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ORIGINAL ARTICLE

Synthesis of benzoylthiourea derivatives and analysis of their antibacterial performance against planktonic *Staphylococcus aureus* and its biofilms

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Significance and Impact of the Study: The study presented herein highlights the antimicrobial and anti-biofilm activity of benzoylthiourea derivatives against a clinical multidrug-resistant *Staphylococcus aureus* isolate. The results may guide the use of these molecules for new strategies to fight staphylococcal infections and to destroy biofilm-forming bacteria in medical devices, such as catheters and prostheses.

Keywords
1,2,4-triazole, biofilm, MBC, MIC, *Staphylococcus aureus*, thiourea.

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Abstract

Following the appearance of several antimicrobial agents to control the spread of infections, two major challenges have emerged: (i) the occurrence and blowout of multiresistant bacteria and the increase of chronic diseases and (ii) difficult-to-eradicate infections. In this study, we tested five benzoylthiourea derivatives for their ability to inhibit and stop bacterial growth and evaluated the possible influence of 1,2,4-triazolyl-benzoylthiourea derivative **4** on the formation and eradication of *Staphylococcus aureus* biofilms. Benzoylthiourea derivatives **4**, **6**, **10**, **11** and **13** were obtained in one or two steps with low cost and subjected to tests to identify their minimum inhibitory concentration (MIC) and minimum bactericidal concentration. *In vitro* tests were also performed to assess their effects on biofilm formation and in preformed biofilms and scanning electron microscopy was used to visualize the effects on biofilm formation. The 1,2,4-triazolyl-benzoylthiourea derivative **4** showed bacteriostatic activity against the *S. aureus* HU25 clinical strain with an MIC of 16 μg ml^{-1}, which is below the toxic concentration (at 2500 μg ml^{-1}, 62·25% of the cells remained viable). Compound **4** also effectively prevented biofilm formation at the three subinhibitory concentrations tested (1/2 MIC, 1/4 MIC and 1/8 MIC) as confirmed by scanning electron microscopy. For breakdown of formed biofilms, the main influence was at a subinhibitory concentration (1/2 MIC). These findings make compound **4** a strong candidate for studies on the development of new antimicrobial and antibiofilm agents.

Introduction

The emergence and spread of antibiotic resistance among pathogens causing infections in humans is an important public health problem. Selective pressure associated with bacterial mechanisms of genetic evolution can imply the acquisition of antimicrobial resistance (Rice 2009). Although it is a representative phenomenon of natural

selection, the acquisition of antimicrobial resistance is accelerated by the misuse of antibiotics (Jindal *et al.* 2015), representing a process of evolution in response to this pressure. Thus, a two-component equation arises: the antibiotic, which inhibits the growth of susceptible micro-organisms, selects the resistance and the genetic determinants of resistance in the selected micro-organisms. When these factors appear together, resistance tends to manifest (Levy and Marshall 2004).

Staphylococcus aureus is the most opportunistic pathogen and of great concern because of its intrinsic virulence and ability to cause a wide variety of infections combined with its ability to adapt to diverse environmental conditions (Lowy 2003). Additionally, some strains of *S. aureus* acquire genes that confer multidrug resistance to antimicrobials (Katayama *et al.* 2000). *Staphylococcus aureus* can also form a biofilm, which improves its resistance mechanisms and is related to several chronic diseases (Karaolis *et al.* 2005). Therefore, the formation of a biofilm in medical devices such as catheters and orthopaedic prostheses is an important virulence factor that is responsible for many different types of infections in the hospital environment.

The main targets evaluated in the engagement against biofilms are blockage of bacterial adhesion to the surface, disruption of bacterial cell communication (quorum sensing) and eradication or treatment of biofilms already formed (Macedo and Abraham 2009).

Thioureas have demonstrated considerable antibacterial activity, mostly against gram-positive strains (Cunha *et al.* 2007; Stefanska *et al.* 2015). Therefore, in this study, we intended to verify the potential antimicrobial and antibiofilm activity of these derivatives against a clinical multidrug-resistant *S. aureus* strain and propose alternatives for the treatment of infections caused by these micro-organisms.

Results and discussion

The design of the new benzoylthiourea derivatives (**4**, **6**, **10**, **11** and **13**) as novel antibacterials was inspired by compounds **I** and **II**, which present a 1,2,4-triazole ring and a thiourea group in their structures, respectively (Fig. 1). Compound **I**, a 1,2,4-triazolyl-ciprofloxacin, has been reported to exhibit antibacterial activity against gram-positive and gram-negative strains (Dolan *et al.* 2016; Plech *et al.* 2015). Compound **I**, when tested against *S. aureus* strains ATCC 25923, ATCC 6538 and MICROBANK 14001, showed MICs of 0·18, 0·091 and 0·046 μmol l^{-1}, respectively, being more effective than the commercial drugs

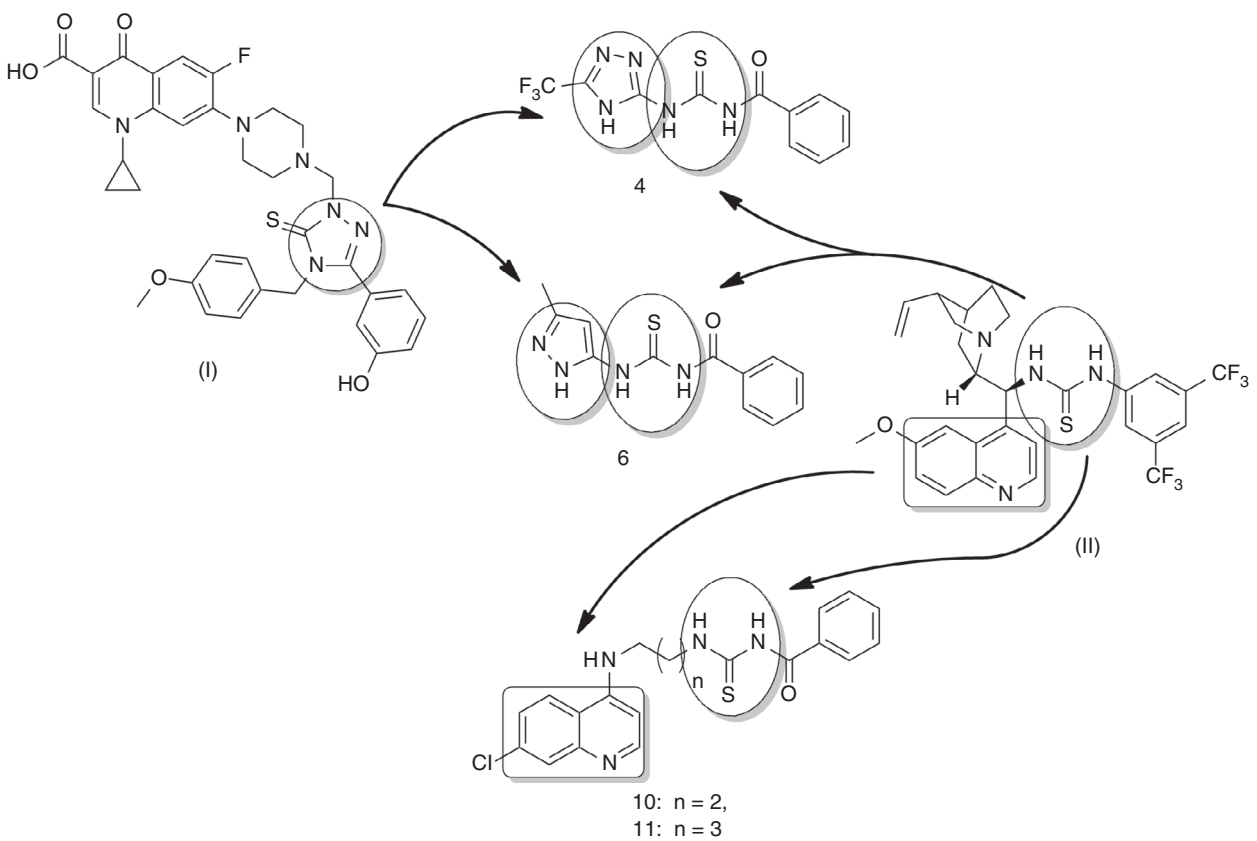
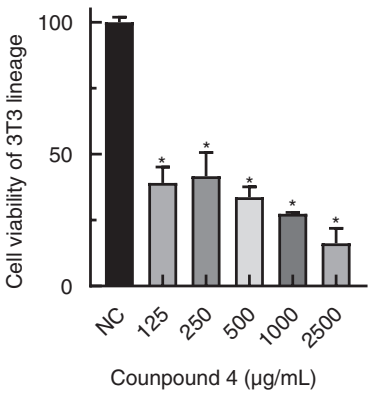


Figure 1 Rational approach to the design of compounds **4**, **6**, **10** and **11**.



Concentration (μg/mL)	Cell Viability (%)
125	51·90
250	31·69
500	30·66
1000	65·46
25000	62·25

Figure 2 Evaluation of the cytotoxic potential of compound **4** at concentrations of 125–2500 μg ml^{-1} against the 3T3 cell line after 24 h of treatment. *$P \leq 0{\cdot}05$ compared to vehicle. Data are presented as the mean (SD). The statistical significance of the differences was assessed by one-way ANOVA, followed by Tukey's *post hoc* test.

ciprofloxacin (against ATCC 25923, MIC = 2·97 μmol l^{-1} and against ATCC 6538, MIC = 0·75 μmol l^{-1}) and vancomycin (MICROBANK 14001, MIC = 0·68 μmol l^{-1}) (Plech *et al.* 2015). The quinoline-thiourea compound **II** was active against a variety of Gram-positive and Gram-negative bacteria, exhibiting activity against methicillin-resistant *S. aureus* with an MIC_{90} 17·74 μmol l^{-1} whereas vancomycin had an MIC_{90} of 1·35 μmol l^{-1} (Dolan *et al.* 2016). 1,2,4-Triazolyl-benzoylthiourea derivative **4** and pyrazolyl-benzoylthiourea derivative **6** were designed by exploring the concepts of molecular hybridization between compounds **I** and **II**, linking the azole ring (blue) to the thiourea group (green). Derivatives **10** and **11** were planned by keeping the quinoline ring (red) attached to the thiourea group (green) through a linker containing 3 or 4 methylene moieties (Fig. 1).

The clinical isolate *S. aureus* HU25 used in this study has been characterized as a strong biofilm former, justified by the presence of the *ica*C and *ica*R genes (data not shown). Among the derivatives **4**, **6**, **10**, **11** and **13** selected for testing in this study, the 1,2,4-triazolyl-benzoylthiourea derivative **4** presented bacteriostatic activity against *S. aureus* with an MIC value of 16 μg ml^{-1}.

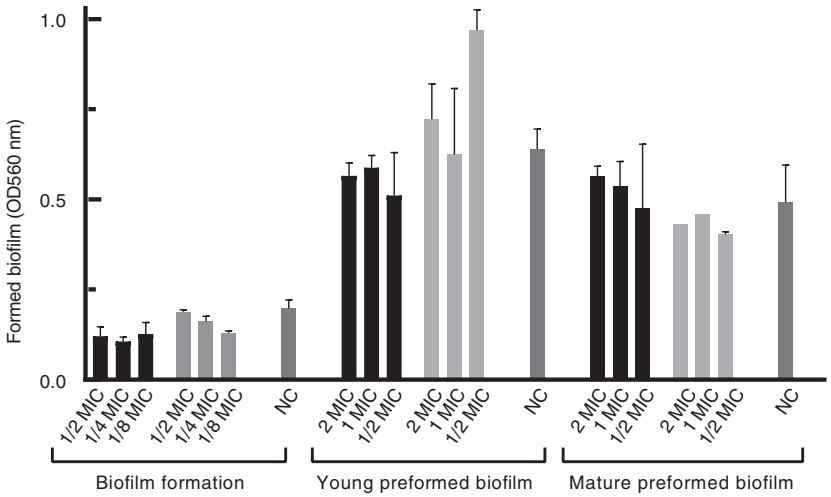
Interestingly, exchange of the 5-trifluoromethyl-1,2,4-triazole moiety (compound **4**) with a 5-methyl-pyrazole moiety (compound **6**) causes loss of the antibacterial activity. The importance of the 1,2,4-triazole ring was confirmed by the loss of activity with compound **13**, which presents a trifluoromethylphenyl bound to the benzoylthiourea group. In addition, quinoline derivatives **10** and **11**, which present methylene spacers between the quinoline ring and benzoylthiourea group, did not show activity against *S. aureus*, *S. epidermidis* or *E. coli* (data not shown). Therefore, the observed activity of derivative **4** could be related to the presence of the 1,2,4-triazole ring, one of the most important heterocycles present in medicinal agents (Kharb *et al.* 2011).

Compound **4** was then tested for its cytotoxicity in a 3T3 cell culture, and it was observed that at 2,500 μg ml^{-1}, 62·25% of the cells remained viable, justifying its safe use as an antimicrobial agent (Fig. 2).

Additionally, compound **4** at the three subinhibitory concentrations (1/2 MIC, 1/4 MIC and 1/8 MIC) showed even greater activity against biofilm formation than vancomycin at the same corresponding concentrations (Graph 1). The antibiofilm activity was confirmed by scanning electron microscopy at 1/2 MIC (8 μg ml^{-1}), as shown in Fig. 3. The image reveals a reduction of cells in the biofilm test, suggesting possible action of the molecule in the phase of bacterial adsorption to the surface.

The experiments with preformed biofilms after 6 h of incubation support that the main influence is at subinhibitory concentrations (1/2 MIC) (Graph 1). Surprisingly, vancomycin at the corresponding concentrations stimulated biofilm formation. This suggests that bacterial cells that had already adhered to the surface tend to increase their aggregation when under the threat of a challenging molecule. However, 1,2,4-triazolyl-benzoylthiourea derivative **4** does not significantly stimulate the breakdown of mature biofilm (24 h of incubation) in contrast with vancomycin activity (Graph 1).

Among the five derivatives tested, only compound **4** showed bacteriostatic activity against the clinical *S. aureus* isolate at a concentration of 16 μg ml^{-1}. It was also observed that in addition to the inhibition activity on planktonic bacteria, compound **4** has an important effect on the biofilm formation of this bacteria. Compound **4** is also safe for use as an antimicrobial agent in humans, as evaluated in 3T3 cell cultures at 2500 μg ml^{-1}, where 62·25% of the cells remained viable. Therefore, the 5-trifluoromethyl-1,2,4-triazole group seems to present strong antibacterial activity. In conclusion, the notable activity of compound **4** against the *S. aureus* HU25 multiresistant



Graph 1 *Staphylococcus aureus* HU25 biofilm formation: young preformed biofilm and mature preformed biofilm under the influence of compound **4** (▬ 1,2,4-triazol thiourea derivate; ▬ vancomycin; ▬ negative control).

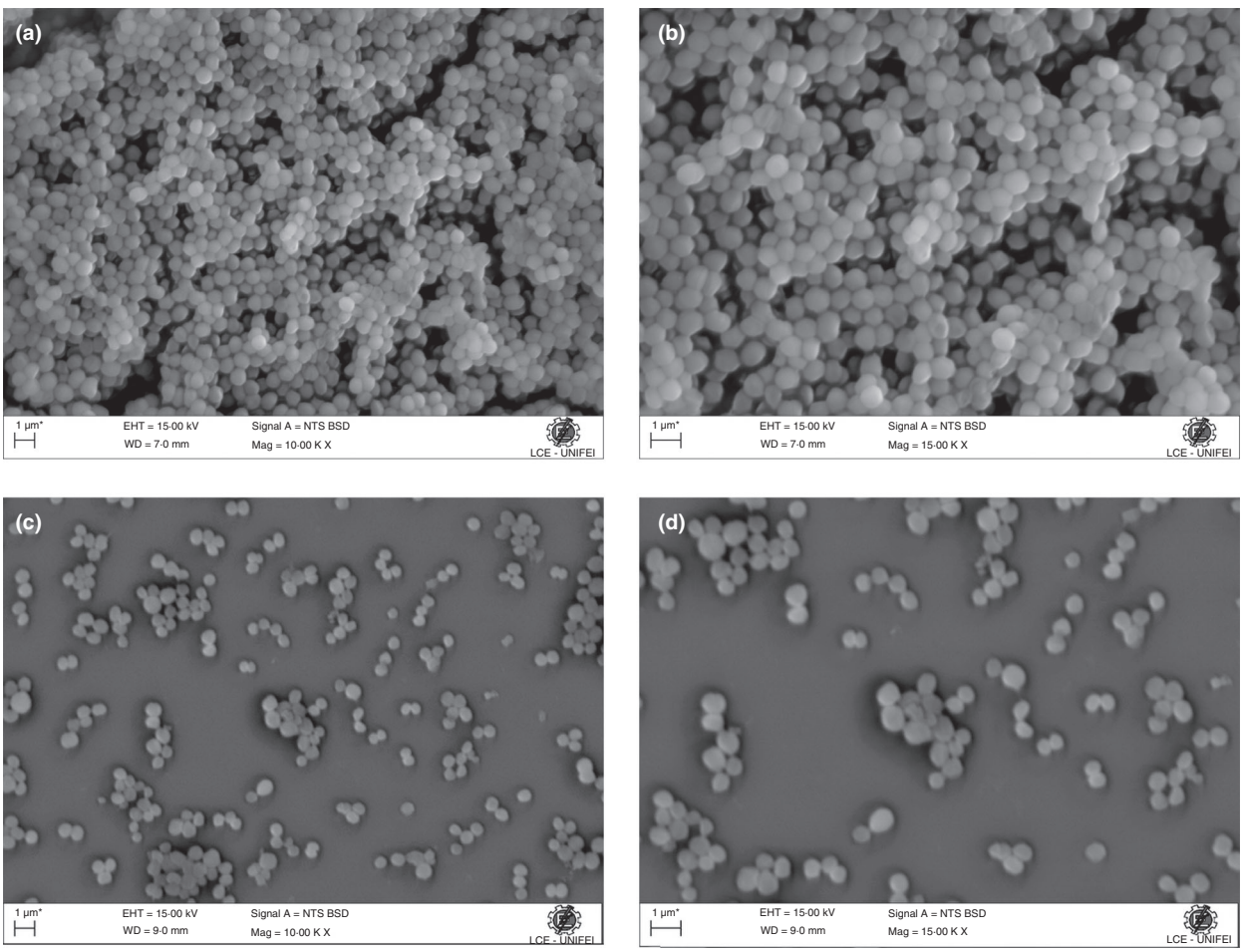


Figure 3 *Staphylococcus aureus* biofilm formation without treatment (a and b) and *S. aureus* HU25 biofilm formation under treatment with compound **4** at 1/2 MIC (c and d). Magnification: a and c = 10 000×; b and d = 15 000×.

clinical isolate indicates that it is a promising antimicrobial agent candidate. These results suggest that this molecule is a promising antimicrobial agent and perhaps a prophylactic agent of biofilm formation due to its role in the biofilm formation phase, although more studies are needed to better assess this possibility.

Methods

Chemistry

Condensation followed by a cyclization reaction of aminoguanidine bicarbonate (**1**) and trifluoroacetic acid (**2**) in toluene under reflux for 24 h was performed to obtain intermediate 5-(trifluoromethyl)-4*H*-1,2,4-triazol-3-amine (**3**) in 91% yield, which was used without purification (Boechat *et al*. 2011). The reaction of **3** with potassium thiocyanate in acetone and then treated with benzoyl chloride under reflux gave rise to the target compound *N*-((5-(trifluoromethyl)-4*H*-1,2,4-triazol-3-yl)-carbamothioyl)benzamide (**4**) in 27% yield (Fatima *et al*. 2016) (Fig. 4).

Raw material 3-methyl-1*H*-pyrazol-5-amine (**5**) was commercially available and submitted to a reaction with potassium thiocyanate in acetone and then treated with benzoyl chloride under reflux to give rise to the target compound *N*-((3-methyl-1*H*-pyrazol-5-yl)carbamothioyl) benzamide (**6**) in 24% yield (Fig. 4).

The solvent-free nucleophilic substitution reaction between raw material 4,7-dichloroquinoline (**7**) and the appropriate alkyldiamine, propane-1,3-diamine or butane-1,4-diamine, refluxed for 4 h, obtained intermediates N^1-(7-chloroquinolin-4-yl)propane-1,3-diamine (**8**) and N^1-(7-chloroquinolin-4-yl)butane-1,4-diamine (**9**), with 90% and 86% yields, respectively (Pinheiro *et al*. 2015). Aminoquinolines **8** and **9** were reacted with potassium thiocyanate in acetone and then treated with benzoyl chloride under reflux to produce the target compounds

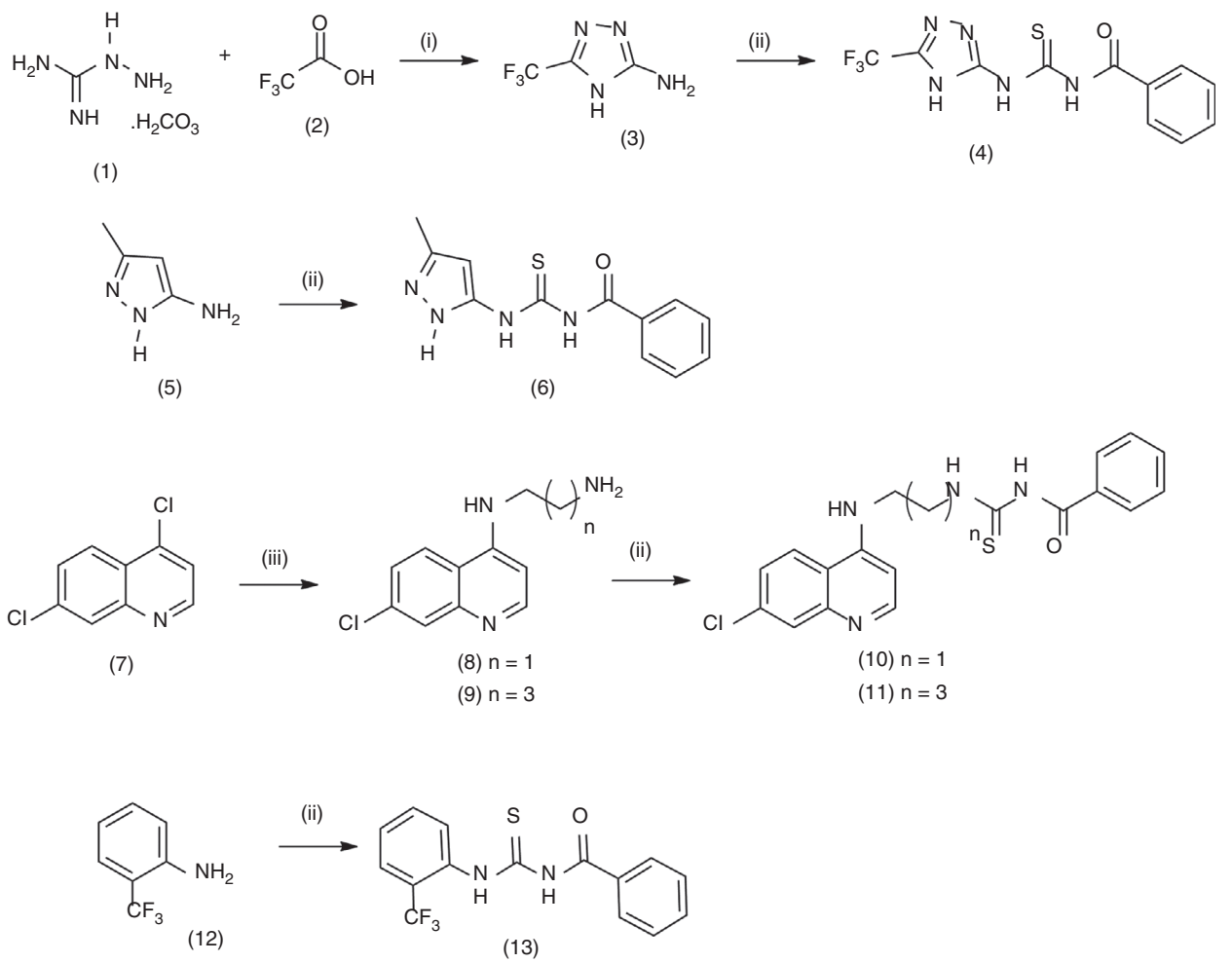


Figure 4 Synthesis of compounds **4**, **6**, **10**, **11** and **13** Reagents and conditions: (i) toluene, reflux, 24 h, 91%; (ii) 1) benzoyl chloride, KSCN, acetone, reflux, 30 min, 2) appropriate amine (**3**, **5**, **8**, **9** or **12**), reflux, 3 h, 10–27%; (iii) appropriate diamine, reflux, 4 h, 86–90%.

N-((3-((7-chloroquinolin-4-yl)amino)propyl)carbamothioyl)benzamide (**10**) and *N*-((4-((7-chloroquinolin-4-yl) amino)butyl)carbamothioyl)benzamide (**11**) in 11% and 10% yields, respectively. (Fatima *et al*. 2016) (Fig. 4).

To study the importance of heterocyclic rings on antimicrobial activity, a non-heterocyclic derivative was also synthesized. From the raw material 2-(trifluoromethyl)aniline (**12**), the derivative *N*-((2-(trifluoromethyl)phenyl)carbamothioyl)benzamide (**13**) was obtained in 27% yield (Fatima *et al*. 2016) (Fig. 4).

Microbiological assays

Bacteria

A multidrug-resistant *S. aureus* (HU25) clinical strain was maintained in 37% (w/v) brain heart infusion (BHI) broth supplemented with 10% (v/v) glycerol at − 80°C.

Minimum inhibitory concentration

Two colonies from a TSA plate were transferred to BHI medium (5 ml) and incubated (4 h/37°C), and the concentration was adjusted to 0·5 McFarland standard with 0·9% NaCl solution. The derivatives in a 1% DMSO dilution were applied to 96-well plates already filled with BHI following serial dilution and addition of the inoculum to 0·5 McFarland standard, finally adding concentrations of derivatives that varied from 512 to 4 μg ml^{-1}. The plate was then incubated for 24 h at 37°C and dyed with 20 μl of 0·01% resazurin to confirm the MIC, which is the lowest concentration of the derivative that inhibits visible growth of the bacterial culture ('M07-A10: Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Tenth Edition,' [s.d.]-CLSI, 2015). Vancomycin, from 8 to 0·0625 μg ml^{-1}, was used as a positive control. All tests were performed in triplicate.

Minimal bactericidal concentration

This test was performed in a Petri dish with TSA medium divided into nine fields, one for each of the eight serial dilutions obtained in the MIC assay (between 512 and 4 μg ml^{-1}) with vancomycin (8 μg ml^{-1}) as a positive control. A total of 10 μl of each dilution was applied, followed by incubation at 37°C/24 h. The field without bacterial growth was considered the minimal bactericidal concentration value (elimination of 99·9% of the bacteria).

Evaluation of the cytotoxic potential in a 3T3 cell culture

A culture of 3T3 lineage cells supplemented with the vital dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was used. The final endpoint was the determination of the IC_{50} (concentration of the compound that inhibits 50% cell growth). Cells (3×10^4 cells per ml) were seeded in a 96-well plate with culture medium (RPMI-1640 supplemented with 10% foetal bovine serum, 1% glutamine and 40 μg ml^{-1} gentamicin). After 24 h, the thiourea derivatives were added at concentrations ranging from 600 to 10 μg ml^{-1}, and the plate was incubated at 37°C/48 h. The plate was then washed, and MTT was applied with another 3 h of incubation and the absorbance at 570 nm was measured with a spectrophotometer to determine cell viability.

Effect of selected substances on the biofilm

Two colonies were resuspended in 2 ml of BHI medium with 1% glucose and incubated at 37°C with shaking at 150 rev min^{-1}. After 20 h, the inoculum was diluted 1 : 100 in BHI with 1% glucose. To analyse the effects of the substances on biofilm formation, 200 μl of each dilution corresponding to subinhibitory concentrations of each selected substance (1/2, 1/4 and 1/8 MIC) was added to a 96-well plate (NUNC) following incubation at 37°C/24 h. After incubation, the first measurement was performed in a spectrophotometer plate at a wavelength of 570 nm to observe the planktonic bacteria present in the medium. The plate was washed with distilled water and maintained at 70°C until dry. A total of 200 μl of violet crystal was added to each well followed by incubation for 1 min/RT. The staining solution was discarded, the plates were dried at 70°C and the optical density was measured at 570 nm (reading 2). Over the preformed biofilm, 200 μl of the bacterial inoculum (1 : 100) was applied to a 96-well plate (NUNC). After 6 h (for young biofilm) or 24 h (for mature biofilm) of incubation, the supernatant was removed, and diluted compounds were applied at 2 MIC, 1 MIC and 1/2 MIC in triplicate and incubated at 37°C/24 h. The plate was washed, dyed with crystal violet and the absorbance was measured as previously described. As a control, vancomycin at 1/2, 1/4 and 1/8 MIC was used with and without inoculum.

Biofilm formation analysis by scanning electron microscopy

Biofilm formation was developed as proposed by Pereira *et al*. (2011), with modifications. The *S. aureus* inoculum was adjusted to a 0·5 McFarland standard. For the growth of biofilms, sterilized glass disks were placed in a polystyrene 24-well plate (Techno Plastic Products AG-TPP, Trasadingen, Switzerland) with 1 ml of BHI broth and inoculated with 0·1 ml of *S. aureus* HU25. The compound was diluted in DMSO, followed by BHI to achieve 1/2 MIC concentrations. The plate was incubated for 24 h at 37°C, and after that period, the disks were removed and fixed for 1 h in 2·5% glutaraldehyde and dehydrated in several ethanol washes (10, 25, 50, 75 and 90% for 20 min and 99·5% for 1 h). The disks were dried overnight at 37°C and coated with gold in a low-pressure

atmosphere with an iron sputter. The surface topographies of the biofilms were visualized and photographed using a scanning electron microscope (Carl Zeiss EVO® MA15 scanning electron microscope).

The results were analysed by the GraphPad Prism 5·1 program using the log-rank test with Kaplan–Meier graphs. Statistical significance was defined as $P < 0{\cdot}05$.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1