(piperidinyl)phenothiazinium chloride (**1b**, IC_{50} = 0.517 μmol L^{-1}). From the IC_{50} values of **1d** and **1e**, it is clearly demonstrated that compounds with benzyl groups show lower toxicity, and 3,7-bis(dibenzylamino)phenothiazinium chloride (**1e**, IC_{50} = 3.957 μmol L^{-1}) is less toxic than 3,7-bis(benzyl(methyl) amino)phenothiazinium chloride (**1d**, IC_{50} = 2.731 μmol L^{-1}). The cytotoxicity (IC_{50} = 8.739 μmol L^{-1}) of 3,7-bis(methyl(phenyl) amino)phenothiazinium chloride(**1c**) was lowest among the tested compounds.

4. Conclusions

A series of phenothiazinium chlorides were synthesized and evaluated for *in vitro* activities against *P. falciparum*, *T. cruzi*, *T. brucei rhodesiense*, and *L. donovani*. 3,7-Di(piperidinyl)phenothiazinium chloride shows IC_{50} of 0.097 μmol L^{-1} against *T. cruzi*; and 3,7-bis (benzyl(methyl)amino)phenothiazinium chloride exhibits IC_{50} of 0.081 μmol L^{-1} against *L. donovani*. The *N*-Aromatic and *N*-benzyl substituted phenothiazinium chlorides are less toxic compounds than the *N*-alkyl substituted compounds.

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