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Synthesis, antibacterial and anti-MRSA activity, in vivo toxicity and a structure–activity relationship study of a quinoline thiourea

Niamh Dolan [a], Declan P. Gavin [a,†], Ahmed Eshwika [c], Kevin Kavanagh [b], John McGinley [a], John C. Stephens [a,*]

[a] Department of Chemistry, Maynooth University, Maynooth, Co. Kildare, Ireland
[b] Department of Biology, Maynooth University, Maynooth, Co. Kildare, Ireland
[c] Department of Microbiology, Pharmacy College, Zawia University, Zawia, Libya

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ABSTRACT

We report the synthesis, antibacterial evaluation of a series of thiourea-containing compounds. 1-(3,5-Bis (trifluoromethyl)phenyl)-3-((*S*)-(6-methoxyquinolin-4-yl)-((1*S*,2*S*,4*S*,5*R*)-5-vinylquinuclidin-2-yl) methyl)thiourea **5**, was the most active against a range of Gram-positive and Gram-negative bacteria, and exhibited bacteriostatic activity against methicillin resistant *Staphylococcus aureus* (MRSA) comparable to that of the well-known antibacterial agent vancomycin. Quinoline thiourea **5** was subjected to a detailed structure–activity relationship study, with **5** and its derivatives evaluated for their bacteriostatic activity against both Gram-negative and Gram-positive bacteria. A number of structural features important for the overall activity of quinoline thiourea **5** have been identified. A selection of compounds, including **5**, was also evaluated for their in vivo toxicity using the larvae of the Greater wax moth, *Galleria mellonella*. Compound **5**, and a number of derivatives, were found to be non-toxic to the larvae of *Galleria mellonella*. A new class of antibiotic can result from the further development of this family of compounds.

The European Centre for Disease Prevention and Control (ECDC) has estimated that on any given day 1 in 18 hospitalised patients are suffering with a healthcare-associated infection (HAI).[1] The four most frequently isolated microorganisms from HAIs are *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), *Enterococcus* spp. and *Pseudomonas aeruginosa* (*P. aeruginosa*).[2] Of the *S. aureus* derived HAIs, 41% are caused by Methicillin Resistant *S. aureus* (MRSA), with 23% of *E. coli* HAIs resulting from cephalosporin-resistant strains.[2] In addition, the ECDC has reported that infections resulting from Gram-negative multidrug-resistant (MDR) bacteria are on the rise.[3,4] In the United States (U.S.) at least 2 million people become infected each year with antibiotic resistant bacteria.[5] Of the 2 million infections, approximately 23,000 people die as a direct result.[5]

This increase in prevalence of antibiotic-resistant bacterial infections and emergence of new resistant strains are only part of the problem. The lack of development of new classes of antibacterial agents also plays a significant role. Of the antibiotics used today almost all of them belong to classes discovered before the 1980s, with the exception of the lipopeptides.[6] Most of the advances that have been made since the 1980s have been achieved through modifications/improvements to existing antibiotic classes.[6] For example, the fluoroquinolones are more effective antimicrobial agents than nalidixic acid.[7] The global spread of antibiotic-resistance and the emergence of bacteria resistant to 'last-resort antibiotics', coupled with the paucity of new classes of antibiotics, means that there is an urgent need for antibacterial research with a focus on the discovery of new antibiotic classes.[5,8]

We employed a building block approach in our search for new antibacterial agents, where we wanted to consider compounds that: (a) bear moieties/blocks that are found in established antimicrobial agents or are known to enhance biological activity and (b) once the blocks are combined, still have the potential to become a new class of antibiotic. This building block approach aimed to include structural moieties, or blocks, such as functional groups bearing fluorine atoms, quinoline bicycles, saturated nitrogen heterocycles and thioureas, which are all recognised motifs in antimicrobial agents.[9,10]

The addition of fluorine is known to enhance drug potency by improving bioavailability, metabolic stability and protein–ligand interactions (e.g., Flurithromycin), whilst the quinoline bicycle is structurally similar to the well-known antibacterial agents, the quinolones.[12,7] Additionally, quinine, a compound most well-known for its antimalarial properties, has also been shown to be bactericidal to a number of Gram-positive and Gram-negative bacteria.[13–15]

* Corresponding author.
† Present address: Sir Robert Kane Building, UCC, College Road, Co. Cork, Ireland.

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Thiourea-based compounds are well-known for their antithyroid activity, however, a vast array of biological activities including antitubercular, insecticidal, rodenticidal, antiviral, antifungal and antibacterial activities have also been associated with thiourea-based compounds.[10,11] A recent study on thiourea-based compounds incorporating a hippuric acid moiety was carried out by Abbas et al.[16] The majority of these compounds exhibited broad spectrum antimicrobial activity with a number of them demonstrating activity comparable to, and in some cases better than, ciprofloxacin.[16]

As such, a number of compounds containing some or all of these moieties/functional groups were chosen and screened for their antibacterial activity (Fig. 1).[17–19]

Each compound was evaluated for their bacteriostatic activity against the Gram-positive bacterium *S. aureus* and the Gram-negative bacterium *E. coli*. The results of the antibacterial screening have been summarised in Table 1 and are expressed as the MIC_{90}, that is, the minimum inhibitory concentration that is required to inhibit 90% of bacterial growth.

Compounds **5** and **7** were found to be the most active of those screened (Table 1). As shown in Table 1, compound **5** exhibited remarkable anti-*Staphylococcal* activity, with a MIC90 of <6.25 μM, which was superior to all other compounds screened. Compound **7** did exhibit higher activity against *E. coli* than compound **5**. However, the almost nanomolar activity of compound **5** against *S. aureus* prompted us to consider compound **5** in more detail. As such, compound **5** was subjected to a detailed structure–activity relationship (SAR) study in order to help understand its impressive biological activity and to identify the moieties/functional groups responsible for this activity.

An initial investigation explored the affect the OMe, CF_3 and thiourea groups had on the biological activity of compound **5**. We generated derivatives **5a**–**c** for this purpose (Scheme 1).

Compound **5a** was synthesised as described by Oliva et al.[20] (Scheme 1). As shown in Scheme 1, the nucleophilic addition of **5a** to 3,5-bis(trifluoromethyl)phenyl isothiocyanate generated compound **5**. Alternatively, the reaction of **5a** with phenyl isothiocyanate gave **5c**. The synthesis of **5b** was carried out using the same method as described for **5**, however, cinchonidine was used in place of quinine in the Mitsunobu reaction (Scheme 1).

Each of the compounds, **5** and **5a**–**c**, were evaluated for their in vitro bacteriostatic activity using the susceptibility assay described by Kelly et al.[21] The three bacteria chosen were, *E. coli*, *P. aeruginosa* and *S. aureus*, as these are three of the most frequently isolated microorganisms from HAIs.[2]

The *S. aureus* and *E. coli* strains used in this study were clinical isolates obtained from St. James Hospital, Dublin, Ireland and *P. aeruginosa* (10145) was obtained from the American Type Culture Collection (ATCC). The results are summarised in Table 2. The results are expressed as the MIC_{50} and MIC_{90} range. Vancomycin hydrochloride (Van·HCl) was chosen as the positive control as it is a well-known antibacterial agent that is often used as a last resort drug in the treatment of drug-resistant infections, such as MRSA.[9] The vancomycin result has also been included in Table 2.

None of the compounds tested displayed significant activity against *P. aeruginosa* (hence these results have been omitted from Table 2). This lack of activity could be due to the intrinsic resistant mechanisms associated with *P. aeruginosa*. For example, the uptake of molecules by *P. aeruginosa* is very slow (in comparison to *E. coli*) due to its inefficient porins.[22] *P. aeruginosa* can also form a capsule providing it with an additional physical barrier to prevent the entry of antibiotics.[23,24] Furthermore, *P. aeruginosa* is well-known for its ability to grow as a biofilm thus aiding its escape from the action of antibiotics.[25] Perhaps one or a combination of these mechanisms is responsible for the lack of bacteriostatic activity observed.

As can be seen in Table 2, compound **5** exhibited good activity against *E. coli*, resulting in an MIC_{50} in the range of 2.63–3.95 μM and 3.95–5.26 μM against *S. aureus*. Compound **5** also inhibited *E. coli* and *S. aureus* growth by 90% at MIC's comparable to that obtained for the reference antibacterial agent, vancomycin hydrochloride (Table 2).

The results from the susceptibility assays of **5a** revealed that it exhibited little or no activity against *E. coli*, *P. aeruginosa* and *S. aureus* (Table 2). This is an interesting result as quinine itself has been shown by others to exhibit bactericidal activity against all three bacteria.[14] However, the small structural differences between **5a** and quinine, that is the replacement of the OH with the NH_2 and **5a** having the opposite stereochemistry to quinine, may be the reasons behind this lack of activity. Alternatively, the lack of activity exhibited by **5a** could be due to the presence of a third basic nitrogen group, the NH_2, in comparison with quinine. When **5a** is in solution it has the potential to become protonated, which, in turn, may prevent it from crossing the lipid membrane of the bacteria. Consequently, **5a** may not be able to inhibit bacterial growth.

As can be seen from Table 2, **5b** exhibited activity against both *E. coli* and *S. aureus*. The MIC_{50} values obtained for **5b** were slightly higher than those exhibited by **5**, with the MIC_{90} values obtained very close to those of **5** (Table 2). Although methoxy groups can be of importance for binding to target sites through their H-bonding ability,[9] these results indicate that for **5**, the presence of the methoxy group is not important for its overall activity.

The effect of removing the two CF_3 groups was investigated using **5c**. Compound **5c** was found to be less active than compound **5**, displaying a 10- and 14-fold decrease in the MIC_{50} range against both *S. aureus* and *E. coli*, respectively (Table 2). These results indicate that the CF_3 groups appear to be very important in the overall activity of **5**.

As previously discussed, the inclusion of fluorine is known to enhance drug potency and in addition can increase the lipophilic character of a compound.[12] A useful way of comparing the lipophilicity of compounds is by calculating their Log*P* value.[9,26] The greater the Log*P* value, the more lipophilic the molecule. The *c*Log*P* of **5** was found to be approximately 7.33, while that of **5c** was found to have a *c*Log*P* of approximately 4.35.[27] Therefore the loss of the CF_3 groups, and hence reduction in lipophilicity, may be impairing the hit compound's ability to cross the cell membranes and bind to its target site.

Having established the importance of the CF_3 and thiourea groups we then continued our exploration of the structure activity relationship of compound **5** by maintaining the CF_3 substituted aryl thiourea core and introducing variation to other parts of the molecule.

In the initial screen (Table 1), compounds **5** and **7** were found to be the compounds that exhibited greatest activity. These compounds share similarities in their structures with both compounds bearing a 3,5-bis(trifluoromethyl)-phenyl thiourea moiety attached to a tertiary amine via a two-carbon chain (Fig. 2). To further explore the importance of these structural similarities compounds **11a**, **11b** and **12** were designed and synthesised. These compounds have lower molecular weights than **5** and **7** yet possess the same connectivity (Fig. 2).

In addition, compounds **13a**, **13b**, **14** and **15** were also generated, which allowed us to explore the significance of the basic tertiary amine group (Scheme 2).

Compounds **11**–**15** were synthesised using a method similar to that described by Andrés et al.,[28] with modifications (Scheme 2).

As with compounds **5**–**5c**, each of the new derivatives, along with compound **7**, were evaluated for their bacteriostatic activity against all three bacteria. The results of which have been summarised in Tables 3 and 4. Any inactive compounds have been excluded from the tables.

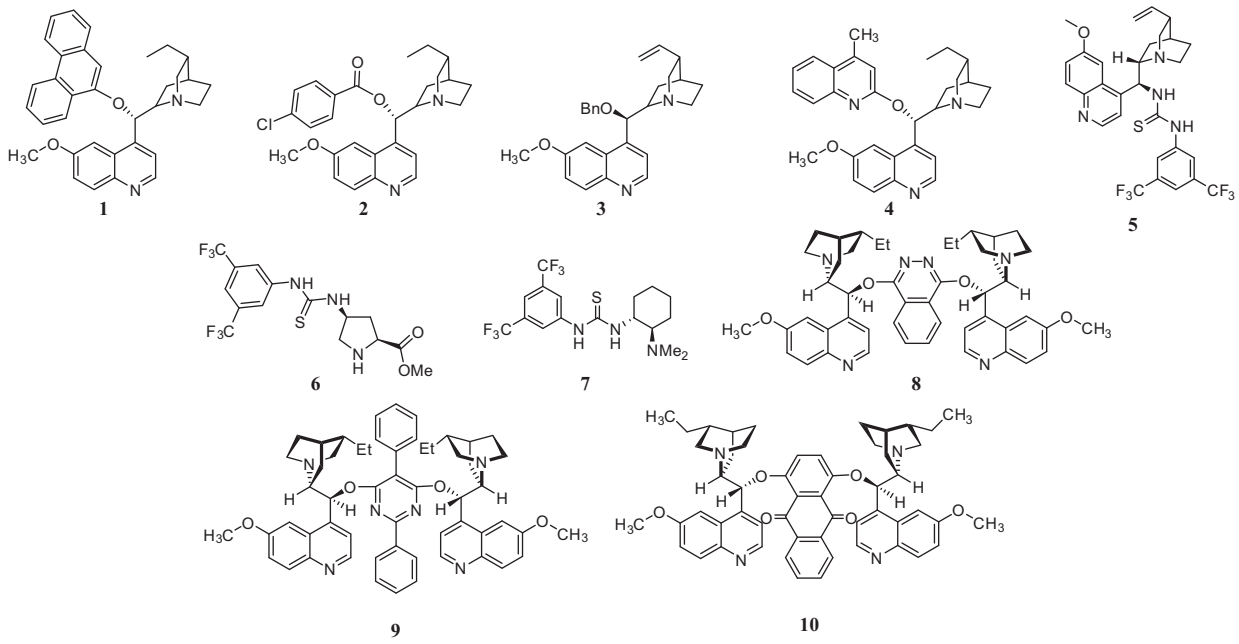
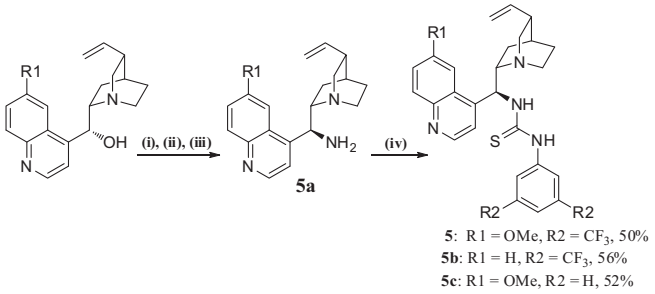


Figure 1. Compounds screened for antibacterial activity.

Table 1
MIC90 values for compounds **1**–**10**

Compound	*S. aureus*[a]	*E. coli*[b]
	MIC_{90} (μM)	MIC_{90} (μM)
1	>200	>200
2	79.44 ± 22.2	>200
3	>200	>200
4	114.8 ± 25.9	>200
5	<6.25	>200
6	>200	>200
7	23.8 ± 0.1	119.9 ± 13.8
8	189.2 ± 2.2	>200
9	>200	>200
10	124.9 ± 35.6[c]	>200

[a] *S. aureus* NCIMB 12702.
[b] *E. coli* NCIMB 9485.
[c] Value given for compound **10** is the MIC_{82}.



Scheme 1. Synthesis of compound **5** and derivatives **5a**–**5c**. Reactants and conditions: (i) PPh_3, DIAD, 0 °C; (ii) DPPA, rt, 12 h and 50 °C, 2 h; (iii) PPh_3, 50 °C, 2 h; (iv) $(CF_3)_2C_6H_3NCS$ or PhNCS, rt, 12 h.

Again, none of the compounds, **7** and **11**–**15**, exhibited activity against the Gram-negative bacterium *P. aeruginosa*. In fact, **13a** exhibited little or no activity against each of the three bacteria examined suggesting that the simple 3,5-bis(trifluoromethyl)phenyl thiourea moiety alone is not the source of activity. As shown in Table 3, **13b** only demonstrated activity against *E. coli* resulting in a MIC_{50} of 158.20–237.31 μM. Although this is a slight improvement on **13a**, the MIC_{50} achieved by **13b** is far removed from that observed for compound **5** (2.63–3.95 μM). Furthermore, **13b** was unable to inhibit any more than 50% of bacterial growth suggesting that the simple thiourea structure, and the addition of a two-carbon chain, is not enough to produce significant antibacterial activity.

Similar to **13a**, compound **11a** demonstrated little or no bacteriostatic activity against all three bacteria. Compound **11b** on the other hand resulted in an MIC_{50} of 96.87–129.16 μM against both *E. coli* and *S. aureus*. Furthermore, **11b** had the ability to inhibit up to 90% of bacterial growth, although a higher concentration was required to do so (Table 4). These results indicate that although the addition of the two-carbon chain bound to a simple dimethyl tertiary amine did not improve bacteriostatic activity (compound **11a**), the addition of a slightly more hydrophobic and slightly larger tertiary amine (diethylamine) was beneficial for activity. What is more, in comparison to **13b**, which was inactive against *S. aureus*, the addition of the $-NEt_2$ group, **11b**, resulted in a compound that can inhibit up to 80% of *S. aureus* growth at a concentration range of 129.16–193.74 μM (see Supporting information).

As shown in Table 3 and **12** (containing a quinuclidine heterocycle, as found in compound **5**) was more active than **13b** and **11b** against both *E. coli* and *S. aureus*. Furthermore, **12** can inhibit the growth of both *E. coli* and *S. aureus* by 50%, at the same concentration, with an increase in concentration resulting in an increase in bacteriostatic activity (Tables 3 and 4).

Table 2
Antibacterial activity of compounds 5 and 5a-5c as MIC_{50} and MIC_{90} ranges

Compound	*E. coli*		*S. aureus*	
	MIC_{50} (μM)	MIC_{90} (μM)	MIC_{50} (μM)	MIC_{90} (μM)
Van·HCl	1.58–2.10	4.21–6.31	1.58–2.10	4.21–6.31
5	2.63–3.95	7.90–10.52	3.95–5.26	10.52–15.78
5a	>309.41	>309.41	>309.41	>309.41
5b	5.54–8.32	8.32–11.08	8.32–11.08	16.62–22.16
5c	40.92–54.56	81.84–109.12	54.56–81.84	163.68–218.24

{Values are the mean of three experiments.}

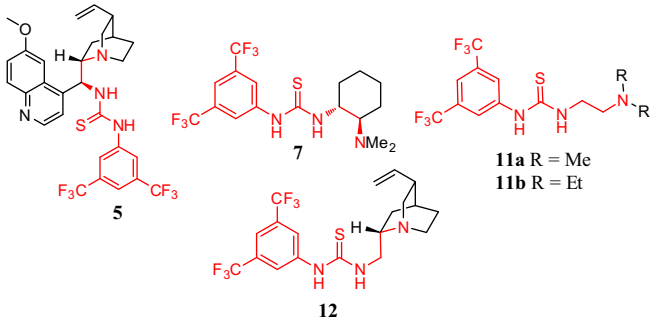
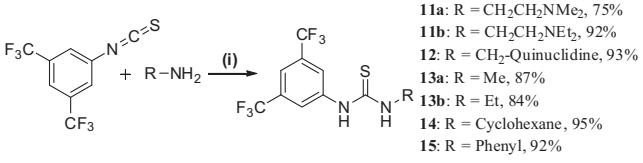


Figure 2. Structural similarity of compounds **1**, **7** and **11**–**12**.



Scheme 2. Synthesis of compounds **11**–**15**. Reactants and conditions: (i) rt, overnight.

The *c*Log*P* of compound **12** was calculated to be approximately 5.63, which is a slight increase in *c*Log*P* compared to that of **11b** (5.09 ± 0.54).[27] Therefore, perhaps this increase in lipophilicity improves activity although the change in *c*Log*P* is marginal. Additionally, these results suggest that the more sterically bulky quinuclidine ring is favourable for activity.

Comparing results for **13b** with **12** suggest that a basic tertiary nitrogen, in combination with steric bulk/lipophilicity, has a beneficial effect on activity. To further investigate this theory compounds **14** and **15** were synthesised, which do not have a basic nitrogen but retained some steric and lipophilic properties (Scheme 2). Both **14** and **15** were evaluated for their bacteriostatic activity. Compound **14** exhibited activity against both *E. coli* and *S. aureus* resulting in MIC_{50} ranges of 101.33–135.10 and 135.10–202.65 μM, respectively (Table 3). Compound **15** on the other hand only exhibited activity against *E. coli*, however, the MIC_{50} obtained was lower than that of **14** (Table 3). Additionally, an increase in **15** concentration resulted in an increase in percentage of growth inhibition, whereas **14** did not inhibit greater than 50% of bacterial growth (Tables 3 and 4). In terms of structure, both **14** and **15** contain a lipophilic six-membered carbon ring with the difference being that, in **15** it is planar and aromatic whereas for **14** it is non-planar and of greater steric bulk (Charton values: Ph = 0.57 and cyclohexyl = 0.87[29]). Both compounds, **14** and **15**, were significantly less active than **12** and **5**, suggesting that the presence of a basic nitrogen is of paramount importance.

Table 4
Anti-MRSA activity of compounds **5**, **5b**, **7**, **11b** and **12** as MIC_{50} and MIC_{90} ranges

Compound	MRSA	MRSA
	MIC_{50} (μM)	MIC_{90} (μM)
5	11.44[a]	17.74[a]
5b	10.81[a]	18.11[a]
7	21.54[a]	32.25[a]
11b	13.47[a]	23.52[a]
12	20.73[a]	31.44[a]
Vancomycin	—	1.35[b]

[a] Clinical isolate, {values are the mean of four experiments}.
[b] Published literature data.[30,31]

As a result of this synthetic and evaluation study, considerable information was attained relating compound structure to biological activity and the outcomes from this SAR exercise are summarised in Figure 3.

The antibacterial activity of several compounds, particularly compounds **5** and **5b**, was quite promising and as a result additional testing against the resistant strain MRSA was performed. The anti-MRSA results for compounds **5**, **5b**, **7**, **11b** and **12**, the top five compounds against *S. aureus*, are shown in Table 4.

All five compounds displayed significant activity levels against MRSA, with MIC_{50} and MIC_{90} values in the low μM range. Compounds **5** and **5b** were the most impressive, exhibiting low MIC_{90} values of 17.74 and 18.11 μM respectively. This compares favourably with the reported anti MRSA MIC_{90} value for vancomycin of 1.35 μM or 2 mg/L (vancomycin is the antibacterial agent commonly used in the treatment of MRSA infection).[30,31] As such, the quinoline thiourea structure, as found in compound **5**, has significant potential as a new antibacterial agent class.

In developing new antibacterial agents it is important to determine, as early as possible, if the antibacterial properties are simply due to the compounds being toxic in nature (i.e., not selective for bacteria). To investigate this possibility, in vivo toxicity studies were carried out, as described by Rowan et al.[32] using the larvae of the Greater wax moth, *Galleria melonella* (*G. melonella*), (Fig. S25 in Supplementary information).

The similarities between the innate immune system of insects and mammals have led to the use of insects as in vivo models for investigating the virulence of many human pathogens including Gram-negative bacteria, Gram-positive bacteria, and fungi.[33–35] The larvae of the Greater wax moth, *G. mellonella*, have been used as an in vivo model in a number of studies to investigate the virulence of human pathogens.[34,35] *G. mellonella* larvae have also been used to evaluate both the therapeutic effect of current and novel antimicrobial agents and the in vivo tolerance of novel antimicrobial agents.[36,37] An investigation into the toxicity of copper(II) and silver(I) complexes by McCann et al.[38] has demonstrated that the level of toxicity exhibited by the test compounds in *G. mellonella* was similar to that observed in Swiss mice.

Table 3
Antibacterial activity of compounds **5**, **7** and **11**–**15** as MIC_{50} and MIC_{90} ranges

Compound	*E. coli*		*S. aureus*	
	MIC_{50} (μM)	MIC_{90} (μM)	MIC_{50} (μM)	MIC_{90} (μM)
Van·HCl	1.58–2.10	4.21–6.31	1.58–2.10	4.21–6.31
5	2.63–3.95	7.90–10.52	3.95–5.26	10.52–15.78
7	60.51–90.77	121.02–181.54	60.51–90.77	90.77–121.02
11b	96.87–129.16	193.74–258.32	96.87–129.16	193.74–258.32
12	42.89–57.19	85.78–114.38	42.89–57.19	85.78–114.38
13b	158.20–237.30	>316.41	>316.41	>316.41
14	101.33–135.10	>270.20	135.10–202.65	>270.20
15	51.50–68.67	206.02–274.69	>274.69	>274.69

{Values are the mean of three experiments.}

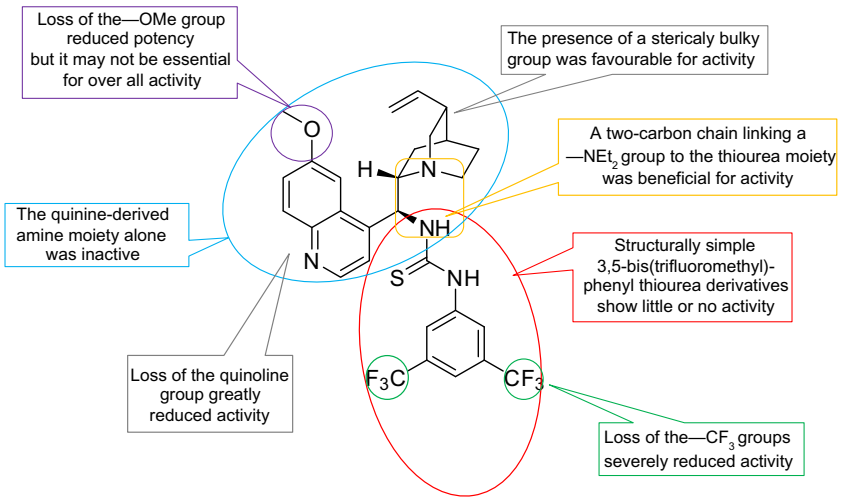


Figure 3. SAR study summary of quinoline thiourea **5**.

Compound **5**, and a selection of derivatives that exhibited antibacterial activity (**5b**, **5c**, **11b**, **12** and **15**) were chosen for the evaluation of their toxicity. Almost all compounds tested displayed a 100% survival rate of *G. mellonella* larvae at a range of concentrations. Compounds **5b**, **5c**, **11b**, **12** and **15**, at a concentration of 1 μg/mL, all displayed a 100% survival rate after 72 h. Increasing the administration dose to 10, 50 and 100 μg/mL did not appear to affect the *G. mellonella* larvae. A 100% survival rate was observed, at each of these concentrations, for every compound tested, including compound **5**. The in vivo toxicity of our most active compound, compound **5**, was also evaluated at the higher concentration of 1000 μg/mL, and once more was found to be non-toxic. A table of results showing survival rates (100% in almost all cases) at 24, 48 and 72 h, and at a range of concentrations, can be found in Supplementary information.

The *G. mellonella* larvae were also monitored for their development, that is, whether or not the larvae proceeded along their normal developmental pathway to form pupae. It was found that after seven days, at each test compound concentration, the number of the *G. mellonella* larvae that had pupated was similar to that observed for the untreated *G. mellonella* ($\geqslant$60%). These results indicate that not only were the compounds non-toxic to the larvae of the Greater wax moth but they also did not appear to interfere with larval development.

In conclusion, we have described the identification of a new quinoline thiourea antimicrobial agent, compound **5**. All compounds were evaluated for their bacteriostatic, and not bactericidal, activity. The antibacterial activity of the quinoline thiourea **5** was discovered when screening selected compounds against *E. coli*, *P. aeruginosa* and *S. aureus*. The compelling activity of compound **5** against *E. coli* and *S. aureus*, activity comparable to vancomycin hydrochloride, necessitated an exploration of its structure activity relationship and additional testing against the resistant strain MRSA. Both compounds **5** and **5b** displayed significant anti MRSA activity, again comparable to vancomycin. Additionally, and importantly, compound **5**, and a number of derivatives, were also found to be non-toxic to *G. mellonella* larvae at concentrations of up to 1000 μg/mL (in the case of compound **5**). Overall, these results suggest that the quinoline thiourea structure, as found in compound **5**, has potential as a new class of non-toxic, anti MRSA agent.

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Supplementary data

Supplementary data (synthetic procedures, compound characterisation data, NMR spectra and biological assay protocols/results) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.11.058.

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