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Synthesis and Antimicrobial and Nitric Oxide Synthase Inhibitory Activities of Novel Isothiourea Derivatives

Zygmunt Kazimierczuk[1], Malgorzata Chalimoniuk[2], Agnieszka Ewa Laudy[3], Rosa Moo-Puc[4], Roberto Cedillo-Rivera[4], Bohdan Jerzy Starosciak[3], and Stanislaw J. Chrapusta[1]

Department of [1]Experimental Pharmacology, Polish Academy of Sciences, 02-106 Warsaw, Poland, [2]Cellular Signaling, Mossakowski Medical Research Center, Polish Academy of Sciences, 02-106 Warsaw, Poland, [3]Department of Pharmaceutical Microbiology, Medical University of Warsaw, 02-007 Warsaw, Poland, and [4]Unidad Interinstitucional de Investigación Clínica y Epidemiológica, Facultad de Medicina, UADY/Instituto Mexicano del Seguro Social, Mérida, Yuc., México

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The reaction of substituted benzylhalides, or of halomethyl derivatives of thiophene or furane, with thiourea or its derivatives yielded the respective isothioureas as hydrohalide salts. The products (a total of 17, including 16 novel compounds) were tested for activity against five Gram-positive and nine Gram-negative bacterial strains, six yeast species and two protozoan species. The most active against Gram-positive bacteria were S-(2,4-dinitrobenzyl)isothiourea hydrochloride (MIC range for four out of five strains tested: 12.5-25 μg/mL) and S-(2,3,4,5,6-pentabromobenzyl)isothiourea hydrobromide (MIC range: 12.5-50 μg/mL). The lowest MICs of novel isothioureas for yeast and Gram-negative bacteria ranged between 50 and 100 μg/mL. Nine novel isothioureas showed appreciable genotoxicity in the Bacillus subtilis 'rec-assay' test, the most potent being S-2-(5-nitrofuran-2-ylmethyl)isothiourea and S-(2-nitrobenzyl)isothiourea. At 10 μM concentration, S-(3,4-dichlorobenzyl)isothiourea hydrochloride and S-(2,3,4,5,6-pentabromobenzyl)isothiourea hydrobromide inhibited Ca^{2+}/calmodulin-dependent (non-inducible) nitric oxide synthase activity in normal rat brain homogenates stronger ($p < 0.05$) than the reference drug 7-nitroindazole (by 78, 76 and 60%, respectively); ten other new isothiourea derivatives significantly inhibited the activity to a lower extent (by 28-60%). These results extend the list of promising isothioureas with substantial activity *in vitro* and suggest that an in-depth study of toxicity, antimicrobial properties *in vivo* and nitric oxide synthase isoform selectivity of selected novel compounds is warranted.

Key words: Isothiourea derivative, Synthesis, Antibacterial activity, Antifungal activity, Anti-protozoal activity, Nitric oxide synthase

INTRODUCTION

Isothioureas are a class of amphiphilic compounds carrying a highly basic isothiourea group of $pK_b \approx 10$. At physiological pH (pH ≈ 7), these compounds exist in a protonated (cationic) form that may be of importance for their specific biological effects. Their synthesis and isolation is simple due to their poor solubility in reaction medium. On the other hand, in solid state, they form salts of usually good water solubility, which makes them particularly attractive for microbiology studies.

Isothioureas are highly interesting compounds from a pharmaceutical/pharmacological point of view as well. The 'parental' unsubstituted thiourea (thiocarbamide) is toxic and is a known carcinogen (see http://msds.chem.ox.ac.uk/TH/thiourea.html). However, a rapidly growing body of evidence demonstrates multiple potentially beneficial biological activities of its derivatives. Some of these compounds show potential as prodrugs of the alcohol deterrent agent cyanamide (Shirota et al., 1997), inhibitors of HIV capsid assembly (Li et al., 2009b), blockers of the CXCR4 chemokine receptors with potential for preventing HIV infection

Correspondence to: Zygmunt Kazimierczuk, Department of Experimental Pharmacology, Mossakowski Medical Research Center, Polish Academy of Sciences, 5 Pawińskiego St., 02-106 Warsaw, Poland
Tel: 4822-593-7613, Fax: 4822-822-1848
E-mail: zkazimierczuk@gmail.com

of target cells (Thoma et al., 2008), anticancer drugs (for review see Li et al., 2009a), hypoglycaemic drugs (Zhang et al., 2009), calcium blockers with potential neuroprotectant and cognition enhancer capability (Perlovich et al., 2009), anti-HCV agents with high selectivity index (Kang et al., 2009), and proapoptotic agents with substantial toxicity toward human glioblastoma cells *in vitro* (Kaminska et al., 2009). However, most attention is being given to these drugs as nitric oxide synthase (NOS) inhibitors with variable NOS isoform selectivity (Szabo et al., 1994; Southan et al., 1995; Handy et al., 1996; Wang et al., 1998; Paquay et al., 1999; Ijuin et al., 2005; Jin et al., 2009) and antimicrobial agents.

First papers on antimicrobial activity of N,S-substituted isothioureas were published in late 1980s (Tait et al., 1989, 1990). Some of those compounds showed substantial activity against Gram-positive and Gram-negative bacteria, but not against fungi tested (*Microsporum canis, Aspergillus niger, A. flavus* and *Candida albicans*). The most potent substances in that series were S-(3,4,5-trichlorobenzyl)-isothiourea and its N,N'-tetramethyl congener. Recently, S-(3,4-dichlorobenzyl) isothiourea and S-(4-chlorobenzyl)isothiourea, but not their S-ethyl, S-nonyl and S-cyclohexyl analogs, were found to interfere with chromosome partitioning and induce spherical and spherical anucleate cells in *Escherichia coli* (Iwai et al., 2004). This effect likely results from their interaction with rod shape-determining proteins (Iwai et al., 2002). Most recently, S-(4-chlorobenzyl)isothiourea has been shown to have substantial activity against many strains of *Pseudomonas aeruginosa* and *Burkholderia cepacia* complex that are important pathogens in cystic fibrosis patients (Nicholson et al., 2009). Anti-protozoal activity of isothiourea derivatives has not been studied until now.

There are three NOS isoforms: an inducible enzyme (iNOS) and the constitutive (Ca^{2+}/calmodulin-dependent) neuronal (nNOS) and endothelial (eNOS) isoforms, first two of which, and particularly iNOS, can cause NO overproduction and, hence, participate in the related tissular damage. In a contrast, eNOS has no known detrimental effect, but plays an important role in maintaining blood pressure and flow. Because of differing roles of these enzymes in physiology and pathology, there is an ongoing search for the respective selective inhibitors (Garvey et al., 1994; Szabo et al., 1994; Babu and Griffith, 1998; Salerno et al., 2002; Castano et al., 2008). This search included also a number of studies on isothiourea derivatives, of which two most investigated and active were, so far, ethylisothiourea and aminoethylisothiourea (Garvey et al., 1994; Southan et al., 1995; Shearer et al., 1997; Salerno et al., 2002; Xu et al., 2003; Paesano et al., 2005; Barocelli et al., 2006).

Below, we describe the synthesis of a series of new compounds of this class, and the results of our preliminary studies *in vitro* of their antibacterial, antifungal and anti-protozoal activities as well as inhibitory activity against Ca^{2+}/calmodulin-dependent NOSs.

MATERIALS AND METHODS

Chemistry

All solvents and reagents were purchased from Sigma-Aldrich. Melting points (uncorr.) were measured in open capillary tubes in a Gallenkamp-5 melting-point apparatus. ^{1}H-NMR spectra were measured with a Varian Gemini 200 MHz (or Varian UNITYplus 500 MHz) spectrometer at 298 °K in D_6(DMSO), using tetramethylsilane as an internal standard. Flash chromatography was performed with Merck silica gel 60 (200-400 mesh). Elemental (C, H, N) analyses of the new compounds were within 0.4% of the respective theoretical values.

Synthesis

3,5-Dinitrobenzylisothiourea hydrochloride (Exemplary synthesis)

To a hot solution of thiourea (400 mg, 5.1 mmol) in anhydrous ethanol (20 mL) 3,5-dinitrobenzylchloride (1.08 g, 5 mmol) was added. The mixture was refluxed for 20 min and then the solvent was partially evaporated to a final volume of about 15 mL. This was left refrigerated overnight. The chromatographically pure crystals that formed (0.75 g, 78% yield) were filtered off and washed with a small volume of cold ethanol-ethyl ether mixture (1:1, v/v). For elemental analysis, a small amount of the product was recrystallized from ethanol.

Biological studies *in vitro*

Antibacterial, antifungal and genotoxicity assays

The microorganisms employed were as follows: (1) Gram-positive bacteria: *Staphylococcus aureus* ATCC 6538P, *Staphylococcus aureus* NCTC 4163, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6633, *Bacillus stearothermophilus* ATCC 7953; (2) Gram-negative bacteria: *Proteus vulgaris* NCTC 4635, *Escherichia coli* ATCC 25922, *E. coli* NCTC 8196, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* NCTC 6749, *Stenotrophomonas maltophilia* ATCC 1363, *Acinetobacter baumannii* 18606, *Bordetella bronchiseptica* ATCC 4617, *Burkholderia*

cepacia ATCC 25416; (3) fungi: *Candida albicans* ATCC 90028, *C. parapsilosis* ATCC 22019, *C. tropicalis* IBA 171, *C. guilliermondii* IBA 155, *C. krusei* IBA 161 and *Saccharomyces cerevisiae* IBA 198. All these microorganisms came from the collection of the Department of Pharmaceutical Microbiology, Medical University of Warsaw (Poland). *Bacillus subtilis* M45 rec^- and H17 rec^+ strains used for genotoxicity study (Sadaie and Kada, 1976; Kada et al., 1980) were kindly donated by Dr. Sadaie of the National Institute of Genetics, Japan.

Antimicrobial activity was examined by the disk-diffusion method and by the minimum inhibitory concentration (MIC) method under standard conditions, using Mueller-Hinton II agar medium (Becton-Dickinson) for bacteria, and RPMI agar medium (Sigma) supplemented with 2% glucose (Sigma) for fungi/yeast, according to the guidelines established by the Clinical and Laboratory Standards Institute (2006a, 2006b). For disk-diffusion assays (both for the antibacterial activity study and the genotoxicity 'rec-assay' test), sterile filter paper (Whatman No. 3) disks of 9 mm diameter were dripped with the tested compound solutions in ethanol or methanol to load 400 μg of a given compound per disk. Except for fluconazole (reference compound), concentration of the tested compounds in solid medium ranged from 0.78 to 400 μg/mL for MIC determinations, and agar plates were inoculated using 2 μL aliquots. The fluconazole MICs for fungi/yeast were determined using Etest gradient strips (AB Biodisk) according to the guidelines established by the manufacturer (Etest Technical Manual, 2000). The final inoculi of all studied organisms were 10^4 colony forming units per mL (CFU/mL), except that the final inoculum of *E. faecalis* ATCC 29212 was 10^5 CFU/mL. Results of the antimicrobial activity tests were read after incubation at 35°C for 18 h for antibacterial activity and the genotoxicity test, and after 24 h incubation at 35°C for antifungal activity study.

Anti-protozoal activity

Susceptibility assays were performed as previously described (Cedillo-Rivera and Munoz, 1992; Cedillo-Rivera et al., 1997). Briefly: 4×10^4 trophozoites of *G. intestinalis,* or 610^3 trophozoites of *E. histolytica,* were incubated for 48 h at 37°C with various concentrations of the tested compounds in modified TYI-S-33 medium. All the compounds, including metronidazole, were added to the incubation medium as solutions in dimethyl sulfoxide (DMSO). For negative control, the trophozoites were incubated in the same medium supplemented with the same amount of DMSO. After the incubation, the cells were washed and cultured for another 48 h in the same medium, but with no drug or DMSO added. Trophozoites were then counted with a haemocytometer. All assays were carried out in triplicates and were repeated three times each. The 50% inhibitory concentrations (IC_{50}) and their respective 95% confidence limits were calculated by probit analysis.

Animals

Specific pathogen-free, intact adult male Wistar rats, 3-4 months of age, 250-300 g body weight, were obtained from the animal facility of the Mossakowski Medical Research Center in Warsaw. The rats were killed by simple decapitation. Their brains were immediately removed, and the cortex and hippocampus were dissected on ice and used fresh for the determination of the NOS inhibitory activity of the novel isothioureas (see below).

Assessment of NOS inhibitory activity

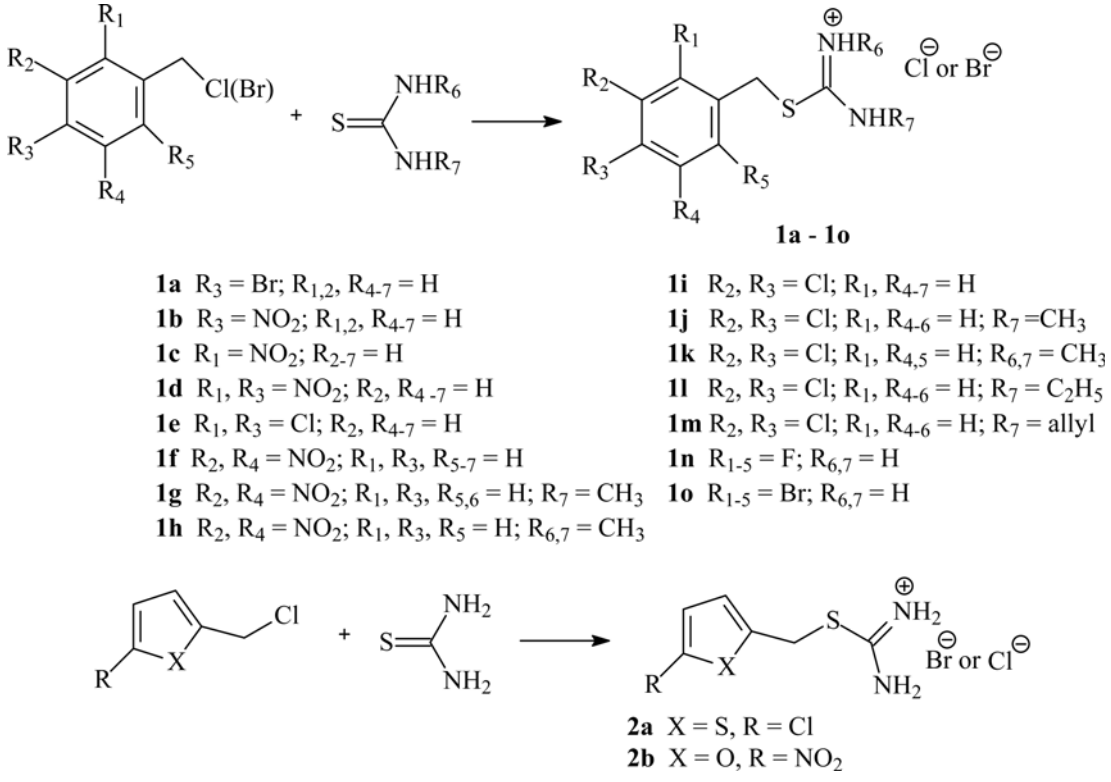
Rat brain tissue samples (cortex + hippocampus) were homogenized in 10 mM Tris-HCl buffer pH 7.4 containing 0.25 M sucrose, 1 mM EDTA, and complete protease inhibitor cocktail (Roche Diagnostics; one mini tablet/10 mL) in a Dounce homogenizer using 14 strokes. Homogenate protein content was determined by the method of Lowry. Homogenate aliquots (200 μg of protein) were incubated for 20 min at 37°C in the solution containing 50 mM Tris-HCl buffer pH 7.4, 100 μM [U-^{14}C]L-arginine (0.2 μCi), 2 mM $CaCl_2$, 1 μM calmodulin, 15 μM FAD, 10 μM tetrahydrobiopterin, 1 mM NADPH, 1 mM EDTA and 1 mM dithiothreitol, in the presence and absence of the neuronal NOS (nNOS) inhibitor 7-nitroindazole (7NI, 10 μM) or of the newly synthesized compounds **1a-1o**, **2a** and **2b** (10 μM); the final volume of all reaction mixtures was 300 μL. The reaction was terminated by adding 1 mL of 100 mM Tris-HCl buffer pH 5.5 containing 10 mM EDTA. Gross cellular debris was removed by centrifugation at 3000 g for 10 min. The resulting supernatant was passed through 1 mL Na^+ form Dowex™ 50W 8 columns, and [^{14}C]L-citrulline was eluted with 2 1 mL H_2O. The eluate was mixed with 10 mL of Bray's scintillation liquid and [^{14}C] radioactivity was measured in an LKB Wallac 1409 counter.

RESULTS AND DISCUSSION

Physicochemical characteristics of the novel isothiourea derivatives and the previously described compound **1i** are presented in Table I.

Table I. Physicochemical properties of the novel isothiourea derivatives

Compound	Formula (M.W.)	M.P. [°C]	Yield [%]	^{1}H-NMR δ [ppm]
1a	$C_8H_{10}Br_2N_2S$ (326.05)	178-180	89	4.50 (s, 2H, C$\underline{H}_2$); 7.37-7.61 (2m, 3H, Ar-$\underline{H}$); 9.13 (bs, 4H, 2×N$\underline{H}_2$)
1b	$C_8H_{10}ClN_3O_2S$ (247.70)	232-234	84	4.74 (s, 2H, C$\underline{H}_2$); 7.72-8.26 (2m, 4H, Ar-$\underline{H}$); 9.43 (bs, 4H, 2×N$\underline{H}_2$)
1c	$C_8H_{10}ClN_3O_2S$ (247.70)	193-195	68	4.79 (s, 2H, C$\underline{H}_2$); 7.62-8.16 (m, 4H, Ar-$\underline{H}$); 9.20 (bs, 4H, 2×N$\underline{H}_2$)
1d	$C_8H_9ClN_4O_4S$ (292.70)	204-206	71	4.99 (s, 2H, C$\underline{H}_2$); 8.10-8.80 (m, 3H, Ar-$\underline{H}$); 9.43 & 9.58 (2bs, 4H, 2×N$\underline{H}_2$)
1e	$C_8H_9Cl_3N_2S$ (271.59)	234-236	85	4.79 (s, 2H, C$\underline{H}_2$); 7.46-7.72 (m, 3H, Ar-$\underline{H}$); 9.41 (bs, 4H, 2×N$\underline{H}_2$)
1f	$C_8H_9ClN_4O_4S$ (292.70)	234-235	78	4.84 (s, 2H, C$\underline{H}_2$); 8.75-8.90 (m, 3H, Ar-$\underline{H}$); 9.42 (bs, 4H, 2×N$\underline{H}_2$)
1g	$C_{10}H_{11}ClN_4O_4S$ (306.72)	99-101	71	2.84 (d, 3H, C$\underline{H}_3$); 4.96 (s, 2H, C$\underline{H}_2$); 8.75-8.85 (m, 3H, Ar-$\underline{H}$); 9.85 & 10.26 (2s, 3H, N$\underline{H}$ & N$\underline{H}_2$)
1h	$C_{10}H_{13}ClN_4O_4S$ (320.75)	221-222	72	2.94 (s, 6H, 2×C$\underline{H}_3$); 4.98 (s, 2H, C$\underline{H}_2$); 8.75-8.83 (m, 3H, Ar-$\underline{H}$); 9.54 & 10.05 (2bs, 2H, 2×N$\underline{H}$)
1i	$C_8H_9Cl_3N_2S$ (271.59)	246-248	88	4.60 (s, 2H, C$\underline{H}_2$); 7.43-7.77 (m, 3H, Ar-$\underline{H}$); 9.40 (bs, 4H, 2×N$\underline{H}_2$)
1j	$C_9H_{11}Cl_3N_2S$ (285.62)	149-151	82	2.86 (s, 3H, C$\underline{H}_3$); 4.68 (s, 2H, C$\underline{H}_2$); 7.40-7.76 (m, 3H, Ar-$\underline{H}$); 9.65 & 10.16 (2bs, 3H, N$\underline{H}$ & N$\underline{H}_2$)
1k	$C_{10}H_{13}Cl_3N_2S$ (299.65)	110-112	69	2.93 (s, 6H, 2×C$\underline{H}_3$); 4.73 (s, 2H, C$\underline{H}_2$); 7.50-7.80 (m, 3H, Ar-$\underline{H}$); 9.80 (bs, 2H, 2×N$\underline{H}$)
1l	$C_{10}H_{13}Cl_3N_2S$ (299.65)	150-152	83	1.06 (t, 3H, C$\underline{H}_3$); 3.32 (q, 2H, C$\underline{H}_2$); 4.68 (s, 2H, C$\underline{H}_2$); 7.42-7.76 (m, 3H, Ar-$\underline{H}$); 9.63 (bs, 3H, N$\underline{H}$ & N$\underline{H}_2$)
1m	$C_{11}H_{13}Cl_3N_2S$ (311.66)	95-97	90	3.97 (d, 2H, C$\underline{H}_2$); 4.72(s, 2H, C$\underline{H}_2$); 5.00-5.13 (m, 2H, C$\underline{H}_2$); 5.74 (m, 1H, C$\underline{H}$); 7.60-7.77 (m, 3H, Ar-$\underline{H}$); 9.75 (bs, 3H, N$\underline{H}$ & N$\underline{H}_2$)
1n	$C_8H_6BrF_5N_2S$ (337.11)	201-203	84	4.67 (s, 2H, C$\underline{H}_2$); 9.29 (bs, 4H, 2×N$\underline{H}_2$)
1o	$C_8H_6Br_6N_2S$ (641.63)	273-276 decomp.	73	4.90 (s, 2H, C$\underline{H}_2$); 9.20 & 9.39 (2bs, 4H, 2×N$\underline{H}_2$)
2a	$C_6H_8Cl_2N_2S_2$ (243.17)	178-180	85	4.81 (s, 2H, C$\underline{H}_2$); 6.99 & 7.04 (2d, 2H, 2×C$\underline{H}$); 9.36 & 9.45 (2bs, 4H, 2×N$\underline{H}_2$)
2b	$C_6H_8BrN_3O_3S$ (282.11)	199	89	4.78 (s, 2H, C$\underline{H}_2$); 6.85 & 7.68 (2d, 2H, 2×C$\underline{H}$); 9.16 & 9.33 (2bs, 4H, 2×N$\underline{H}_2$)



Secheme 1. Synthesis of isothiourea derivatives

The substituted benzylisothioureas **1a-1o** and the heterocyclic isothioureas **2a** and **2b** were synthesized by heating the respective bromide or chloride with thiourea in anhydrous ethanol (Scheme 1). In most cases the reaction product precipitated as the respective chromatographically pure hydrochloride or hydrobromide after cooling down the reaction mixture. Flash column chromatographic purification of the product was only necessary for the compounds **1g**, **1h**, **1k** and **1m**.

In a majority of the few earlier studies on the antibacterial activity of isothiourea derivatives, the range of the bacterial genera used included only a small number of microorganisms. In the present study, antimicrobial activity of the new isothiourea derivatives was tested against much wider range of bacteria, including four species (five strains) of Gram-positive cocci and eight species (nine strains) of Gram-negative rods, and against six yeast species. Detailed results of these tests are given in Tables II, III and IV, respectively.

Overall, twelve of the novel isothioureas showed antibacterial activity *in vitro*. As a rule, they were more active against Gram-positive than against Gram-negative bacteria. Generally speaking, the lowest MIC values with respect to both bacteria types were found for S-2,4-dinitrobenzylisothiourea (**1d**) and S-2,3,4,5,6-pentabromobenzyl-isothiourea (**1o**). The two derivatives were highly effective against *B. subtilis, B. stearothermophilus and S. aureus*, and were more active against *S. aureus* strains than any other thiourea derivative tested until now (Khan et al., 2008; Zhong et al., 2008), and compounds **1f**, **1g** and **2a**, showed high activity against the *Bacillus* spp. and *Staphylococcus* spp. used (MIC: 12.5-50 μg/mL, growth inhibition area: 16-28 mm diameter). The activities against *S. aureus* of these five compounds were similar to that of the reference compound nitrofurantoin (Table II). A majority of novel isothiourea derivatives were also active against Gram-negative rods, eleven of them being active both against *Enterobacteriaceae* and non-fermentative rods. Compounds **1d**, **1i** and **1o** showed substantial activity against a majority of the Gram-negative bacteria employed, with MIC values ranging from 12.5 to 200 μg/mL. The activities of these derivatives against *E. coli* were similar to those of the previously published thiourea derivatives (Khan et al., 2008). However, their activities against *P. aeruginosa* and *B. cepacia* were somewhat lower than that reported for the prototype substituted isothiourea derivative S-(4-chlorobenzyl)isothiourea (*cf.* Nicholson et al., 2009). Nevertheless, it would be interesting to see if and which of the new compounds, similarly to the other substituted S-(benzyl)isothioureas (Nicholson et al., 2009), show synergy with conventional antibacterial agents. Of the bacteria utilized in the present study, *P. aeruginosa* and *E. faecalis* showed the lowest susceptibility to all studied compounds (Table III).

Intriguingly, S-(3,4-dichlorobenzyl)isothiourea (**1i**), which has been previously reported to be highly active against *E. coli* (MIC: 3.13 μg/mL; Iwai et al., 2004), did not demonstrate similar activity in our study (MIC = 200 μg/mL). The reason for the discrepancy may be the different *E. coli* strain utilized. We employed standard *E. coli* strains ATCC 25922 and NCTC 8196, which are widely used as control Gram-negative bacteria for antibiotic susceptibility assays and testing of disinfectants, respectively. The *E. coli* K-12 MG1655

Table II. Activity of selected novel isothioureas against Gram-positive bacteria[a]

Bacterium	MIC [μg/mL] (diameter of growth inhibition area, mm)[b]										
	1d	**1e**	**1f**	**1g**	**1h**	**1i**	**1m**	**1o**	**2a**	**2b**	Nitro-furantoin[c]
Staphylococcus aureus ATCC 6538P	12.5 (28)	200 (16)	25 (28)	25 (26)	200 (16)	100 (20)	200 (17)	12.5 (13)	50 (28)	200 (23)	25 (24)
Staphylococcus aureus NCTC 4163	25 (28)	200 (17)	50 (26)	50 (27)	200 (16)	200 (19)	200 (18)	12.5 (13)	50 (27)	400 (19)	25 (23)
Enterococcus faecalis ATCC 29212	200 (-)	400 (14)	400 (13)	200 (17)	>400 (13)	200 (16)	200 (21)	50 (-)	>400 (11)	>400 (14)	12.5 (22)
Bacillus subtilis ATCC 6633	12.5 (30)	200 (14)	25 (22)	200 (19)	50 (20)	200 (18)	200 (18)	12.5 (11)	50 (20)	400 (23)	12.5 (28)
Bacillus stearothermophilus ATCC 7953	12.5 (33)	200 (17)	25 (22)	50 (24)	50 (22)	200 (20)	100 (20)	12.5 (11)	12.5 (16)	100 (25)	12.5 (27)

[a]Compounds that showed no activity by both the disk-diffusion method and the MIC method were omitted in the table.
[b]Denotes no growth inhibition around disk
[c]Reference compound, 300 μg per disk (Mast Diagnostics, UK)

($F^-\lambda^-$) strain used by Iwai et al. was genetically manipulated and has been cured of bacteriophage λ and F plasmid by means of UV light and acridine orange, respectively (Blattner et al., 1997). Since the mutations present in the genotype of that strain were likely acquired early in its history and are present in most K-12 strains (see http://www.genome.wisc.edu/resources/strains.htm), the basis for the increased susceptibility of this particular strain to compound **1i** is obscure.

Table III. Activity of selected novel isothioureas against Gram-negative bacteria[a]

Bacterium	MIC [μg/mL] (diameter of growth inhibition area, mm)[b]											
	1a	**1d**	**1e**	**1f**	**1g**	**1i**	**1j**	**1m**	**1o**	**2a**	**2b**	Nitro-furantoin[c]
Proteus vulgaris NCTC 4635	400 (11)	50 (23)	200 (20)	100 (24)	100 (26)	200 (20)	400 (28)	200 (22)	100 (-)	200 (28)	400 (17)	100 (17)
Escherichia coli ATCC 25922	400 (13)	50 (24)	200 (17)	50 (21)	100 (22)	200 (18)	200 (20)	200 (22)	25 (12)	400 (26)	400 (19)	6.25 (24)
Escherichia coli NCTC 8196	400 (13)	50 (25)	100 (18)	50 (21)	200 (19)	200 (20)	200 (26)	200 (17)	25 (12)	>400 (25)	>400 (15)	6.25 (24)
Klebsiella pneumoniae ATCC 13883	400 (11)	50 (21)	100 (18)	200 (20)	200 (19)	100 (20)	200 (20)	200 (18)	50 (-)	400 (19)	>400 (14)	25 (23)
Pseudomonas aeruginosa NCTC 6749	200 (13)	400 (11)	400 (14)	200 (12)	>400 (-)	100 (14)	400 (25)	>400 (-)	>400 (-)	>400 (28)	>400 (-)	>400 (-)
Stenotrophomonas maltophilia ATCC 1363	200 (14)	100 (20)	100 (17)	100 (19)	200 (17)	50 (19)	200 (38)	100 (33)	12.5 (12)	100 (28)	>400 (13)	>400 (-)
Burkholderia cepacia ATCC 25416	100 (18)	>400 (16)	100 (19)	>400 (14)	>400 (13)	50 (20)	400 (13)	400 (12)	>400 (-)	100 (29)	>400 (13)	>400 (-)
Acinetobacter baumannii ATCC 18606	200 (18)	100 (22)	100 (21)	200 (19)	200 (21)	100 (22)	400 (22)	200 (21)	100 (-)	400 (25)	400 (16)	200 (14)
Bordetella bronchiseptica ATCC 4617	100 (14)	100 (20)	100 (22)	100 (18)	200 (15)	50 (17)	100 (28)	200 (28)	50 (-)	50 (21)	400 (14)	>400 (-)

[a]Compounds that showed no activity by both the disk-diffusion method and the MIC method were omitted in the table.
[b]Denotes no growth inhibition around disk
[c]Reference compound, 300 μg per disk (Mast Diagnostics, UK)

Table IV. Antifungal activity of selected novel isothioureas[a]

Yeast strain	MIC [μg/mL] (diameter of growth inhibition area, mm)[b]													
	1a	**1d**	**1e**	**1f**	**1g**	**1i**	**1j**	**1k**	**1l**	**1m**	**1o**	**2a**	**2b**	Flucon-azole[c]
Candida albicans ATCC 90028	400 (11)	>400 (-)	200 (19)	>400 (-)	200 (-)	400 (18)	400 (20)	400 (12)	400 (13)	200 (22)	50 (-)	400 (13)	400 (15)	2 (43)
Candida parapsilosis ATCC 22019	400 (13)	>400 (-)	200 (22)	400 (13)	200 (-)	400 (18)	400 (21)	400 (16)	400 (14)	200 (12)	100 (-)	400 (-)	400 (15)	2 (32)
Candida tropicalis IBA 171	400 (11)	>400 (-)	200 (18)	>400 (-)	200 (-)	400 (20)	>400 (18)	400 (16)	400 (14)	200 (21)	50 (-)	400 (-)	>400 (-)	0.38 (39)
Candida guilliermondii IBA 155	400 (13)	400 (16)	200 (22)	400 (12)	200 (-)	200 (22)	400 (20)	200 (20)	200 (17)	200 (23)	100 (-)	400 (15)	200 (14)	0.75 (40)
Candida krusei IBA 161	400 (15)	400 (14)	200 (24)	400 (15)	200 (-)	200 (21)	200 (21)	200 (18)	200 (21)	100 (25)	50 (-)	400 (16)	400 (15)	>256 (16)
Saccharomyces cerevisiae IBA 198	400 (11)	>400 (-)	200 (19)	400 (11)	200 (-)	200 (20)	200 (26)	200 (16)	200 (17)	100 (28)	50 (-)	200 (17)	200 (15)	>256 (12)

[a]Compounds that showed no activity by both the disk-diffusion method and the MIC method were omitted in the table.
[b]Denotes no growth inhibition around disk
[c]Reference compound; filter paper disks (Bio-Rad) used for the disk-diffusion method contained 25 μg of fluconazole per disk, whereas Etest gradient strips (AB Biodisk) were used for the determination of fluconazole MICs.

A few N-substituted thiourea derivatives have been reported to have substantial antifungal activity (del Campo et al., 2004; Rodriguez-Fernandez et al., 2005). Sensitivity to those compounds differed considerably between molds and yeast. In contrast to that of molds, the growth of *S. cerevisiae* was not inhibited by any of the eight N-benzoyl-N'-alkylthioureas derivatives examined so far (del Campo et al., 2004). Until now, only a low activity against *C. albicans* of a few thiourea derivatives was reported (Eissa and Moneer, 2004). In our study, the sensitivity of *Candida* to isothiourea derivatives was investigated using five *Candida* strains that are known human pathogens. Fifteen of the studied compounds showed no (**1b**, **1c**, **1h** and **1n)** or low activity (MIC: ≥200 μg/mL), and one compound (**1o**) showed a moderate activity (MIC: 100 μg/mL) against all these pathogens. However, the activity of derivatives **1m** and **1o** against the nonpathogenic *S. cerevisiae* IBA 198 and fluconazole-resistant yeast strain *C. krusei* IBA 161 exceeded that of fluconazole (MIC: 50-100 μg/mL versus >256 μg/mL, respectively), see Table IV.

There was an apparent disparity between the MICs of compound **1o** for the yeast strains tested and the total lack of such activity of this derivative in the corresponding disk-diffusion test. One should remember that the pentabromobenzyl group present in its molecule (Scheme 1) confers substantial hydrophobic effect. Hence, the negative result of the latter test might have reflected poor water solubility of this compound. However, inactivation of this compound, e.g. by oxidation or bacterial catabolism under highly aerobic conditions of the disk-diffusion test could be at play as well. Generally, MIC method is considered more reliable than the disk-diffusion assay for examination of antimicrobial activity (CLSI, 2006a).

Genotoxicity of the new isothiourea derivatives was tested using the genetically modified *B. subtilis* strains H17 (rec^+) and M45 (rec^-). The latter strain is devoid of the recombination-based DNA repair mechanism and is, therefore, much more susceptible to genotoxic damage. As evidenced by the difference in the diameter of the growth inhibition area between the rec^- strain and its rec^+ counterpart, S-(2-nitrobenzyl)isothiourea (**1c**) and S-2-(5-nitrofuran-2-ylmethyl)isothiourea (**2b**) showed the highest and substantial genotoxicity, and seven more of the novel isothiourea derivatives (**1d**, **1f**, **1g**, **1h**, **1j**, **1l** and **1o**) exerted an appreciable genotoxic effect. Compound **1o** produced the smallest areas of growth inhibition in this test (Table V), which was consistent with its poor solubility in water.

Table V. Genotoxicity of the novel isothiourea derivatives[a]

Compound tested	Diameter of growth inhibition area (mm)	
	Bacillus subtilis (H17 rec^+)	*Bacillus subtilis* (M45 rec^-)
1a	16	16
1b	24	25
1c	–[b]	23
1d	37	44
1e	23	23
1f	31	36
1g	29	35
1h	28	33
1i	23	24
1j	14	20
1k	15	15
1l	14	18
1m	19	19
1n	11	11
1o	11	15
2a	24	24
2b	28	40
4-nitroquinoline N-oxide[c]	12	24

[a]By the "rec-assayn" test without enzymatic activation (8)
[b]No growth inhibition area around disk
[c]Reference compound, 2 μg per 9 mm diameter disk

Table VI. *In vitro* susceptibility of *Giardia intestinalis* and *Entamoeba histolytica* to novel isothiourea derivatives

Compound tested	*Giardia intestinalis*		*Entamoeba histolytica*	
	IC_{50} [μg/mL]	95% confidence limits	IC_{50} [μg/mL]	95% confidence limits
1a	6.76	6.75-6.78	7.92	7.91-7.94
1b	2.23	2.22-2.24	9.14	9.12-9.14
1c	1.21	1.20-1.22	5.80	5.78-5.82
1d	2.48	2.47-2.50	3.42	2.41-3.43
1e	2.24	2.23-2.25	9.89	9.85-9.94
1f	4.01	4.00-4.01	8.28	8.26-8.30
1g	1.23	1.22-1.24	4.44	4.43-4.45
1h	3.52	3.51-3.53	3.57	3.53-3.62
1i	2.08	2.07-2.09	5.39	5.37-5.40
1j	1.27	1.25-1.29	6.44	6.43-6.45
1k	9.70	6.67-9.73	5.16	5.15-5.16
1l	1.14	1.13-1.16	7.44	7.43-7.45
1m	6.76	6.74-6.77	6.08	6.07-6.09
1n	6.98	6.96-6.99	21.95	21.85-21.96
1o	5.48	5.47-5.49	16.58	16.51-16.64
2a	6.86	6.83-6.89	10.86	10.84-10.89
2b	1.59	1.58-1.60	6.84	6.83-6.85
Metronidazole[a]	0.210	0.150-0.270	0.06	0.05-0.07

[a]Reference compound

Anti-protozoal activity of the novel isothioureas is shown in Table VI. The activity against *G. intestinalis* of twelve of these compounds was ≥10 times lower than that of metronidazole that is the drug of choice for the treatment of giardiasis and amoebiasis. Derivatives **1c**, **1g**, **1j**, **1l** and **2b** showed considerable while moderate activity (IC_{50} < 2 μg/mL) against *G. intestinalis*, whereas the activity against *E. histolytica* of all the novel compounds tested was >50 times lower than that of metronidazole. To the best of our knowledge, this is the first report demonstrating anti-protozoal activity of isothiourea derivatives.

We tested inhibition of NOS activity by the novel isothioureas using routine procedure that employs homogenates of normal brain tissue from adult male rats as a source of NOS, in the presence of Ca^{2+} ions and calmodulin; the results of the test are shown in Fig. 1. Since iNOS is normally absent from mature rat brain (Jesko et al., 2003), the total activity measured reflected combined activities of the constitutive isoforms nNOS and eNOS. The commercially available NOS inhibitor 7NI, which selectively inhibits nNOS at the concentration used in our study (10 μM), blocked about 60% of the total NOS activity in the homogenates, and most of the new compounds studied showed substantial inhibition of this activity as well. It is worth noting that compounds **1i** and **1o** showed significantly stronger inhibition of the activity than 7NI (Fig. 1) at 10 μM. This difference suggests that they inhibit both nNOS and eNOS at this concentration, and raises the possibility that specific inhibition of one of these isoforms might be possible at a lower concentration. The latter possibility may be of importance for possible future *in vivo* studies, because compound **1o** at 10 μM shows substantial toxicity in primary cultures of normal rat cortical astrocytes (Kaminska et al., 2009).

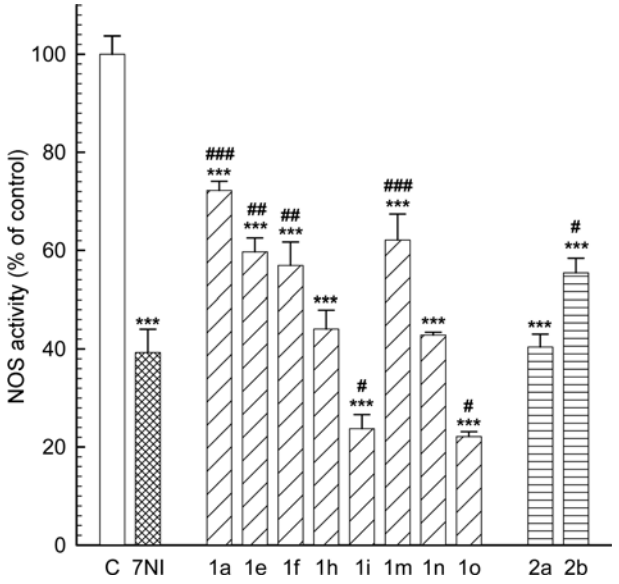


Fig. 1. Effects of selected most active isothioureas (10 μM) on Ca^{2+}/calmodulin-dependent NOS activity in the homogenate of normal adult rat brain. Results shown are the mean ± S.E.M. from three separate experiments (each performed in triplicate). $^{***}p < 10^{-4}$ *vs.* the control (C) value, Dunnett's test; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *vs.* the 7-nitroindazole (7-NI) value; Dunnett's test, with the control (C) value omitted.

Significant inhibition of iNOS has been reported for aminoethylisothiourea and ethylisothiourea (Garvey et al., 1994; Southan et al., 1995). However, the inhibitory potency declined sharply if the side alkyl chain was substituted or exceeded two carbon atoms, e.g. for the corresponding propyl and butyl congeners, and substitution of ethylisotiourea nitrogens with amino or alkyl groups also resulted in decreased potency (Southan et al., 1995). Whereas a trend for similar effect was apparent also in our study, the presence of the massive, but highly hydrophobic pentabromobenzyl S-substituent in the derivative **1o** appeared to enhance the inhibitory activity of the novel compounds against Ca^{2+}/calmodulin-dependent NOS activity (Fig. 1).

To summarize, the results of this study extend the list of promising isothioureas with substantial antimicrobial and anti-NOS activity *in vitro*. Hence, it seems that a more detailed study of the toxicity, antimicrobial properties *in vivo* and NOS isoform selectivity of selected novel isothiourea derivatives is warranted.

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