Novel 2,4-disubstituted quinazoline analogs as antibacterial agents with improved cytotoxicity profile: Modification of the benzenoid part

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Abstract

Bacterial resistance to currently used antibiotics demands the development of novel antibacterial agents with good safety margins and sufficient efficacy against multi-drug resistant isolates. We have previously described the synthesis of *N*-butyl-2-(butylthio)quinazolin-4-amine (**I**) as an optimized hit with broad-spectrum antibacterial activity and low cytotoxicity. In addition, we have identified a potential growing vector for this series of compounds. Herein, we describe further hit optimization which includes systematic diversifications of both the benzenoid part and the substituents at position 6 and 7 of compound **I**. Growing of the molecule beside the core modifications yielded several compounds with remarkable anti(myco)bacterial activity against a panel of pathogenic bacteria, including drug-resistant strains. Compound **12** showed a 2-4 fold improvement in activity than **I** against *S. aureus Newman*, *S. pneumoniae DSM-20566* and *E. faecalis DSM-20478*. The compounds also showed a good safety profile towards human HepG2 cells.

Keywords: Quinazoline; thieno[2,3-*d*]pyrimidine; pyrazolo[3,4-*d*]pyrimidine; antibacterial; cytotoxicity

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The inappropriate and excessive use of antibiotics, without medical supervision, led to the increase and the emergence of lethal human infections that are resistant to multiple antibiotics. The prevalence of multidrug-resistant bacterial pathogens has increased the mortality and morbidity of infectious diseases, and remains among the major health problems around the globe._ENREF_1[1-5]

Staphylococci and enterococci are Gram-positive bacterial pathogens that are the causative agents for several hospital-and community-acquired infectious diseases. *Staphylococcus aureus* causes different types of infections, ranging from simple infections in skin and soft tissues to more serious diseases such as infective endocarditis, sepsis and pneumonia.[6, 7] The spread of methicillin-resistant *S. aureus* (MRSA) is on escalation and presents a major threat. Vancomycin-resistant strains of *S. aureus* have been also isolated from hospitalized patients.[8-11] Furthermore, the enterococcal species *Enterococcus faecium* and *Enterococcus faecalis* are the most common enterococcal species cultured from patients, accounting for more than 90% of clinical enterococcal isolates. *E. faecalis* infections by gentamicin-resistant strains are regarded as one of the most problematic hospital infections.[12-15] *Streptococcus pneumoniae* is a Gram-positive bacterial pathogen and a causative agent of respiratory tract infections such as meningitis, sinusitis, pneumonia and acute otitis media. It produces numerous virulence factors involved in the progress of the disease.[16] Several reported cases of endocarditis were caused by penicillin-resistant *S. pneumoniae* (PRSP) strains.[17] Over the past years, *S. pneumoniae* has developed resistance to several classes of antibiotics, including macrolides, beta-lactams, fluoroquinolones, lincosamides, tetracyclines and trimethoprim-sulfamethoxazole.[18] Consequently, the need for new antibacterial drugs is now of paramount importance.

Quinazoline is a well-known scaffold exhibiting a wide range of different biological activities including anticancer,[6, 19] anti-inflammatory[20-22] and antimicrobial activities.[23, 24] Among the reported bioactive quinazoline derivatives, some compounds showed significant antibacterial activity against both, Gram-positive and Gram-negative bacterial pathogens.[25-32] In addition, other derivatives have shown promising antitubercular activity.[33-35] A series of 2-substituted quinazoline derivatives with broad-spectrum antibacterial activity through the inhibition of the transcription/translation in different bacterial species was previously reported.[36] In an earlier study, Harris *et al.* developed a number of 5-substituted-2,4-diaminoquinazolines that revealed inhibitory activity against the bacterial dihydrofolate reductase (DHFR) enzyme in *S. aureus* and in *Escherichia coli*. However, this series of synthesized compounds lacked specificity, as the compounds did not only inhibit bacterial DHFR but they were also active against its bovine liver counterpart. Optimization of the substituents on the quinazoline resulted in higher selectivity towards the bacterial enzyme.[37] In another recently published study, some quinazolin-4-ones were also reported as cell wall biosynthesis inhibitors, through their ability to bind to the penicillin-binding protein 22 (PBP)2a.[38] Another series of N^2,N^4-disubstituted quinazoline-2,4-diamine derivatives was reported in the literature to have antibacterial activity against MRSA.[39]

Recently, we reported the synthesis and antibacterial activity of an optimized hit compound **I** (**Figure 1**) with promising antibacterial activity against Gram-positive bacteria, including drug-resistant strains, the Gram-negative bacterium *E. coli* TolC as well as *Mycobacterium smegmatis*, with MIC values ranging from 2-8 µg/mL.[40] Importantly, compound **I** showed also a good safety profile rationalized by the decreased toxicity against A549 and HepG2 cells, in addition to the lack of hemolytic activity at concentrations up to 500 µM. Noteworthy, its sulfur analog **(II)** was inactive. We also identified position 6 as a potential growing vector for this series of compounds.

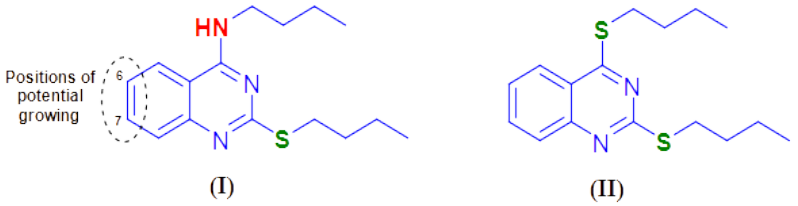


Figure 1: Optimized hit compound **I** showing the potential growing position and its inactive analog compound **II**

Herein, we describe further structure-activity relationship (SAR) exploration and optimization of compound **I** while maintaining a safe cytotoxicity profile (Figure 2). The modifications adopted include variable substitutions at positions 6 and 7 **(III)**. These substituents include halogens, which are known to efficiently influence various properties such as membrane permeability, intramolecular interactions, pharmacokinetic properties and others.[41, 42] Additionally, analogs with different aryl and heteroaryl substitutions at position 6 or 7 were also prepared. Another group of compounds was prepared by replacing the benzenoid part of the quinazoline ring with the two isosteric heterocycles - thiophene **(IV)** and pyrazole **(V)** – to give thieno[2,3-*d*]pyrimidine and pyrazolo[3,4-*d*]pyrimidine scaffolds. Finally, we further derivatized compound **(II)** at its position 6 **(VI)** in a trial to recover its antibacterial potency.

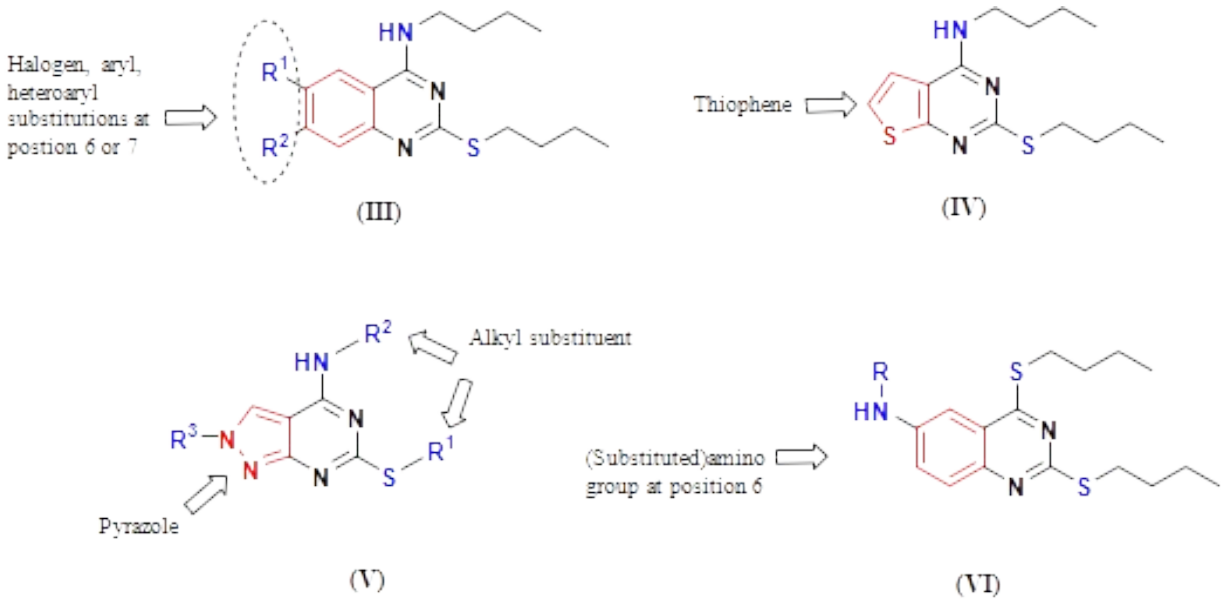
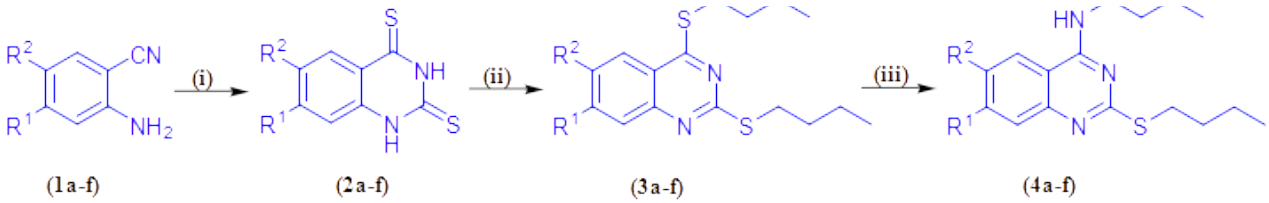


Figure 2: General structures of the target compounds: quinazoline derivatives (**III** and **VI**), thieno[2,3-*d*]pyrimidine derivatives **(IV)** and pyrazolo[3,4-*d*]pyrimidine derivatives (**V**)

The antibacterial activity of all newly synthesized target compounds was tested in vitro against both drug-sensitive and drug-resistant bacteria.

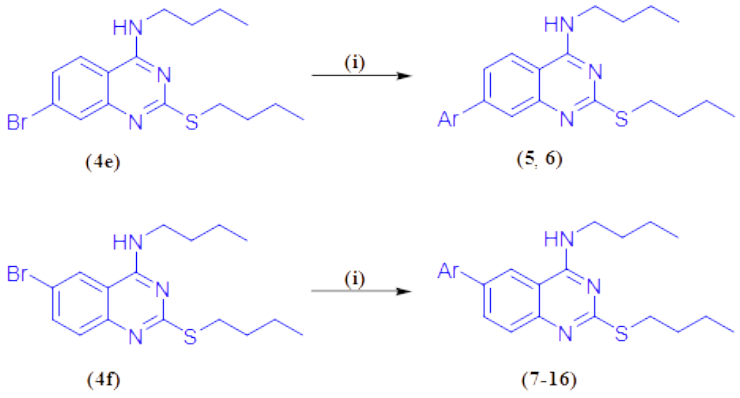
The preparation of the quinazoline-2,4-dithione analogs **2a-f** was achieved by the reaction of the *ortho*-aminobenzonitrile derivatives **1a-f** with carbon disulfide in pyridine at 70 °C. The 2,4-bis(butylthio)-quinazoline derivatives **3a-f** were obtained by the alkylation of compounds **2a-f** with 1-bromobutane, in the presence of K_2CO_3. The obtained 2,4-bis(butylthio)-quinazoline derivatives **3a-f** were then reacted with n-butylamine which underwent a regioselective substitution at position C4[43-45] to afford the 4-*N*-butyl derivates **4a-f**. (Scheme 1).

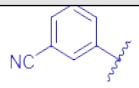
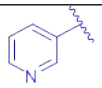
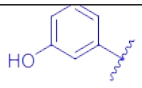


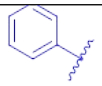
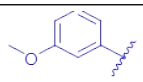
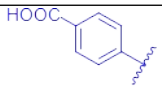
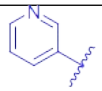
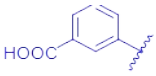
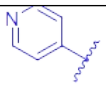
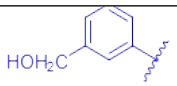
Compound no.	R^1	R^2
(1-4)a	F	H
1-4)b	H	F
(1-4)c	Cl	H
(1-4)d	H	Cl
(1-4)e	Br	H
(1-4)f	H	Br

Scheme 1: Synthesis of compounds **2a-2f**, **3a-3f** and **4a-4f**. Reagents and reaction conditions: **(i)** Carbon disulfide, pyridine, 70 ºC, 6 h (**2a-f**, yield: 72% - 91%) **(ii)** 1-Bromobutane, K_2CO_3, acetone, overnight reflux (**3a-f**, yield: 81% - 89%) **(iii)** n-Butylamine, overnight reflux (**4a-f**, yield: 84% - 95%).

The arylated quinazoline derivatives **5-16** were obtained through Suzuki coupling by reacting compounds **4e** and **4f** with the respective aryl boronic acid derivative, in the presence of Cs_2CO_3 and ($Pd(dppf)Cl_2$). The solvent used was a mixture of water/ethanol/toluene (4:10:10) (**Scheme 2**).

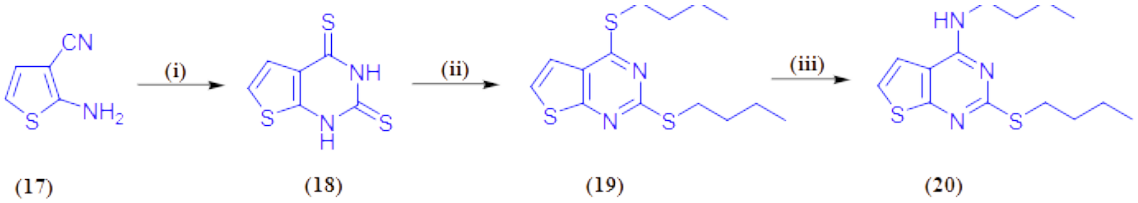


Compound no.	Ar	Compound no.	Ar
5		**11**	
6		**12**	

7		**13**	
8		**14**	
9		**15**	
10		**16**	

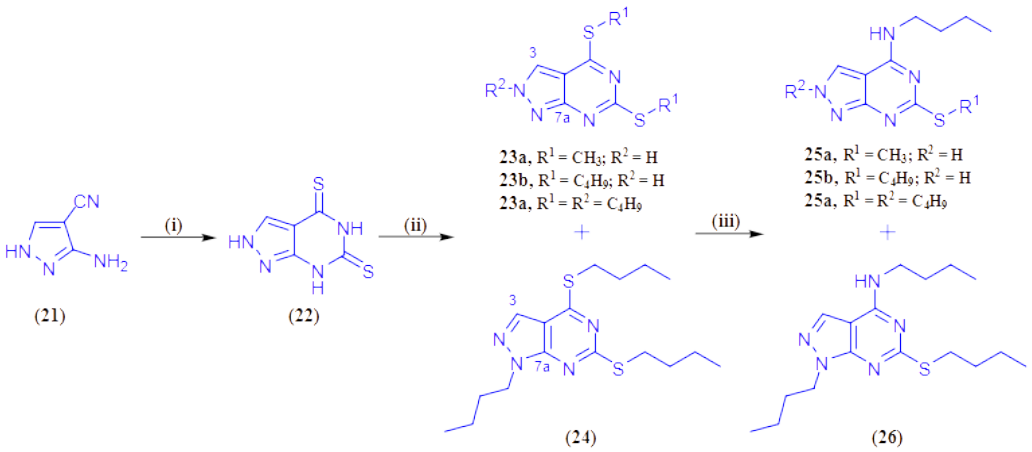
Scheme 2: Synthesis of compounds **5-16**. Reagents and reaction conditions: **(i)** Arylboronic acid, Cs_2CO_3, Pd(dppf)Cl_2, water/ethanol/toluene (4:10:10), overnight reflux (**5-16**, yield: 37% - 76%)

Other bicyclic heterocycles were also prepared using similar synthetic routes. The synthesis of thieno[2,3-*d*]pyrimidine-2,4(1*H*,3*H*)-dithione **18** was achieved by the reaction of 2-aminothiophene-3-carbonitrile **17** with carbon disulfide in pyridine. The 2,4-Bis(butylthio)thieno[2,3-*d*]pyrimidine **19** was obtained by alkylation of compound **18** with 1-bromobutane, in the presence of K_2CO_3. Finally, the reaction of n-butylamine with compound **19** afford the 4-*N*-butyl derivates **20** (Scheme 3).



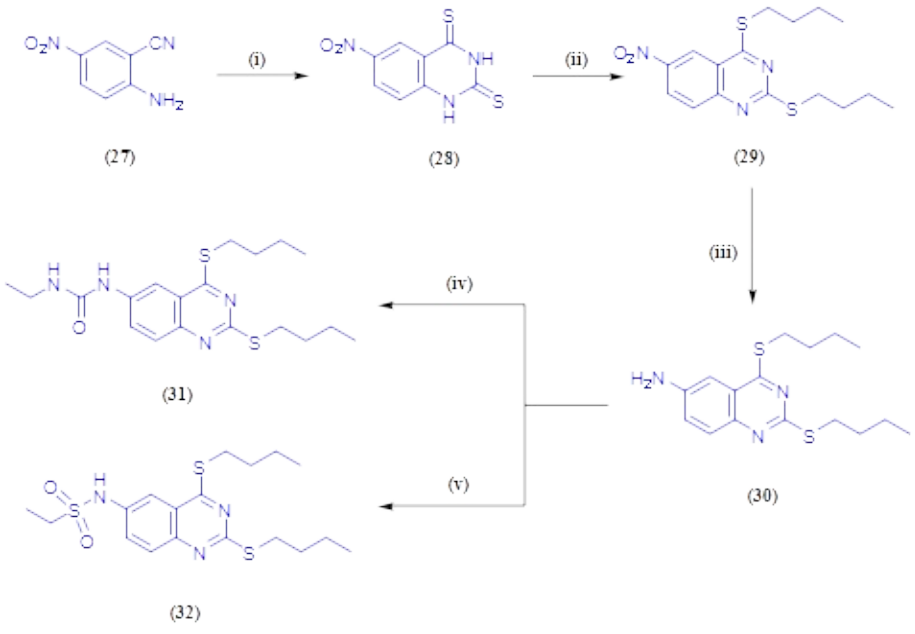
Scheme 3: Synthesis of compounds **18-20**. Reagents and reaction conditions: **(i)** Carbon disulfide, pyridine, 70 ºC, 6 h (**18**, yield: 73%) **(ii)** 1-Bromobutane, K_2CO_3, acetone, overnight reflux (**19**, yield: 88%) **(iii)** n-Butylamine, overnight reflux (**20**, yield: 89%)

Similarly, the reaction of 3-amino-1*H*-pyrazole-4-carbonitrile (**21**) with carbon disulfide yielded the pyrazolo[3,4-*d*]pyrimidine-4,6-dithione derivative **22**. Compound **22** was reacted with methyl iodide to afford **23a**. However, when compound **22** was reacted with n-butylbromide, a mixture of dibutyl and tributyl derivatives was obtained (**23b, 23c** and **24**) (Scheme 4). From the same reaction, two different trialkyl isomers were obtained. Both isomers were isolated and their structures were elucidated using 2D-HMBC and 2D-NOESY NMR experiments. In the HMBC spectrum, compound **23c** showed a clear cross peak between the α-CH_2 and C3, while compound **24** showed a clear cross peak between α-CH_2 and C7a (**Figure S1, Supplementary Information**). In the NOESY spectrum, compound **23c** showed a clear cross peak between the α-CH_2 and C3-H which was not seen in compound **24** (**Figure S2, Supplementary Information**). Compounds **23a-c** and **24** were reacted with n-butylamine and afforded compounds **25a-c** and **26**, respectively (Scheme 4).



Scheme 4: Synthesis of compounds **21**, **22**, **23a-c**, **24**, **25a-c** and **26**. Reagents and reaction conditions: **(i)** Carbon disulfide, pyridine, 70 ºC, 6 h (**22**, yield: 68%) **(ii)** Alkylhalide, K_2CO_3, acetone, overnight reflux **(iii)** n-Butylamine, overnight reflux (**25a-c** and **26**, yield: 85% - 93%)

In order to synthesize the 6-aminosubstituted-2,4-dibutylthioquinazolines, a reaction of 2-amino-5-nitrobenzonitrile (**27**) with carbon disulfide was performed to afford 6-nitroquinazoline-2,4(1*H*,3*H*)-dithione (**28**). This was followed by di-S alkylation by heating with 1-bromobutane in presence of K_2CO_3 to give compound **29**. The nitro-compound **29** was then reduced to its corresponding 6-aminoquinazoline derivative **30**. Reacting compound **30** with ethyl isocyanate and ethanesulfonylchloride yielded the ethyl urea derivative (**31**) and the ethanesulfonamide derivative (**32**), respectively (**Scheme 5**).



Scheme 5: Synthesis of compounds **28-32**. Reagents and reaction conditions: **(i)** Carbon disulfide, pyridine, 70 ºC, 8 h, (**28**, yield: 75%) **(ii)** 1-bromobutane, acetone, K_2CO_3, overnight reflux, **(iii)** Fe powder, ethanol:water (2:1), conc. HCl, stirring at RT for 5 h then reflux for 1 h at 65 ºC, (**30**, yield: 70%) **(iv)** ethyl isocyanate, DMF, stirring 2 h, (**31**, yield: 73%) **(v)** ethanesulfonylchloride, pyridine, stirring 2 h (**32**, yield: 88%)

In order to study the general chemical stability of the synthesized compounds, three representative compounds (**4a**, **7** and **25b**) were selected and tested in both solid and soluble forms. The compounds were dissolved in DMSO and incubated for two days at room temperature, to test the stability in solution. The three compounds were also exposed in solid state to γ-irradiation at a single absorbed dose of 25 kGy.[46-49] The results revealed no change in the physico-chemical properties, including the color, form and solubility. No change in the R_f values of the tested compounds and no additional spots were observed. In addition, LC-MS experiments revealed no change in the mass nor in the purity of any of the tested compounds after their incubation in DMSO or even after irradiation.

All synthesized target compounds were tested for their *in vitro* antibacterial activity against ten strains. The bacterial strains included the Gram-positive bacteria *S. aureus* Newman strain (methicillin-sensitive *S. aureus*, MSSA), two methicillin-resistant *S. aureus* strains (N315 and Mu50; MRSA) with *S. aureus* Mu50 displaying additionally increased tolerance to vancomycin (vancomycin-intermediate *S. aureus*, VISA), *S. pneumoniae* DSM-20566, penicillin-resistant *S. pneumoniae* (PRSP) DSM-11865, *E. faecium* DSM-20477, and *E. faecalis* DSM-20478. The compounds were also evaluated for their antibacterial activity against the Gram-negative bacterium *E. coli* (DSM-11116 wild type strain and an *E. coli* K12 TolC-deficient mutant strain), as well as *M. smegmatis* mc^2155. The screening results are presented in **Table 1** (Table S1 in **Supplementary Information** is showing the data in µM concentration unit).

Table 1: MIC values of the synthesized analogs.

Compound*	**Minimum Inhibitory Concentration**[a] (µg/mL)									
	S. aureus			***S. pneumoniae***		***E. faecium*** DSM-20477	***E. faecalis*** DSM-20478	***E. coli***		***M. smegmatis*** mc^2155
	Newman	N315 (MRSA)	Mu50 (MRSA/ VISA)	DSM-20566	DSM-11865 (PRSP)			DSM-1116	K12 Δ*tolC*	
9	8	8	8	4	16	8	8	>128	>128	>128
12	2	>128	>128	1	16	4	2	>128	>128	16
20	8	32	>128	8	8	>128	>128	>128	8	16
25a	64	64	64	>128	>128	>128	>128	>128	64	>128
25b	>128	>128	>128	>128	>128	>128	>128	>128	4	>128
26	>128	>128	>128	8	>128	>128	>128	>128	>128	>128
30	8	4	4	4	8	8	32	>128	>128	32
32	>128	>128	>128	4	4	8	8	>128	>128	>128
I	8	8	4	4	4	4	4	>128	2	8
Vancomycin	0.5	1	8	0.5	1	1	0.5	> 64	> 64	8
Linezolid	1	2	2	0.5	2	4	2	> 64	16	4

[a] Values are from two independent experiments.
* Compounds **4a-4f**, **5-8**, **10**, **11**, **13-16**, **25c**, **31** had MIC values higher than 128 µg/mL against all tested strains.

In order to further explore and optimize the hit compound **I** while maintaining a safe cytotoxicity profile, several modifications were adopted. These modifications included variable substitutions at positions 6 or 7 with either halogens or further extending with aryl or hetero aryl systems. Besides, replacing the benzenoid part of the quinazoline with other heterocycles.

The insertion of halogens (either fluorine, chlorine, or bromine) at position 6 or 7 on the quinazoline scaffold of compound **I** (**4a-f**) diminished the antibacterial activity. The electron-withdrawing effect of the halogens was detrimental since all compounds exhibited MIC values higher than 128 µg/mL against the tested strains.

The second group of compounds were those substituted at positions 6 or 7 with aryl or heteroaryl rings (**5-16)**. Most of the selected aryl/heteroaryl rings had polar features to minimize the gain in lipophilicity and associated solubility problems. The plain phenyl at position 6 or 7 (compounds **7** and **5**, respectively) abolished the antibacterial activity. Next, we tried to introduce a carboxylic acid group at *meta*- and *para*- position of the phenyl ring (compounds **8** and **9**, respectively). Although the *p*-COOH derivative (**8**) was completely inactive, shifting of the -COOH to the *meta* position (**9**) dramatically revived the activity against the tested Gram-positive bacteria to give one of the most potent compounds in the present series. Compound **9** showed remarkable antibacterial activity against all tested Gram-positive bacteria, including the drug-resistant *S. aureus* and *S. pneumoniae* strains, with MIC values ranging from 4-16 µg/mL. However, compared to the initial frontrunner compound **I**, compound **9** did not show activity against *M. smegmatis* mc^2155. Replacing the *m*-COOH with the less acidic phenolic OH (compound **12)** slightly enhanced the potency in comparison with compound **9**, with compound **12** displaying MIC values of 1-4 µg/mL against *S. aureus*, *S. pneumonia*, and *E. faecalis*, and it also showed activity against *M. smegmatis* (MIC 16 µg/mL). Unfortunately, although being active against drug-sensitive strains and PRSP, **12** was inactive against the MRSA/VISA strains. Neither the alcoholic OH (compound **10**) nor the cyano function (compound **11**) succeeded to replace the phenolic group while maintaining the potency. Moreover, using the *meta*-methoxy phenyl (compounds **13**) was also not beneficial. Replacing the phenyl in compound **7** with the 3- or 4-pyridyl (**14** and **15,** respectively) or 4-pyrazolyl (**16**) failed to regain the high potency of compound **I**. In conclusion, the acidic function (*m*-COOH and *m*-OH) in the 6-aryl extension on the quinazoline ring seemed a strict requirement for high potency in this cluster of compounds.

Replacing the benzenoid part of the quinazoline compound **I** with a thiophene gave the thieno[2,3-*d*]pyrimidine analogue **20**, which displayed good antibacterial activity against *S. aureus* Newman strain, *S. pneumoniae* and the efflux-deficient *E. coli* strain with MIC in the one-digit µg/mL range. However, activity against *Enterococcus* spp. and VISA was completely abolished.

The benzenoid part was also replaced with the bioisostere pyrazole represented in compounds **25a-c** and **26**. This modification in general did not seem to be beneficial, with compounds **25a**, **25b** and **26** showing only moderate antibacterial activity against selected strains of the test panel, while **25c** with n-butyl substitution at position 2 was inactive against all tested strains. Shortening the S-butyl of **25b** into methyl as in compound **25a** decreased the antibacterial activity against *E. coli* TolC (MIC = 64 µg/mL for **25a** vs. 4 µg/mL for **25b**), while marginally improved the antibacterial activity against the three strains of *S. aureus* (MIC = 64 µg/mL for **25a** and >128 µg/mL for **25b**), including the drug-resistant strains. Moving the n-butyl chain to position 1 (**26**) led to a virtually inactive compound but **26** unexpectedly inhibited the growth of *S. pneumoniae* with an MIC of 8 µg/mL.

To further explore the antibacterial activity of compound **II**, we investigated the effect of adding some polar functional groups like amino, sulfonamide and urea at position 6 of the quinazoline nucleus. The presence of an amino group at position 6 (**30**) gave one of the most active compounds in this study with similar potency to compound **I,** but it lacked activity against *E. coli* K12 Δ*tolC*. Compound **30** showed a broad antibacterial activity against the tested Gram-positive bacteria, including the drug-resistant strains, as well as *M. smegmatis* (MIC values ranging from 4-32 µg/mL).

Replacing the amino group in **30** with ethylsulfamoyl moiety (**32**), resulted in an analog with potent narrow-spectrum activity against *S. pneumoniae* (MIC 4 µg/mL) and *Enterococcus* spp. (MIC 8 µg/mL), while replacing the 6-amino group in **30** with an ethylureido residue (**31**) diminished the antibacterial activity.

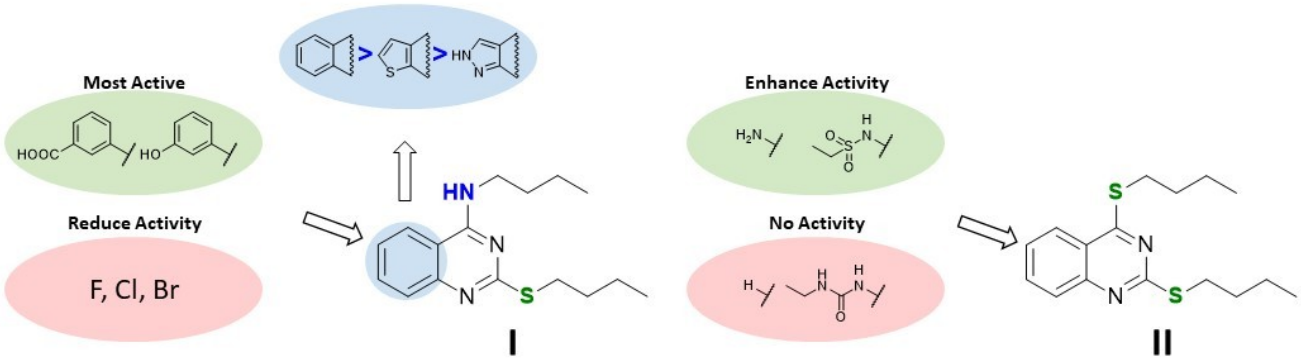


Figure 3: SAR summary

Cytotoxicity of the most potent compounds (**9**, **12**, **20**, **30** and **32**) was determined using human HepG2 hepatocellular carcinoma cells (Table 2). Compounds **9**, **20**, **30** and **32** did not show any cytotoxic activity in the tested concentration range (half-inhibitory concentrations, IC_{50} > 37 µg/mL). Only compound **12** showed moderate cytotoxicity towards HepG2 cells with IC_{50} of 15.3 µg/mL. However, the IC_{50} of compound **12** is still about 3-15 folds higher than its MICs on *S. aureus, S. pneumoniae, E. faecium and E. faecalis* (MICs = 2, 1, 4 and 2 µg/mL, respectively). These results indicate that the compounds possess promising and selective antibacterial activity.

Table 2: Growth inhibitory activity against HepG2 cells

Compound no.	HepG2 cells IC_{50} (µg/mL)*
9	>37
12	15.3
20	>37
30	>37
32	>37

*Results are from two independent experiments and IC_{50} is given as average value (SD <10%).

In conclusion, we report the synthesis of novel *N*-butyl-2-(butylthio)-halogenated quinazolin-4-amine derivatives (**4a-f**), *N*-butyl-2-(butylthio)-arylquinazolin-4-amine derivatives (**5-16**), *N*-butyl-2-(butylthio)thieno[2,3-*d*]pyrimidin-4-amine (**20**), 4,6-disubstituted-pyrazolo[3,4-*d*]pyrimidines (**25a-c** and **26**) and 2,4-bis(butylthio)quinazolin-6-amine derivatives (**30**-**32**). All synthesized target compounds were evaluated for their *in vitro* antibacterial activity against both Gram-negative and Gram-positive bacteria, in addition to *M. smegmatis*. The tested panel of bacteria included drug-resistant strains (MRSA, VISA, PRSP). Among the synthesized compounds, compounds **9**, **12**, **20**, **30** and **32** were the most active compounds against Gram-positive pathogens, with MIC values ranging from 1-16 µg/mL. Compound **12** also showed enhanced potency against *S. aureus Newman*, *S. pneumoniae DSM-20566* and *E. faecalis DSM-20478.* According to the SAR of the synthesized compounds, it was found that the presence of an acidic functional group on a phenyl ring at position 6 of the quinazoline ring is beneficial for the antibacterial activity. In addition, replacing the benzenoid part of the quinazoline ring with thiophene or pyrazole rings led to the discovery of new potential antibacterial

thieno[2,3-*d*]pyrimidine and pyrazolo[3,4-*d*]pyrimidine analogs with the thieno[2,3-*d*]pyrimidine being more potent. It was also found that the insertion of a (substituted-)amino group at position 6 of the inactive 2,4-di-thiobutyl quinazoline could help to regain antibacterial activity of the analogs (**30** and **32**), Interestingly, the quinazoline derivatives **9**, **30** and **32** and the thieno[2,3-*d*]pyrimidine derivative **20** showed no cytotoxicity in HepG2 cells. Only compound **12** displayed moderate cytotoxicity at a concentration higher than its MICs against four of the tested bacterial strains. Overall, the applied modification approaches, molecule growing and scaffold replacement were successful to produce novel promising antibacterial quinazoline analogs with a favorable selectivity index.

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