Synthesis of N-Benzenesulfonylbenzotriazole Derivatives, and Evaluation of their Antimicrobial Activity

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Received May 05, 2008: Revised May 12, 2008: Accepted May 12, 2008

Abstract: A series of benzenesulfonyl compounds, **1a-i**, containing a BZT moiety was synthesized and characterized, and their antifungal and antibacterial activities were investigated. Compounds **1a** and **1d** showed the highest activity against *Escherichia coli* ATCC 25922; in addition **1a** presented bactericidal activity against *E. coli* and *Staphylococcus aureus* at 8.6 mM. The ability of **1a** to generate superoxide anion ($O_2^{\cdot-}$) was measured and it showed more stimuli in *S. aureus* compared to sulfathiazole, indicating that **1a** can be involved in oxidative stress of bacteria. None of the compounds inhibited the growth of the dermatophytes strains at the tested concentration (250 μg/ml).

Keywords: Benzenesulfonyl, Benzotriazole, Antibacterial, Antifungal.

INTRODUCTION

As part of our current studies on new antiinfective drugs, we designed and prepared a library of N-benzenesulfonyl derivatives of bioactive heterocycles. The first series of compounds, where the benzotriazole (BZT) moiety was selected, is presented here and its H at N1 was replaced by substituted-benzenesulfonyl groups (BS). A preliminary antibacterial screening was carried out against representative strains of Gram positive and Gram negative bacteria. A search for their antifungal properties was also evaluated.

The chemistry of BZT has been extensively studied and it is usually simple and easy to understand. Many of its derivatives are commercially available or can be synthesized by already known procedures [1,2]. There are thousands of publications available on BZT derivatives. Even more, a comprehensive database with information originated from Katrizky's work on this topic has been constructed and can be accessed from his homepage [3]. Substitution and addition reactions have been used to introduce a BZT group in a molecule. Generally, an N-substituted BZT can be obtained by displacement of a halogen in alkyl, aryl, acyl, or sulfonyl halides; a hydroxyl in alcohols; and an alkoxyl in acetals or ketals. Other important routes involve additions of BZT to aldehydes, imines, iminuim salts and to enamines [1,4]. For the synthesis of BS-BZT, several methods have been described. They can be prepared by reacting benzotriazole, or some of its derivatives with sulfonyl chlorides in the presence or not of solvent or base [5-11]; or by addition of BZT to the double-bond of (4S,5R,6R)-diphenylmethyl (E)-4,5,6,7-tetrahydroxy-2-heptenoate in the presence of 4-toluensulfonic acid [12].

From a pharmacological point of view, BZT moieties are present in compounds with antimycobacterial [13], antinflamatory [14], antiviral [15], antiproliferative and antitumoral [16], anticonvulsant [17], and diuretic [18] activities; actions of BZT derivatives on central nervous system are also known [19]. Furthermore, Dixit *et al*. found that the introduction of BZT groups afforded potent oxazolidinones with *in vitro* activity comparable or superior to linezolid against resistant and susceptible Gram positive bacterial pathogens. These oxazolidinones act by inhibition of bacterial protein synthesis in the initial stages [20]. On the other hand, antibacterial and antifungical activities associated to BZT heterocycle have been reported by Nanjunda *et al*. [4]. Similar results were also reported by Tiwari *et al.* who found that indole derivatives substituted with BZT moiety show antibacterial activity as well [21].

Also, several bioisosters of BZT molecules have been studied for their antimicrobial activities and many of them have been reported to act through the inhibition of the (TCS). Recently, the TCSs have attracted attention due to their potential as novel antibacterial targets, especially those required for regulation of bacterial growth and virulence in pathogenic microorganisms [22]. In this sense, new compounds as benzimidazoles, benzothiazoles, benzoxazines and benzoxazoles are effective in inhibiting the action of a bacterial histidine protein kinase [22].

On the other hand, the BS moiety is also present in an important variety of biological active compounds [23]. It is also worth to point out that the BZT is considered to have low toxicity [24]. Finally, many BS-BZT have been synthesized but their pharmacological properties have not been explored.

All this information was evaluated to design and select the BS-BZT scaffold as the first series of compound to be prepared and tested as antimicrobials. Herein, we present the first systematic approach to the development of a prototype of this class of compounds as anti-infective agents.

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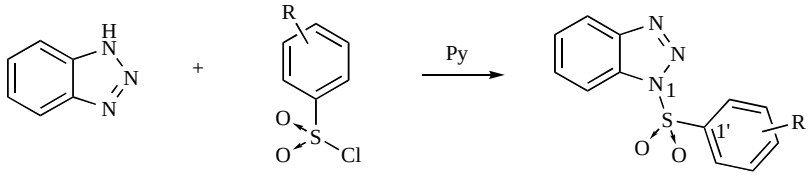

Scheme 1.

RESULTS AND DISCUSSION

Chemistry

BS-BZTs were prepared as shown in Scheme (**1**). Substituents at the 3- or 4-position of the BS group were carefully selected and accounted for electronic and/or lipophilic differences within the series.

Compounds **1a-i** (Table **1**) were prepared by the addition of a solution of BZT in anhydrous pyridine to the appropriate commercially available benzenesulfonyl chloride under nitrogen atmosphere. The reaction mixture was heated until clear solution was obtained and then stirred for two hours. Addition of water precipitated the desired product, which in turn was filtered out and exhaustively washed with acidic solution and then cold water to give the product with good yields (70-90%). Table **1** shows the structure of compounds **1a-i** along with their melting point (m.p.) and yield based on purified compounds.

The chemical structure of compounds **1a-i** was characterized by EIMS, HRMS, IR and ^{1}H and ^{13}C NMR. High-Resolution MS spectra showed the molecular ion [M^{+}] that corresponds to the calculated mass. The cleavage of the BS group, as one of the fragmentation pathway at EIMS spectra, confirmed the presence of both BS and BZT moieties in structure **1**. Moreover, the spectra showed a typical fragmentation of a N1-substituted-benzotriazole [25]. The FT-IR displayed characteristic absorptions for sulfanilamide groups in the regions 1330-1360 cm^{-1} (νSO_2 asim) and 1140-1180 cm^{-1} (νSO_2 sim), as well as other typical signals for the BZT moiety. The structural characterization of compounds **1a** and **1b** agrees with the previously reported data [6,11].

There are several reports where N1 and N2 isomers of BZT have been described as products of many of the synthetic procedures previously mentioned [26]. Also, *ab initio* calculations predict almost equal stability in the gas phase for both regioisomers [27]. In terms of biological relevance, Sanna *et al.* reported a better antimycobacterial activity for the N1 isomer of a benzotriazolylacrylonitrile derivative [28]. By NMR experiments and analytical data we have carefully characterized the BS-BZT series, and the data suggest that only the N1 isomer was obtained. The substitution pattern at the ^{1}H NMR spectra is characteristic of an asymmetric substitution on the BZT moiety. Moreover, protons H-4, H-5, H-6 and H-7 shifted to lower field compared to the unsubstituted BZT confirming the presence of the BS substituent at N1. The ^{13}C NMR showed ten signals corresponding to ten magnetically and chemically different Cs.

Quality controls of the BS-BZT were performed to assure the stability of this series of compounds during the biological screening. The experiment was performed in polyethylene glycol 400 and ethanol (70:30) solutions at room temperature for 72h. All the solutions were analyzed by TLC at initial time and at 72h. In all cases only the corresponding initial compound was detected.

Biological Activity

An exploratory and preliminary antibacterial and antifungal activity was performed for the synthesized of **1a-1i** compounds against sensitive standard strains.

In vitro antibacterial activity of compounds **1a-e** and **1h** was determined against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213. The minimum inhibitory concentration (MIC) was determined by macrobroth dilution methods [29a] and it is expressed as milimolar concentration (mM). Suitable solvent control (polyethylene glycol 400 and ethanol) and standard drug controls were also

Table 1. Structure, Melting Point and Yield for the BSBZT Series

Compounds, 1	*R*	m.p. (°C)[a]	yield (%)
a	H	117-118 (124-126) [5-7]	85
b	4-CH_3	134-135 (133-137) [8-11]	85
c	4-NO_2	158-159	90
d	4-$NHCOCH_3$	110-111	70
e	4-F	163-164	85
f	4-Cl	147-148	90
g	4-Br	148-149	90
h	4-OCH_3	130-131	80
i	3-NO_2	149-150	85

[a]Numbers in parenthesis correspond to the m.p. informed in the cited references.

Table 2. Area Under the Curve (AUC) of 1a in *Staphylococcus aureus* ATCC 29213

Compounds 1a (mM)	AUC_{0-5} (RLU min) ±SEM*
0	2.588±0.063
8.6	12.518±2.084
4.3	3.490±2.518
2.15	0.94±0.537

*p<0.05 respect to the control without **1a**.

run simultaneously. It was selected sulfisoxazole, a commercially available antimicrobial agent, as a positive reference due to its structural similarity with the BS moiety. The BZT was also included to compare its activity with the new analogs. From this series only compounds **1a** (8.6mM), and **1d** (2.15mM) have shown an inhibitory activity against *E. coli* in the assayed range (0-17.6mM). The MIC for **1a** and **1d** is higher than sulfisoxazole (1.9mM) but lower than BZT (17.2mM). The only compound that showed inhibition against *S. aureus* ATCC 29213 was **1a** (8.6mM), and again with a MIC higher than sulfisoxazole (1.9mM), and lower than BZT (17.2 mM).

Based on the results for compound **1a**, more studies were performed in order to investigate its biological properties. The bactericidal activity was explored by the time-kill method according to CLSI. [29b] Kill curves are constructed by plotting the cfu/mL surviving at each time point in the presence and absence of the antimicrobial agent. The results showed a reduction of growth (log_{10} cfu/mL) by approximately 3 and 6 orders of magnitude for **1a** against *E. coli* and *S. aureus*, respectively.

To further explore the mode of action for this class of compounds, we evaluate their ability to generate O2 The oxygen radicals contribute to alter the bacterial physiology [30], and the oxidative stress has been demonstrated to be involved in the mechanism of action of diverse antibiotics [31]. Suspensions of S. aureus ATCC 29213 were incubated with different concentrations of 1a. The highest stimulus was observed with 8.6 mM (MIC) and the value of the AUC was 12.518, an increment of 5-fold respect to control. Again, a commercially available antimicrobial sulfamide was selected as a positive reference based on structural similarities but not on a mechanistic point of view. The AUC for sulfathiazole at the MIC was 2.927±0.042, similar to that obtained in the absence of antibiotic (Table **2**). These results indicate that the effect of 1a in the respiratory metabolism of the *S. aureus* was higher than the reference drug (sulfathiazole).

The enhanced $O_2^{\cdot -}$ generation induced by **1a** could be an interesting result if it is taken into account that TCSs are often involved in stress responses. TCSs will produce factors required for transcription of genes needed for growth or survival under stress conditions [33, 34].This mechanism could explain the MIC of **1a** being comparable to the reference compound but having a higher effect on the oxidative stress (Table **2**). Even more, Quin *et al.* [22a] and Ohemeng *et al.* [22b], demonstrated that BZT-related heterocycles are inhibitors of histidine kinase.

The antifungal activity was also determined for this series of compounds. For the *in vitro* antifungal activity the following dermatophytes strains were used: *Aspergillus flavus* ATCC 9170, *Aspergillus fumigatus* ATCC 26934, *Aspergillus niger* ATCC 9092, *Microsporum gypseum* C115, *Trichophyton mentagrophytes* ATCC 9972, *Trichophyton rubrum* C137, *Saccharomyces cerevisiae* ATCC 9763, *Cryptococcus neoformans* ATCC 32264, *Candida albicans* ATCC 10231, and *Candida tropicalis* ATCC 7349. Only the most representative compounds, **1a-d** were assayed and BZT was used for comparison and Ketoconazole as positive reference. None of the new compounds inhibited the growth of the strains in the tested concentration at 250 μg/mL.

CONCLUSIONS

Nine BS-BZT derivatives were synthesized and structurally characterized. Six of them were tested against reference strains of *S. aureus* and *E. coli*. Compounds **1a** and **1d** were the most active against *E. coli* ATCC 25922 and **1a** presented bactericidal activity against *E. coli* and *S. aureus* at 8.6 mM. According to the results obtained in the present investigation, **1a** generated more stimuli of $O_2^{\cdot -}$ in *S. aureus* compared to sulfathiazole, indicating that **1a** is more toxic for the respiratory metabolism of the bacteria.

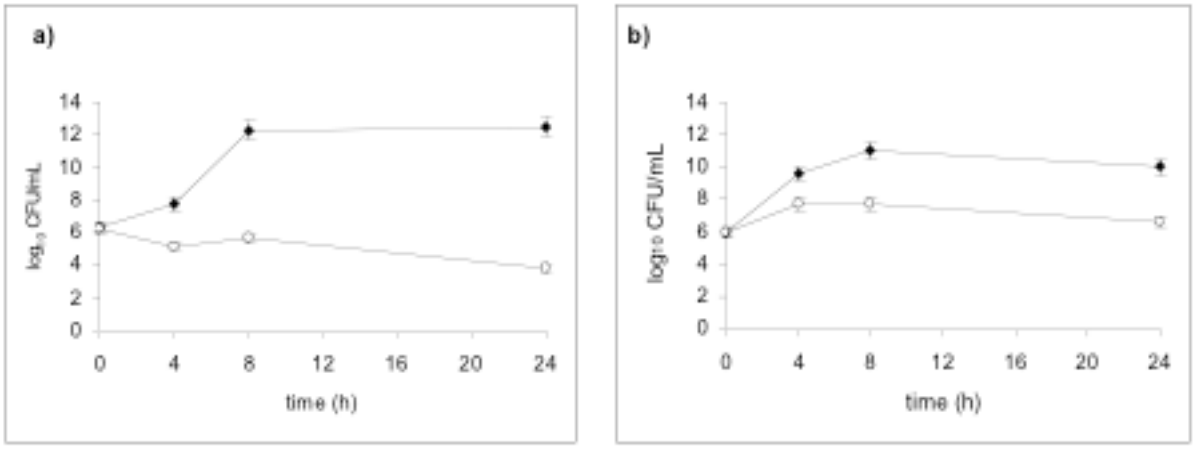

Fig. (1). Time-kill kinetics of **1a** 8.6 mM (○); control (♦). **a**) against *S. aureus* ATCC 29213. **b**) against *E. coli* ATCC 25922.

These results have shown the potential of BS-BZT analogs as prototype for the development of drugs and for the treatment of infectious caused by bacteria. Iterative medicinal chemistry and structure-based drug design are in progress.

EXPERIMENTAL

Melting points (m.p.) were determined by Electrothermal apparatus, by microcapillary methods and are uncorrected. TLC was performed on analytical silica-gel 60 F_{254} plates RP-18 of 0.25 mm (Merck), with dichloromethane/acetone (2:1) as solvent system. Tripteine soy agar and Mueller–Hinton Broth (Britania), Sabouraud-dextrose agar, and Sulfisoxazole (Merck), DMSO (Sintorgan), Ethanol (Cicarelli), PEG (Parafarm), lucigenin, and ketoconazole (SIGMA). Infrared spectra were recorded on a Nicolet spectrophotometer as potassium bromide disks and the reported wavelength are given in cm^{-1}. NMR spectra were recorded on a Bruker (200.00 MHz for ^{1}HNMR, 50.00 for ^{13}CNMR) instrument in $CDCl_3$ solutions. The chemical shift values are reported in ppm (δ). The high-resolution mass spectra (HRMS) were recorded on a Waters Micromass Q-Tof Micro mass spectrometer with a lock spray source. EIMS were collected on a Finnigan 3300F-100 mass spectrometer at 70 eV by direct inlet. Determinations by chemiluminescence were performed at room temperature in a BioOrbit chemiluminometer mod 1253.

General Procedure for Preparation of 1-(Benzenesulfonyl)-1,2,3 Benzotriazole Derivatives

A solution of BZT in anhydrous pyridine (1 mmol) was added to the corresponding commercially available substituted benzenesulfonyl chloride (1.1 mmol) under nitrogen atmosphere. The reaction mixture was heated at 65°C until clear solution and then stirred for two hours. Addition of water and cooling to 0ºC precipitates the desired product, which in turn is filtered off. Finally, the solid was washed with 0.1 M HCl (3x10mL), cold water (3x10mL), and cold ethanol (1x10mL), and then dried under vacuum.

Benzotriazole (BZT). IR (cm^{-1}, KBr)

3246 (νNH); 3060 and 3092 (νCH aromatic); 1622 (νCC aromatic, asim); 1457 (νCC aromatic, sim); 1308 (νCN). **^{1}H NMR ($CDCl_3$, 200 Hz):** 12.66 (br s, 1H); 7.99-7.93 (m, 2H); 7.47-7.35 (m, 2H). **^{13}C NMR ($CDCl_3$, 60 Hz):** 139.21; 126.47; 115.34.

1-(benzenesulfonyl)benzotriazole (1a)

BZT (0.45 g, 3.8 mmol), benzenesulfonyl chloride (0.98 g, 3.8 mmol), gave **1a** (78%) as a white solid, m.p. 117-118ºC. **IR (cm^{-1}, KBr):** 3092, 3069, 1605, 1481, 1313, 1386, 1194. **^{1}H NMR ($CDCl_3$, 200 Hz):** 8.15-8.06 (m, 4H); 7.71-7.61 (m, 2H); 7.58-7.44 (m, 3H). **^{13}C NMR ($CDCl_3$, 60 Hz):** 145.4; 137.05; 135.16; 131.61; 130.29; 129.64; 127.86; 125.86; 120.56; 111.96. **HRMS:** 260.0494 (Calcd 259.285); molecular formula, $C_{12}H_{10}N_3O_2S$. **MS (70 eV, m/z):** 259 (M^+), 141 (M^+- BZT), 119 (BZT), 91 (BZT-N_2), 64 (SO_2).

1-(4-Methylbenzenesulfonyl)benzotriazole (1b)

BZT (0.70 g, 5.9 mmol), p-toluensulfonyl chloride (1.61 g, 6.0 mmol), gave **1b** (80%) as a white solid, m.p. 134-135ºC. **IR (cm^{-1}, KBr):** 3089, 3057, 2975, 2925, 1606, 1482, 1313, 1386, 1191. **^{1}H NMR ($CDCl_3$, 200 Hz):** 8.22-7.92 (m, 4H); 7.69-7.61 (m, 1H); 7.51-7.43 (m, 1H); 7.34-7.29 (m, 2H); 2.39 (s, 3H). **^{13}C NMR ($CDCl_3$, 60 Hz):** 146.78; 145.45, 134.03; 131.62; 130.33; 130.24; 128.00; 125.82; 120.56; 112.05; 21.766. **HRMS:** 274.064 (Calcd 273.311); molecular formula, $C_{13}H_{12}N_3O_2S$. **MS (70 eV, m/z):** 274 (M^+), 155 (M^+-BZT), 119 (BZT), 91 (BZT-N_2), 64 (SO_2).

1-(4-Nitrobenzenesulfonyl)benzotriazole (1c)

BZT (0.58 g, 4.9 mmol), p-nitrobenzenesulfonyl chloride (1.49 g, 5.0 mmol), gave **1c** (90%) as a light yellow solid, m.p. 158-159ºC. **IR (cm^{-1}, KBr):** 3113, 3101, 1607, 1483, 1313, 1540, 1395, 1271, 1187. **^{1}H NMR ($CDCl_3$, 200 Hz):** 8.37-8.29 (m, 4H); 8.12-8.08 (dd, 2H); 7.76-7.67 (m, 1H); 7.56-7.48 (m, 1H). **^{13}C NMR ($CDCl_3$, 60 Hz):** 151.42; 145.48; 142.39; 131.55; 130.95; 129.39; 126.43; 124.87; 120.99; 111.79. **HRMS:** 305.0331 (Calcd 304.283); molecular formula, $C_{12}H_9N_4O_4S$. **MS (70 eV, m/z):** 304 (M^+), 186 (M^+- BZT), 119 (BZT), 91 (BZT-N_2), 64 (SO_2).

1-(4-Acetylbenzenesulfonyl)benzotriazole (1d)

BZT (0.46 g, 3.9 mmol), N-acetylsulfanilyl chloride (1.22 g, 4.0 mmol), gave **1d** (70%) as a white solid, m.p. 110-111ºC. **IR (cm^{-1}, KBr):** 3367, 3105, 3085, 2932, 2858, 1710, 1588, 1395, 1318, 1179. **^{1}H NMR ($CDCl_3$, 200 Hz):** 8.14-7.96 (m, 5H); 7.72-7.63 (m, 3H); 7.53-7.44 (m, 1H); 2.21 (s, 3H). **^{13}C NMR ($CDCl_3$, 60 Hz):** 168.85; 144.46; 132.49; 131.65; 130.85; 130.42; 129.53; 126.00; 120.45; 119.48; 112.15; 24.24. **HRMS:** 315.99 (Calcd 313.336); molecular formula, $C_{14}H_{12}N_4O_3S$. **MS (70 eV, m/z)**: 133 (M-BZTSO$_2$), 119 (BZT), 91 (BZT-N_2), 64 (SO_2).

1-(4-Fluorobenzenesulfonyl)benzotriazole (1e)

BZT (1.0 g, 8.4 mmol), 4-fluoro-benzenesulfonyl chloride (2.35 g, 8.5 mmol), gave **1e** (90%) as a white solid, m.p. 163-164ºC. **IR (cm^{-1}, KBr):** 3100, 3074, 1391, 1192, 1008, 1586, 1490, 1311. **^{1}H NMR ($CDCl_3$, 200 Hz):** 8.20-8.08 (m, 4H); 7.72-7.64 (m, 1H); 7.54-7.44 (td, 1H); 7.22-7.17 (m, 2H). **^{13}C NMR ($CDCl_3$, 60 Hz):** 169.17 ($^{C4'}J_{HF}$= 310 Hz); 145.43; 131.06; 131.17($^{C2'}J_{HF}$= 11.4 Hz); 130.45; 130.37; 125.99; 120.72; 117.43 ($^{C3'}J_{HF}$= 27 Hz); 111.93. **HRMS:** 276.963 (Calcd 277.275); Molecular formula, $C_{12}H_8N_3O_2FS$.

1-(4-Chlorobenzenesulfonyl)benzotriazole (1f)

BZT (0.46 g, 3.9 mmol), 4-chloro-benzenesulfonyl chloride (1.15 g, 3.9 mmol), gave **1f** (90%) as a white solid, m.p. 147-148ºC. **IR (cm^{-1}, KBr):** 3097, 3076, 1393, 1193, 758, 1581, 1476, 1312. **^{1}H NMR ($CDCl_3$, 200 Hz):** 8.11-8.02 (m, 4H); 7.71-7.63 (m, 1H); 7.53-7.45 (m, 3H). **^{13}C NMR ($CDCl_3$, 60 Hz):** 145.46; 142.22; 135.47; 131.62; 130.50; 130.07; 129.37; 126.05; 120.74; 111.93. **HRMS:** 292.992 (Calcd 293.731); Molecular formula, $C_{12}H_8N_3O_2SCl$.

1-(4-Bromobenzenesulfonyl)benzotriazole (1g)

BZT (0.41 g, 3.4 mmol), 4-bromo-benzenesulfonyl chloride (1.16 g, 3.5 mmol), gave **1g** (90%) as a white solid, m.p.

148-149ºC. **IR (cm^{-1}, KBr):** 3090, 3079, 1588, 1476, 1395, 1310, 1191, 601. **^{1}H NMR ($CDCl_3$, 200 Hz):** 8.11-8.02 (m, 4H); 7.71-7.63 (m, 1H); 7.53-7.45 (m, 3H). **^{13}C NMR ($CDCl_3$, 60 Hz):** 145.46; 142.22; 135.47; 131.62; 130.50; 130.07; 129.37; 126.05; 120.74; 111.93. **HRMS:** 336.944 (Calcd (Br^{79}), 336.18 and (Br^{81}), 338.942; molecular formula, $C_{12}H_8N_3O_2BrS$.

1-(4-Methoxybenzenesulfonyl)benzotriazole (1h)

BZT (0.31 g, 2.6 mmol), 4-methoxy-benzenesulfonyl chloride (0.76 g, 2.7 mmol), gave **1h** (80%) as a white solid, m.p. 130-131ºC. **IR (cm^{-1}, KBr):** 3096, 3090, 2945, 2854, 1389, 1179, 1592, 1495, 1308. **^{1}H NMR ($CDCl_3$, 200 Hz):** 8.13-8.03 (m, 4H); 7.69-7.61 (m, 2H); 7.51-7.43 (m, 2H); 7.00-6.93 (m, 3H). **^{13}C NMR ($CDCl_3$, 60 Hz):** 164.97; 143.36; 130.47; 130.07; 130.01; 128.34; 125.67; 120.53; 114.89; 112.09; 55.83. **HRMS:** 289.041 (Calcd 289.311); Molecular formula, $C_{13}H_{11}N_3O_3S$.

1-(3-Nitrobenzenesulfonyl)benzotriazole (1i)

BZT (0.57 g, 4.8 mmol), 3-nitro-benzenesulfonyl chloride (1.48 g, 5.0 mmol), gave **1i** (85%) as a white solid, m.p. 149-150ºC. **IR (cm^{-1}, KBr):** 3102, 3089, 1602, 1484, 1316, 1529, 1395, 1354, 1195. **^{1}H NMR ($CDCl_3$, 200 Hz):** 8.94-8.92 (t, 1H); 8.53-8.45 (tt, 2H); 8.16-8.08 (m, 2H); 7.84-7.69 (m, 2H); 7.57-7.49 (m, 1H). **^{13}C NMR ($CDCl_3$, 60 Hz):** 151.23; 139.15; 133.41; 131.28; 130.08; 10.90; 129.94; 129.48; 126.40; 123.14; 120.93; 111.88. **HRMS:** 305.044 (Calcd 304.283); molecular formula, $C_{12}H_9N_4O_4S$.

Microbiology

In Vitro Antibacterial Activity

The microorganisms used for the study included three clinical isolates of *E. coli* resistant to ampicillin provided by the Hospital Tránsito Cáceres de Allende, Buchardo 1250, (5000) Córdoba, Argentina) and the quality control strains *E. coli* ATCC 25922 and *S. aureus* ATCC 29213. Resistance in the clinical isolates was determined by Kirby Bauer Disk Diffusion method according to the guidelines of Clinical and Laboratory Standards Institute (CLSI) [31]. The solutions of the synthesized compounds were prepared in polyethylene glycol 400 and ethanol (70:30) at concentrations of 34.4, 17.2, 8.6, 4.3, 2.2, 1.1, 0.6, 0.3, 0.2, 0.1, 0.05, 0.03 mM by diluting in Mueller Hinton Broth (MHB). Sulfisoxazole was used as reference drug. This drug solution was prepared according to the guidelines of CLSI.

The bacterial suspensions used for inoculation were prepared at 10^5cfu/ml by diluting fresh cultures at MacFarland 0.5 density (10^8cfu/ml). Bacterial suspensions were inoculated to the two-fold diluted solution of the compounds. The lowest concentration of the compound that completely inhibits its macroscopic growth was determined and minimum inhibitory concentrations (MICs) were reported.

Bactericidal activity was measured by time-kill curves as recommended by the CLSI. Aliquots of exponentially growing bacteria (*E. coli* ATCC 25922, *S. aureus* ATCC 29213) were suspended in fresh MHB at approximately 10^6 cfu/ml and were exposed to **1a** 8.6mM for 0, 4, 8 and 24 h at 35°C. Total bacterial cfu/mL (log_{10}) was determined after 18 h of incubation at 35°C. Bactericidal activity was defined as a reduction of 99.9% ($\geq$3 log_{10}) in the total count of the original inoculum.

Oxidative Stress

The effect of production of superoxide anion by bacteria was study in *S .aureus* ATCC 29213. Bacterial suspensions were prepared in trypticase soy agar (TSA), incubated for 18 h at 37º C and suspended in buffer phosphate saline pH 7. Bacterial absorbance was adjusted to an OD of 1.5 and 0.1 mL was incubated with 0.1 mL lucigenin (75 µg/mL); then 0.1 mL of **1a** or sulfathiazole was added. Control production of $O_2^{\cdot -}$ by *S. aureus* was determined by means of assays without drugs. The relative light unities (RLU) were measured for five minutes by chemiluminescence and the results were expressed as mean area under the curve (AUC). The results of three assays were expressed as the mean $\pm$ SEM and $p < 0.05$ was considered significant.

In Vitro Antifungal Activity

To determine the antifungal activity, the following microorganisms were used in the present study: from the American Type Culture Collection (ATCC) (Rockville, MD, U.S.A.): *Aspergillus flavus* (ATCC 9170), *Aspergillus fumigatus* (ATCC 26934), *Aspergillus niger* (ATCC 9092), *Trichophyton mentagrophytes* (ATCC 9972), *Saccharomyces cerevisiae* (ATCC 9763), *Cryptococcus neoformans* (ATCC 32264), *Cryptococcus neoformans* (ATCC 32264), *Candida albicans* (ATCC 10231), and *Candida tropicalis* (ATCC 7349); and from clinical isolates provided by the Centro de Referencia Micológica (CEREMIC, Facultad de Ciencias Bioquímicas y Farmacéuticas, Suipacha 531, (2000) Rosario, Argentina): *Microsporum gypseum* (C115), Trichophyton rubrum (C137), *Saccharomyces cerevisiae* (ATCC 9763) , *Cryptococcus neoformans* (ATCC 32264), *Candida albicans* (ATCC 10231), and *Candida tropicalis* (ATCC 7349). The yeasts used were cultivated on Sabouraud-dextrose agar (Merck, 5438) for 48 h at 37° C. Cell suspension in sterile distilled water was adjusted to give a final concentration of $1x10^6$ to $5\ x10^6$ yeast cells/ml, standardized with 0.5 on the McFarland scale (530 nm). Inoculated of 5ml with the yeast cells or spore suspensions were added to Sabouraud-dextrose agar media. The antifungal agent ketoconazole (Sigma) was included in the assay as positive control. Drug-free solution was also used as a blank control. Tubes were incubated at 37 °C for 24 to 72 h for yeasts and at 25 °C for 5 to 15 days (up to 15 days for dermatophyte strains) according to the control fungus growth. MIC was defined as the lowest compound concentration, showing no visible fungal growth after the incubation period. Fungal growth was analyzed after an appropriate incubation period, specific to each fungi. Each assay was repeated three times.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial support granted by CONICET and SECYT-UNC. MCB and LYH were supported by CONICET fellowship program (Argentina). We are grateful to Prof. Susana Zacchino (National University of Rosario, Argentina, ANPCyT PICT R 260) for

the antifungal evaluation of the compounds. We also thank Drs. McCurdy and Carvalho (Department of Medicinal Chemistry, The University of Mississippi) for the HRMS.

REFERENCES

[1] Katritzky, A.R.; Xiangfu, L.; Yang, J.Z.; Denisko**,** O.V. *Chem. Rev*., **1998**, *98*, 409.

[2] Katritzky, A.R.; Rogovoy, B.V. *Chem. Eur. J.*, **2003**, *9*, 4586.

[3] Katritzky, A.R. http://ark.chem.ufl.edu.

[4] Nanjunda Swamy, S.; Basappa; Sarala, G.; Priya, B.S.; Gaonkar, S.L.; Shashidhara Prasad, J.; Rangappa, K.S. *Bioorg. Med. Chem. Lett*., **2006**, *16*, 999.

[5] Fuheng, C.; Wei, L. *Yingyong Huaxue*, **1991**, *8*, 70.

[6] Katritzky, A.R.; Shobana, N.; Pernak, J.; Afridi, A. S.; Fan, W-Q. *Tetrahedron*, **1992**, *48*, 7817.

[7] Mucheli, M.V.R.; Kudav, N.A. *Chem. & Ind*., **1985**, *3*, 92.

[8] Katritzky, A.R.; Kurz, T.; Zhang, S.; Voronkov, M.; Steel, P. J. *Heterocycles*, **2001,** *55*, 1703.

[9] Katritzky, A.R.; Rodriguez-Garcia, V.; Nair, S.K. *J. Org. Chem*, **2004**, *69*, 1849.

[10] Nishikawa, M.; Inaba, Y.; Furukawa, M. *Chem. Pharm. Bull.*, **1983**, *31*, 1374.

[11] Katritzky, A.R.; Fan, W-Q. *J. Het. Chem.*, **1990**, *6*, 1543.

[12] Rodríguez, R.; Nogueras, M.; Cobo, J.; Low, J.N.; Glidewell, C. *Acta Cryst*., **2005**, *E61*, 2795.

[13] (a) Sanna, P.; Carta, A.; Gherardini, L.; Nikookar, M.E.R. *Il Farmaco*, **2002**, *57*, 79. (b) Sanna, P.; Carta, A.; Nikookar, M.E.R. *Eur. J. Med. Chem*, **2000,** *35*, 535. (c) Dixit P.P.; Patil V.J.; Nair, P.S.; Jain, S.; Sinha, N.; Arora, S.K. *Eur. J. Med. Chem.*, **2006,** *41*, 423. (d) Sanna P.; Carta, A.; Gherardini, L.; Esmail, M.; Nikookar, R. *Farmaco*, **2002,** *57*, 79.

[14] Letavic, M.A.; Dombroski, M.A.; McClure, K.F.; Laird, E.R. *US Patent*: **2003,** 6,664,395 B2.

[15] Carta, A.; Loriga, G.; Piras, S.; Paglietti, G.; Ferrone, M.; Fermeglia, M.; Pricl, S.; La Colla, P.; Secci, B.; Collu, G.; Loddo, R. *Med. Chem.*, **2006,** *2*, 577.

[16] (a) Carta, A.; Sanna, P.; Palomba, M.; Vargiu, L.; La Colla, M.; Loddo, R. *Eur. J. Med. Chem*., **2002,** *37*, 891. (b) Carta, A.; Palomba, M.; Boatto, G.; Busonera, B.; Murreddu, M.; Loddo, R. *Farmaco*, **2004,** *59*, 637. (c) Al-Soud, Y.A.; Al-Masoudi, N.A.; Ferwanah, A.R. *Bioorg. Med. Chem*., **2003**, *11*, 1701.

[17] Dawood, K.M.; Abdel-Gawad, H.; Rageb, E.A.; Ellithey, M.; Mohamed, H.A. *Bioorg. Med. Chem*., **2006**, *14*, 3672.

[18] Boido, A.; Vazzana, I.; Sparatore, F.; Cappello, B.; Diurno, V.; Matera, C.; Marrazzo, R.; Russo, S.; Cenicola, M.L.; Marmo, E. *Farmaco*, **1989**, *44*, 257.

[19] Chojnacka-Wojcik, E.; Klodzinska, A.; Tatarczynska, E.; Paluchowska, M.H. *Pol. J. Pharmacol*., **1999**, *51*, 405.

[20] Dixit, P.P.; Nair P.S.; Patil, V.J.; Jain, S.; Arora, S.K.; Sinha, N. *Bioorg. Med. Chem. Lett*., **2005**, *15*, 3002.

[21] Tiwari, R.K.; Singh, D.; Singh, J.; Yadav, V.; Pathak, A.K.; Dabur, R.; Chhillar, A.K.; Singh, R.; Sharma, G.L.; Chandra, R.; Verma, A.K. *Bioorg. Med. Chem. Lett*., **2006,** *16*, 413.

[22] (a) Quin, ZQ.; Zhang, J.; Xu, B.; Chen, L.; Wu, Y.; Yang, X.; Shen, X.; Molin, S.; Danchin, A.; Jiang, H.; Qu, D. *B.M.C. Microbiology***, 2006**, *6*, 96. (b) Ohemeng, K.A.; Nguyen, V.N. US Patent, **1999**, 5,942,532. (c) Barret, J.F.; Hoch, J.A. *Antimicrob. Agents Chemother*., **1998,** *42*, 1529. (d) Weidner-Wells, M.A.; Ohemeng, K.A.; Nguyen, V.N.; Fraga-Spano, S.; Macielag, M.J.; Werblood, H.M.; Foleno, B.D.; Webb, G.C.; Barrett, J.F.; Hlasta, D.J. *Bioorg. Med. Chem. Lett.*, **2001**, *11*, 1545. (e) Hilliard, J.J.; Goldschmidt, R.M.; Licata, L.; Baum, E.Z.; Bush, K. *Antimicrob. Agents Chemother*., **1999**, *43*, 1693.

[23] Williams, D.A.; Lemke, T.L. Foye's Principles of Medicinal Chemistry" 5ª Ed. Lippincott Williams & Wilkins: Philadelphia, **2002**.

[24] Health Council of the Netherlands: Dutch expert committee on occupational standards (DECOS). *1,2,3-Benzotriazole*. The Hague: Health Council of the Netherlands, **2000**.

[25] Lawrence, R.; Waight, E.S. *Org. Mass Spectrom*., **1970**, *3*, 367.

[26] Katritzky, A.R. *et al. J. Chem. Soc., Perkins Trans* 1, **1987**, 2673.

[27] Katritzky, A.R.; Yannakopoulou, K. *J. Org. Chem*., **1990**, 55, 5683.

[28] Sanna, P.; Carta, A.; Nikookar, M.E.R.. *Eur. J. Med. Chem*., **2000,** *35*, 535.

[29] (a) Clinical and Laboratory Standards Institute (CLSI) (formerly NCCLS): Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically; Approved Standard - Seventh Edition. CSLI document M7-A7. Clinical and Laboratory Standards Institute, Wayne, Pennsylvania, USA, **2006**. (b) Clinical and Laboratory Standards Institute. M26-A Methods for Determining Bacterial Activity of Antimicrobial Agents. Clinical and Laboratory Standards Institute, Wayne, Pennsylvania, USA. **2007**.

[30] Albesa, I.; Becerra, M.C.; Battán, P.C.; Páez, P.L. *Biochem. Biophys. Res. Commun.*, **2004**, *317*, 605.

[31] Becerra, M.C.; Páez P.L.; Laróvere, L.E.; Albesa, I. *Mol. Cel. Biochem.*, **2006**, 285, 29.

[32] Dalton, T.L.; Scott, J.R. *J. Bacteriol*., **2004**, *186*, 3928.

[33] Gardete, S.; Wu, S.W.; Gill, S.; Tomasz, A. *Antimicrob. Agents Chemother*. **2006,** *50*, 3424.