Research Paper

Antimicrobial activity of allylic thiocyanates derived from the Morita-Baylis-Hillman reaction

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Abstract

Bacterial resistance to commonly used antibiotics has been recognized as a significant global health issue. In this study, we carried out the screening of a family of allylic thiocyanates for their action against a diversity of bacteria and fungi with a view to developing new antimicrobial agents. Allylic thiocyanates bearing halogenated aryl groups, which were readily obtained in two steps from the Morita-Baylis-Hillman adducts, showed moderate-to-high activity against selective pathogens, including a methicillin-resistant *S. aureus* (MRSA) strain. In particular cases, methyl (*Z*)-3-(2,4-dichlorophenyl)-2-(thiocyanomethyl)-2-propenoate exhibited antimicrobial activity comparable to the reference antibiotic Imipenem.

Key words: antimicrobial, allylic thiocyanate, Morita-Baylis-Hillman reaction, MRSA.

Introduction

Despite the great efforts that have been put forth to discover novel types of antimicrobial agents, the battle against pathogenic microorganisms is far from being settled owing to the emergence of drug-resistant strains (Foss *et al.*, 2011; Kohanski *et al.*, 2010; Lee and Collins, 2012; Silveira *et al.*, 2006; Walsh, 2000). Bacterial resistance to commonly used antibiotics is recognized as a significant global health issue, being related to a considerable number of cases where the conventional chemotherapeutic treatment of microbial infections has failed. Consequently, the search for novel classes of molecules associated with higher potency, an expanded spectrum of activity and an improved safety profile is urgently required (Diekema and Jones, 2001; Reck *et al.*, 2005).

Many organic compounds containing the S-C-N framework are related to potent biological activity (Allan *et al.*, 1997; Liu *et al.*, 2008; Regan *et al.*, 1967). In particular, the antifungal and antibacterial profiles of thioureas, isothioureas and thiosemicarbazones have been well documented (Chimenti *et al.*, 2011; Iwai *et al.*, 2007; Pavan *et al.*, 2010; Saeed *et al.*, 2010; Umamatheswari *et al.*, 2011). However, the biological activity related to the thiocyanate functionality is much less frequently reported (Capon *et al.*, 2004; Elhalem *et al.*, 2002; Liñares *et al.*, 2007).

In the course of our research interest (Meier *et al.*, 2012; Sá, 2003; Sá *et al.*, 2007) in the chemistry of Morita-Baylis-Hillman (MBH) adducts (Basavaiah *et al.*, 2010; Basavaiah and Veeraraghavaiah, 2012; Singh and Batra, 2008) and the antimicrobial activity of synthetic derivatives (Silveira *et al.*, 2012), we have been able to readily install the S-C-N framework through chemical synthesis in aqueous medium under mild conditions to give the corresponding allylic isothiouronium salts or thiocyanates in excellent yields. Herein, we present our results for the screening of a family of allylic thiocyanates against bacteria and

fungi strains with a view to developing novel antibiotic agents.

Materials and Methods

General experimental procedures

Analytical reagent grade chemicals and solvents were obtained commercially and used without further purification. Melting points were determined using a Microquímica MQPF301 hot plate apparatus and are uncorrected. Infrared spectra were acquired with a Perkin-Elmer FT-IR 1600 spectrometer (range 4000-400 cm^{-1}) using KBr for solids and film for liquid samples. Elemental analyses were conducted on a CHNS Carlo Erba EA-1110 microanalyzer and the analytical results were within ± 0.4% of the theoretical values for all compounds. ^1H NMR spectra were recorded at 400 MHz and splitting patterns were designated as s (singlet), d (doublet), t (triplet), m (multiplet). Coupling constants (*J*) were determined in Hertz (Hz). ^{13}C NMR spectra (fully decoupled) were recorded at 100 MHz. Chemical shifts were recorded in parts per million (ppm, δ) relative to $CDCl_3$ (at 7.26 ppm for ^1H NMR, and at 77.2 ppm for ^{13}C NMR) as the internal standard. Column chromatography was performed using silica gel (70-230 mesh) and hexane/ethyl acetate as the eluent. TLC analysis was performed on silica gel plates. Allylic bromides 1a-m and thiocyanates 2a-m (Figure 1) were synthesized according to the described methods (Ferreira *et al.*, 2009; Sá *et al.*, 2006, 2008; Silveira *et al.*, 2012). Purification of 2a-m in a short plug of silica gel furnished pure products as crystalline solids in all but two cases (2f and 2h), where the product remained oily. The physical and spectral data for allylic bromides 1a-d,f,g,i,k-m (Ferreira *et al.*, 2009; Sá *et al.*, 2006) and thiocyanates 2a-m (Sá *et al.*, 2008; Silveira *et al.*, 2012) are in accordance with spectral data in literature. Data for novel bromides 1e, 1h, and 1j:

Methyl (Z)-2-(bromomethyl)-3-(2-nitrophenyl)-2-propenoate (1e). Yield 83%; yellow solid, mp 63.0-64.0 °C. IR (KBr) v_{max}/cm^{-1} 3042, 2948, 1713, 1518, 1435, 1338, 1273, 1201, 1152. ^1H NMR (400 MHz, $CDCl_3$): δ 3.88 (s, 3H), 4.12 (s, 2H), 7.60 (t, *J* 8.0 Hz, 1H), 7.69-7.78 (m, 2H), 8.04 (s, 1H), 8.20 (d, *J* 8.0 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 25.8, 52.8, 125.4, 130.08, 130.13, 130.2, 130.5, 134.2, 139.8, 147.4, 165.8.

Methyl (Z)-2-(bromomethyl)-3-(4-fluorophenyl)-2-propenoate (1h). Yield 91%; clear yellow oil. IR (neat):

v_{max}/cm^{-1} 3071, 2950, 1717, 1629, 1599, 1509, 1437, 1271, 1230, 1154. ^1H NMR (400 MHz, $CDCl_3$): δ 3.84 (s, 3H), 4.33 (s, 2H), 7.12 (t, *J* 8.5 Hz, 2H), 7.53-7.56 (m, 2H), 7.74 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 26.6, 52.6, 116.1 (d, *J* 22.0 Hz), 128.4, 130.4 (d, *J* 3.6 Hz), 131.9 (d, *J* 8.1 Hz), 141.8, 163.2 (d, *J* 250.6 Hz), 166.5.

Methyl (Z)-2-(bromomethyl)-3-(2-bromophenyl)-2-propenoate (1j). Yield 78%; white solid, mp 83.5-84.5 °C. IR (KBr) v_{max}/cm^{-1} 3083, 3011, 2956, 1709, 1625, 1582, 1448, 1357, 1285, 1215, 1156. ^1H NMR (400 MHz, $CDCl_3$): δ 3.90 (s, 3H), 4.24 (s, 2H), 7.26 (t, *J* 7.8 Hz, 1H), 7.42 (t, *J* 7.8 Hz, 1H), 7.63 (d, *J* 7.8 Hz, 1H), 7.68 (d, *J* 7.8 Hz, 1H), 7.84 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 26.3, 52.7, 124.5, 127.7, 129.7, 130.3, 130.8, 133.1, 134.8, 141.8, 166.1.

Microorganisms

Screening of the *in vitro* antimicrobial activity of allylic thiocyanates 2 against a series of standard strains was carried out (Table 1), including Gram-positive (*Bacillus cereus* ATCC 9634, methicillin-sensitive *Staphylococcus aureus* ATCC 25923, methicillin-resistant *Staphylococcus aureus* ATCC 33591) and Gram-negative (*Pseudomonas aeruginosa PA01*) (Stover *et al.*, 2000) bacteria. Strains of *C. albicans* (ATCC 90028) were kindly provided by the University of São Paulo (USP, Brazil) and *Candida tropicalis* were isolated from the oral cavity of patients with removable dentures (Freire *et al.*, 2010).

Antibacterial profile of thiocyanates 2 by the agar diffusion test

This section follows the instructions from Clinical and Laboratory Standards Institute (CLSI), with modifications (Watts *et al.*, 2008). The bacterial strains grown on nutrient agar (Himedia, Mumbai, India) at 37 °C for 24 h were suspended in a saline solution (0.85% NaCl) and adjusted to a turbidity equivalent to the 0.5 McFarland standard (10^8 CFU mL^{-1}). The inoculum was applied (1:10 v/v) to a Müeler-Hinton agar (Himedia, Mumbai, India) into Petri dishes (90 mm diameter), which were previously prepared with a lower layer of agar. Wells (6 mm diameter) were punched in the inoculated Müeler-Hinton agar and filled with 100 μL of 1 mg mL^{-1} solution of the test compounds. The dissolution of the test compounds in water was obtained by adding 1% (v/v) DMSO, which was used as
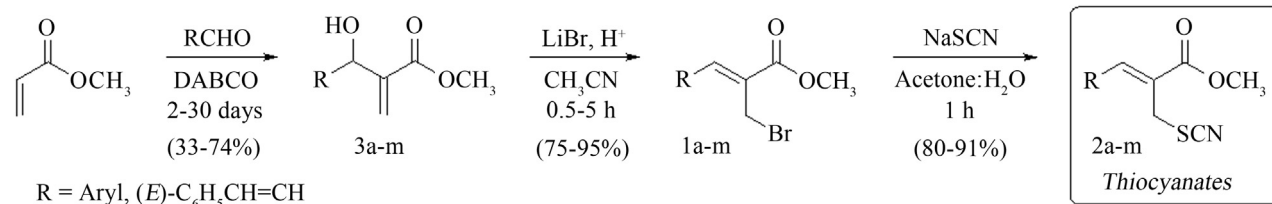
Figure 1 - Three-step preparation of allylic thiocyanates 2a-m (chemical yields are in brackets).

negative control that did not affect the growth of microorganisms, according to the control experiments.

Plates were incubated at 37 °C for 24 h. The experiments were conducted in triplicate. Imipenem was used as a positive control because we found that this drug had an excellent *in vitro* activity against the MRSA strain tested. In order to visualize the activity, 8 μL of a triphenyltetrazolium chloride solution (0.1%), together with bacteriological agar (1%), were added and the plates were incubated again at 37 °C for 4 h. Antibacterial activity was evaluated by measuring the diameter of the inhibition zone in millimeters (Anesini and Perez, 1993). The activity of each substance was compared with the positive control. The substance was classified as no inhibitor (diameter < 25% of the positive control), weak inhibitor (diameter between 25% and 50%), moderate inhibitor (diameter between 50% and 75%), good inhibitor (diameter between 75% and 99%) or excellent inhibitor (diameter equal or higher than 100%).

Determination of minimal inhibitory concentration (MIC)

The test used for determining the MIC for bacterial strains was the microdilution method (Mann and Markham, 1998; Watts *et al.*, 2008) with modifications of the original procedure, including the addition of the colorimetric growth indicator resazurin, which is reduced when viable bacterial cells are present, as previously described (Cursino *et al.*, 2011). Serial three-fold dilutions starting at 50 μM of each test compound were prepared by vortexing in DMSO at room temperature. The Luria-Bertani broth (LB - Himedia, Mumbai, India) was inoculated with the test bacteria to yield a final cell density around 1 log cycle lower than the cell density required to reduce resazurin (usually 2.5×10^5 cell mL^{-1}). The inoculum density was confirmed by plate counts. A sterile 96-well microtiter plate was set up with each of the test bacteria as follows: 100 μL inoculum + 100 μL test compound (0.1-50 μM); 100 μL inoculum + 100 μL DMSO (growth positive control); and sterile medium + 100 μL DMSO (negative control). The well contents were thoroughly mixed. Two trays were prepared for each organism and incubated at 37 °C for 3 h. After incubation, 30 μL of resazurin solution (6 μg mL^{-1}) was added to all wells. After a second incubation for 4 h at 37 °C, the wells were visually analyzed for color change. Inhibition was considered when the bacterial count did not increase after the incubation time and resazurin was not reduced to pink color; the highest dilution which remains blue was considered the MIC.

In vitro antifungal activity

The *in vitro* antifungal activity of thiocyanates 2 was assessed by either agar diffusion test or broth microdilution in accordance with the CLSI guidelines (Pfaller *et al.*, 2002). The test compounds 2 were suspended in DMSO and diluted in the RPMI 1640 medium with L-glutamine (Life Technologies, Grand Island, NY), which was previously buffered to pH 7.0 with 0.165 M MOPS and supplemented with 18 g L^{-1} of glucose. To activate the strains, they were subcultured on Sabouraud's dextrose agar for 24 h at 35 °C. A suspension of yeast cells with a turbidity of 0.5, according to the McFarland standard, was prepared in distilled water, adjusted in a Neubauer chamber (Brand, Wertheim, Germany) and diluted to the final inoculum concentration in the range of 0.5×10^3 to 2.5×10^3 CFU mL^{-1}. All assays were performed in triplicate. The microtiter plates containing the test compounds and the inoculum were incubated at 35 °C for 24 h. Minimum inhibitory concentration (MIC) values were assessed visually by two separate investigators and defined as the lowest concentration that resulted in no growth. Itraconazole was used in the experiments as the antifungal standard.

Results and Discussion

A diversity of *S*-substituted allylic derivatives has been successfully prepared as reported in previous publications (Sá *et al.*, 2008, 2010; Silveira *et al.*, 2012). Allylic bromides 1 are the precursors for thiocyanates 2, obtained in high yields through halide displacement by the thiocyanate anion (NCS$^-$) in aqueous medium at room temperature (Figure 1). The key bromides 1 are readily prepared by treating α-methylene-β-hydroxyesters 3 (MBH adducts) with LiBr in acidic medium at room temperature (Ferreira *et al.*, 2009; Sá *et al.*, 2006). This two-step transformation (MBH reaction followed by bromination) is routinely performed in multigram scale with an overall yield of 65-85% (13 examples) and thus offers a practical route to produce the starting material 1.

Screening of the *in vitro* antimicrobial activity of allylic thiocyanates 2 against a series of standard strains was carried out (Table 1). Moderate antimicrobial activity was found for toluoyl- (2f), naphthyl- (2g), and fluorophenyl- (2h) substituted allylic thiocyanates, while much more significant results were observed for the bromo and chloro analogues (2i-m). In particular, the three chloro-substituted thiocyanates screened (2k-m) were highly active, with inhibition zones of the same magnitude or even greater than the positive control Imipenem (Table 1). It is also worth mentioning the higher potency associated with the 2,4-dichloro-substituted thiocyanate 2m compared with both the 4- and 2-monosubstituted analogues 2k and 2l, which is indicative of a synergistic effect of the chloro substitution. Most importantly, a handful of compounds exhibited moderate-to-high activity against both methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA). Multidrug-resistant organisms pose important treatment challenges. Perhaps no organism has received more attention than MRSA (Kallen *et al.*, 2010). Unfortunately, the growing incidence of MRSA has been met with insufficient efforts to combat this important public health

Table 1 - *In vitro* antimicrobial activity of thiocyanates 2 against selected strains[a,b].

#	R	*C. albicans*	*P. aeruginosa*	*B. cereus*	MSSA	MRSA
2a	C_6H_5	NT	NA	NA	NA	NA
2b	$4\text{-}CH_3OC_6H_4$	NT	NA	NA	NA	NA
2c	$3, 4\text{-}(OCH_2O)C_6H_3$	NT	NA	NA	NA	NA
2d	$(E)\text{-}C_6H_5CH{=}CH$	NT	NA	NA	NA	NA
2e	$2\text{-}NO_2C_6H_4$	NT	NA	NA	NA	NA
2f	$4\text{-}CH_3C_6H_4$	NA	NT	12 ± 3	12 ± 2	NA
2g	$2\text{-}C_{10}H_7$	NA	NT	13 ± 1	15 ± 1	15 ± 1
2h	$4\text{-}FC_6H_4$	13 ± 1	NT	12 ± 3	15 ± 2	NA
2i	$4\text{-}BrC_6H_4$	13 ± 1	NT	17 ± 1	18 ± 2	17 ± 1
2j	$2\text{-}BrC_6H_4$	13 ± 1	NT	NA	21 ± 1	14 ± 1
2k	$4\text{-}ClC_6H_4$	22 ± 2	NT	NA	19 ± 2	15 ± 1
2l	$2\text{-}ClC_6H_4$	NA	NA	18 ± 2	21 ± 2	NA
2m	$2, 4\text{-}Cl_2C_6H_3$	19 ± 2	NA	19 ± 1	25 ± 6	19 ± 1
	Imipenem	NT	30 ± 1	20 ± 1	21 ± 3	19 ± 2
	Itraconazole	28 ± 2	NT	NT	NT	NT

[a]Antimicrobial activity, expressed as inhibition zone diameter in millimeters (mm), of chemical compounds against the pathological strains based on agar well diffusion assays at 1 mg mL^{-1}. The experiment was carried out in triplicate and the average zone of inhibition was calculated.
[b]NA = Not active; NT = not tested.

issue. Therefore, the continued emergence of MRSA demands an urgent need to develop new, more effective antibiotics (Morell and Balkin, 2010).

Among the thiocyanates screened against MRSA, the dichloro-substituted analog 2m was found to be the most active, with a remarkable potency that is comparable to Imipenem. Interesting, *P. aeruginosa* was the sole strain that was not sensitive to any of the thiocyanates 2 tested. This observation is suggestive of a selective action against Gram-positive rather than Gram-negative bacteria (Pieri *et al.*, 2011).

Antifungal activity of thiocyanates 2a-m was also studied for the first time, and the results are shown in Table 1. Most of the compounds were inactive against *C. albicans*, but the chloro derivatives 2k and 2m exhibited a moderate-to-good action compared to Itraconazole as the positive reference.

MIC determination for the selected chloro derivatives 2k, 2l, and 2m was carried out using a microdilution assay (Watts *et al.*, 2008) modified with resazurin growth indicator that made easily to establish the size of inhibition zones. These compounds showed good-to-high potency (3-6 μM) against both strains of *S. aureus* and the results are compiled in Table 2. As anticipated from the agar diffusion method in Table 1, chloro derivatives 2k and 2m also presented good antifungal activity (6-25 μM) against both *Candida* species under evaluation.

In the development of promising new antitubercular agents, we have recently reported that 4-chloro-substituted thiocyanate 2k, as well as the two bromo-substituted analogues 2i and 2j, were highly active against replicating and non-replicating forms of *Mycobacterium tuberculosis* (*Mtb*) H$_{37}$Rv, with relatively low toxicity toward VERO cells (Silveira *et al.*, 2012). In the current screening for antimicrobial activity (Tables 1 and 2), these three compounds were also potent against some of the strains under study. On the other hand, the chloro-derivatives 2l and 2m, which were inactive against *Mtb* (MICs > 128 μM) (Silveira *et al.*, 2012), were found to be potent inhibitors of MSSA and, in the case of 2m, of MRSA. Therefore, some of the thiocyanates 2 are more likely to be related to a broad-spectrum of activity, including against *Mtb*, Gram-positive bacteria and fungi (2i, 2j, and 2k), while other analogues can be considered as powerful agents against a selective group of bacteria and fungi but not *Mtb* (2l and 2m).

Conclusions

Allylic thiocyanates bearing halogenated aryl groups, in particular the dichloro-substituted analog 2m, were found to be active against a diversity of pathogen strains. Both MRSA and MSSA as well as the two *Candida* species were susceptible to some of the thiocyanates screened, but the *P. aeruginosa* strain tested was not. The analogues identified as the most active possess a bromoaryl or a chloroaryl group, and the presence of a second chlorine

Table 2 - Selected antimicrobial activity of thiocyanates 2 (expressed as MIC [μM])[a].

#	R	*C. albicans*	*C. tropicalis*	MSSA	MRSA
2k	4-ClC6H4	12.5	25.0	NT	NT
2l	2-ClC6H4	NT	NT	3.0	6.0
2m	2,4-Cl2C6H3	6.0	12.5	2.5	5.0
	Imipenem	NT	NT	1.5	2.5
	Itraconazole	4.4	0.2	NT	NT

[a] NT = not tested.

atom in the aromatic ring enhanced the potency. In addition to their remarkable antimicrobial activity, the straightforward preparation of allylic thiocyanates from inexpensive and readily available chemicals will allow the full exploitation of this promising class of compounds as future leads for drug development.

Acknowledgments

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