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Compounds Containing 2,3-Bis(phenylamino) Quinoxaline Exhibit Activity Against Methicillin-Resistant *Staphylococcus aureus*, *Enterococcus faecalis*, and Their Biofilms

Gilda Padalino[1,2] | Katrina Duggan[3] | Luis A. J. Mur[2] | Jean-Yves Maillard[3] | Andrea Brancale[4] | Karl F. Hoffmann[2]

[1]Swansea University Medical School, Swansea, UK | [2]Department of Life Sciences (DLS), Aberystwyth University, Aberystwyth, UK | [3]School of Pharmacy and Pharmaceutical Sciences, Cardiff University, Cardiff, UK | [4]Department of Organic Chemistry, UCT Prague, Prague, Czech Republic

Correspondence: Gilda Padalino (gilda.padalino@swansea.ac.uk)

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ABSTRACT

Antimicrobial resistance remains a global issue, hindering the control of bacterial infections. This study examined the antimicrobial properties of 2,3-N,N-diphenyl quinoxaline derivatives against Gram-positive, Gram-negative, and Mycobacterium species. Two quinoxaline derivatives (compounds 25 and 31) exhibited significant activity against most strains of *Staphylococcus aureus*, *Enterococcus faecium*, and *Enterococcus faecalis* tested, with MIC values ranging from 0.25 to 1 mg/L. These compounds also showed effective antibacterial activity against methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *E. faecium*/*E. faecalis* (VRE) strains. They demonstrated comparable or superior activity to four current antibiotics (vancomycin, teicoplanin, daptomycin, and linezolid) against a wide range of clinically relevant isolates. Additionally, they were more effective in preventing *S. aureus* and *E. faecalis* biofilm formation compared to several other antibiotics. In summary, these two quinoxaline derivatives have potential as new antibacterial agents.

Introduction

Antimicrobial resistance (AMR) leads to treatment failure, increased mortality and morbidity as well as spiralling costs for healthcare providers and governments (Salam et al. 2023). With an estimated 10 million deaths attributable to AMR by 2050, this public health threat remains a worldwide concern (O'Neill 2016). Recently, an in-depth evaluation from Murray and colleagues showed that 4.95 million deaths were associated with drug-resistant infections globally in 2019 (Murray et al. 2022). Among the potential interventions to effectively combat the rise of AMR are those that involve the identification and development of novel antimicrobials (Courtenay et al. 2019). Novel antimicrobials can help combat the rising threat of antibiotic-resistant bacteria, ensure effective treatment options for bacterial infections exist and, more broadly, support diverse public health initiatives. However, the gap in innovation for developing new antibacterials stems from a combination of scientific, economic and regulatory challenges (Hegemann et al. 2023). Addressing this gap requires a multifaceted approach that involves stakeholders working across healthcare and academic sectors; an example of such an approach is highlighted in this study.

Heterocyclic structures, contained within natural or synthetic products, are increasingly being used as components of new therapeutics. Amongst these, the quinoxaline core represents an important scaffold associated with many biologically—and

pharmacologically—active properties useful for treating both non-communicable diseases and infectious agents (Ajani 2014; Pereira et al. 2015). For example, quinoxaline derivatives possess potent antiparasitic activities against *Leishmania* (Silva de Jesus Passaes et al. 2023), *Trypanosoma* (Rodrigues et al. 2014), *Plasmodium* (Kümpornsin et al. 2023) and *Schistosoma* (Ingram-Sieber et al. 2014) species.

During our search for broadly-active anthelmintics to control the neglected tropical disease Schistosomiasis, we recently designed, synthesised and evaluated a small library of quinoxaline analogues against *Schistosoma mansoni*, *Schistosoma japonicum* and *Schistosoma haematobium* (Padalino et al. 2021). While these compounds demonstrated anti-schistosomal potencies at nanomolar concentrations, they also displayed structural similarities to previously-described, antibacterial, quinoxaline-containing compounds (El-Atawy et al. 2019; Keri et al. 2018; Montana et al. 2021).

With the increasing concerns around the emergence of resistance (Yadav and Kapley 2021; Chait, Vetsigian, and Kishony 2012) and tolerance (Brauner et al. 2016; Kester and Fortune 2014) to currently used antibiotics, we decided to further investigate the broader antibacterial potential of this family of quinoxaline-containing compounds. Here, we first tested a small number of 2,3-*N*,*N*-diphenyl quinoxaline derivatives against a wide panel of bacterial strains (Gram-positive bacteria, Gram-negative bacteria and *Mycobacterium smegmatis*) to gather preliminary information about the structure–activity relationship (SAR). We subsequently progressed more detailed antibacterial screens with selected compounds against defined strains of clinical relevance, particularly against antibiotic-resistant isolates.

We used both a standard microdilution broth assay to determine minimal inhibitory concentrations (MIC) and a biofilm test to measure minimal biofilm eradication concentrations (MBEC). Indeed bacterial biofilms are less susceptible to antibiotics (Zhao, Sun, and Liu 2023a) and are clinically more relevant (Zhao, Sun, and Liu 2023a; Donlan 2001).

By doing so, our results demonstrate the relevance of two of our synthesised quinoxaline derivatives (compound **25** and compound **31**) against a range of bacteria, warranting further investigations.

Methods

The Workflow of Antibacterial Investigations Described in This Study

The present study was performed in four phases. In Phase 1, a library of 15 compounds (13 synthesised compounds and the 2 initial building blocks) was initially tested against nine bacterial strains (see the full list in Table S1). In Phase 2, only the six most promising compounds derived from Phase 1 were investigated against 18 bacterial species originating from clinical settings. In Phase 3, the antibacterial activities of the two most active compounds (derived from Phase 2) were next explored against a further 41 clinical strains. In Phase 4, the effects of pre-exposure to subminimum inhibitory concentrations (MIC) were evaluated for the two most active compounds (derived from Phase 2).

The organisms used in this study (full list included in Table S1) were obtained from hospital Laboratories (e.g., SACU_Bead_No 23306, available from the Specialist Antibacterial Chemotherapy Unit - Public Health Wales, Cardiff), the American Type Culture Collection (e.g., ATCC 700699, 12301 Parklawn Drive, Rockville, MD 20852, USA) and the National Collection of Type Cultures (e.g., NCTC 12201, Central Public Health Laboratory, 61 Colindale Avenue, London NW9 5HT).

Phase 1: Compound Preparation

A library of 15 compounds (13 synthesised compounds and the two initial building blocks, described in Padalino et al. (Padalino et al. 2021), was prepared in 100% methanol (MeOH) at 2.5 mg/mL final concentration. A full list of compounds can be found in Table S1.

Phase 1: Bacterial Growth Conditions

All procedures were carried out in a biosafety level (BSL) 2 cabinet. A fresh subculture of each of the nine bacterial species (full list in Table S1) was prepared by streaking onto a fresh agar plate and incubating at 37°C for 24 h. The agar plates were prepared with high salt Lysogeny Broth medium (HSLB, 4 g), agar (2 g) and water (200 mL) for all strains except *Mycobacterium smegmatis* $mc^2$15, which required supplementation of the solid growth medium with 0.2% v/v glycerol and 0.05% v/v Tween-80. Bacteria were stored on agar plates at 4°C until needed and replaced weekly.

Before use, a single colony of each bacterium was removed from the agar plates using a sterile loop and inoculated in a fresh growth medium. Lysogeny broth (LB) medium was used for all strains except *M. smegmatis* $mc^2$15, which required supplementation with 0.2% (v/v) glycerol and 0.05% (v/v) Tween-80. Cultures were incubated for 24 h (or 48 h for *M. smegmatis*) at 37°C with aeration at 200 rpm until they reached an OD_{600} between 0.8 and 1.0 (assessed using spectrophotometer BioTek Synergy 4). In the case of low OD_{600}, the cultures were left to incubate further; if the OD_{600} was higher than 1.0, then a dilution was performed. Once optimal OD_{600} were reached, each bacterial culture was diluted with LB medium to approximately 1.0×10^5 CFU/mL.

Phase 1: Determination of in Vitro Antibacterial Activity Against Bacteria Isolates

The MIC was determined using the broth microdilution method in a 96-well plate containing fresh LB medium except for *M. smegmatis*, which was supplemented with 0.05% Tween 80% and 0.2% glycerol (Wiegand, Hilpert, and Hancock 2008a; Bhowmick et al. 2022; Baptista et al. 2018). A full list of bacteria isolates is included in Table S1.

A primary screen was carried out at both 125.0 and 62.5 mg/L to keep the MeOH content below 10% v/v. A secondary, dose–response titration (125.00, 62.50, 31.25, 15.63, 7.81,

3.91 mg/L or even lower concentrations when appropriate) was performed only for compounds able to inhibit the visible growth of bacteria in the primary screen. In each assay, all compounds were tested in triplicate against the nine bacteria strains; both primary and dose–response assays were performed twice.

The OD_{600} was measured at the beginning (initial reading) and at the end (after incubation at 37°C for 24 or 72 h for *M. smegmatis*l—final readings) of dose–response titration. Those readings were compared to calculate the MIC (as mg/L), defined as the lowest concentration of compound that inhibits 90% of the growth of the organism studied.

Phase 2 and 3: Determination of in Vitro Antibacterial Activity Against Clinically Relevant Bacteria Strains

A sub-selection of compounds (**2Cl-Q**, **22 f**, **25**, **31**, **32** and **35**) was selected for Phase 2 of the study (Table S2). Here, the broth microdilution assay was performed in Mueller-Hinton broth (MHB) according to the ISO-20766 international standard (Standardization IOf 2019) and clinical significance of MIC was interpreted using the current EUCAST breakpoints (https://www.eucast.org/clinical_breakpoints/).

Each compound was prepared in dimethyl sulfoxide (DMSO instead of MeOH used in Phase 1) and then diluted in water to create stock solutions at lower concentrations (0.008 to 128 mg/L). For some bacteria (*S. pneumoniae*, *H. influenzae* and *Neisseria* species), the MHB was supplemented with 5% lysed horse blood and nicotinamide adenine dinucleotide (β-NAD).

The MIC values (expressed in mg/L) were determined as the lowest concentration that, under defined in vitro conditions (incubation at 34°C to 37°C), prevented visible growth of bacteria within a defined period (for 18–24 h).

During Phase 3, four antibiotics (vancomycin, teicoplanin, linezolid and daptomycin) with known activity against Gram-positive agents were used for activity comparison (Table S3A). The MIC values (expressed as mg/L) of each known antibiotic were interpreted using the current EUCAST breakpoints (Standardization IOf 2019) (https://www.eucast.org/clinical_breakpoints/ l—summarised in Table S3B).

Phase 4

In this final stage of the study, only the two most active compounds (**25** and **31**) were further investigated to determine potential bacterial emerging resistance following exposure, their minimum biocidal concentrations (MBCs) and their minimum biofilm eradication concentrations (MBECs).

Phase 4: Determination of MIC Following Pre-Exposure to Quinoxaline Analogues 25 and 31

Fresh stocks of compounds **25** and **31** were resuspended in 1 mL DMSO and further diluted to 512 mg/L in deionised water (final DMSO concentration below 1% v/v). *S. aureus* ATCC 29213, *E. faecalis* ATCC 29212 (vancomycin sensitive) and *E. faecalis* NCTC 12201 (vancomycin-resistant) (see details in Table S1) were stored in cryopreservation beads (Fisher Scientific, Loughborough, UK) at −80°C and sub-cultured onto tryptone soya agar (TSA) for a maximum of two subcultures before use.

To initiate liquid cultures of each strain for repeat MIC determinations, MHB supplemented with cations to a final concentration of 20 mg/L $CaCl_2$ and 10 mg/L $MgCl_2$ was inoculated with two to three bacterial colonies and incubated at 37°C for 16–24 h. Suspensions were centrifuged at 3000xg for 20 min to pellet the bacteria; the pellets were resuspended in fresh cation-adjusted MHB to reach a cell density between 1.5 and 5×10^6 CFU—Colony Forming Units/mL.

Following the ISO-20766 international standard (Standardization IOf 2019), assays were initiated in 96-well microtiter plates where descending twofold dilutions of compounds (quinoxaline analogues or antibiotic controls) were included in a final volume of 50 µL; negative control wells remained compound-free but contained 50 µL of MHB instead. Aliquots of adjusted bacterial inoculum (50 µL) were added to each well except for the negative control wells where 50 µL of MHB was added instead, giving a final cell concentration of approximately 5×10^5 CFU/mL (in 0.8% v/v DMSO, the highest concentration used). The plates were incubated at 37°C for 16–24 h. The lowest concentration of compound that inhibited cell growth was determined by visual inspection and recorded as the MIC (mg/L). The experiments were conducted in triplicate and the most frequently occurring MIC was recorded.

To determine MIC following compound pre-exposure, bacterial cultures were exposed to compounds **25** and **31** (at half of the MIC concentration, determined above) for a period of 16–24 h at 37°C in total volumes of 10 ml in 50 mL Falcon™ centrifuge tubes (Fisher Scientific, UK), using the cell densities and culture conditions described in the MIC protocol. Bacterial cultures were incubated with agitation at 200 rpm. Following incubation, the cultures were centrifuged at 3,000 x g and the pellets were resuspended in neutraliser solution (Lecithin 10 g/L, Tween80 30 g/L, Sodium Thiosulphate 20 g/L, L-Histidine 1 g/L, Saponin 30 g/L, Sodium Dodecyl Sulphate 5 g/L in deionised water) and vortexed. The suspensions were re-centrifuged, the cell pellets resuspended in tryptone sodium chloride (TSC) and the cell densities adjusted to $1.5–5 \times 10^6$ CFU/mL.

MIC determination of compounds **25** and **31** were subsequently determined as described above using the cultures that had been pre-exposed to sub-MIC levels of the test compounds.

In addition, a selection of known antibiotics (ampicillin, imipenem, vancomycin, levofloxacin, ciprofloxacin, trimethoprim/sulfamethoxazole, cefotixin, gentamicin, erythromycin, tetracycline, rifampicin and benzylpenicillin) was diluted and prepared as described in BS EN ISO20776-1:2020 (British Standards Institution 2020). Antibiotic-loaded 6 mm paper discs (Oxoid, Basingstoke, UK) were applied to the surfaces of the agar plates seeded with each of the three bacteria strains (stated above) before (control) and after pre-exposure to test compounds **25** and **31**. Plates were incubated for 18 ± 2 h at 37°C. Zones of inhibition were measured using a calliper

and recorded. The antibiotic susceptibility profiles of the three bacteria strains before and after pre-exposure to the quinoxaline derivatives to the known antibiotics selection were compared to the current EUCAST breakpoints (https://www.eucast.org/clinical_breakpoints/) (Ekkelenkamp et al. 2022).

Phase 4: Determination of Minimum Biocidal Concentration (MBC)

Following MIC determination (described in the section above), the entire contents of the wells corresponding to the MIC level and all higher concentrations for which no visible growth was observed, were removed by pipette and plated onto TSA plates. The plates were incubated at 37°C for 16–24 h. The MBC was recorded as the lowest concentration of test compounds for which there were no colonies.

Phase 4: Determination of Minimum Biofilm Eradication Concentration (MBEC)

The standard ASTM E2799 (International A. 2012) assay was used to determine the MBEC of compounds **25** and **31** as well as several antibiotics (vancomycin, rifampicin, linezolid, teicoplanin and sparfloxacin). Briefly, bacterial cultures (1.5×10^5 CFU/mL) were added to 96-well Calgary biofilm devices (Innovotech, Canada); plastic lids containing 96 pegs were subsequently added to each 96-well base. The entire Calgary device was incubated at 37°C for 16–24 h in an orbital shaker to allow the initial biofilm establishment on a surface. The biofilm-containing pegs were rinsed in TSC to remove planktonic cells and transferred to a challenge plate containing test compounds **25** and **31** as well as five selected antibiotics serially diluted across the plate. Following another 24 h incubation, the pegs were placed in neutralising broth (Lecithin 10 g/L, Tween-80 30 g/L, Sodium Thiosulphate 20 g/L, L-Histidine 1 g/L, Saponin 30 g/L, Sodium Dodecyl Sulphate 5 g/L in deionised water) for 10 min and then transferred to a recovery plate containing 100 μL of sterile tryptone soya broth (TSB). An effectiveness test of the neutralising broth was performed in accordance with ASTM E2799 to validate the use of the neutralising broth. The recovery plates were placed in a sonicating water bath for 30 ± 5 min to disaggregate the biofilms. The lids containing the pegs were discarded and replaced with standard lids. The plates were incubated at 37°C for 16–24 h to allow biofilm growth and the MBECs were determined qualitatively by recording the lowest concentration of antibiotic/compound that prevented cell growth (i.e., absence of turbidity).

Evaluation of Cell Morphology Following Exposure to Test Compounds

Bacterial cultures (*S. aureus* ATCC 29213 and *E. faecalis* NCTC 12201) were grown for 16–24 h at 37°C in cation-adjusted MHB and resuspended in TSC to approximately 1×10^9 CFU/mL.

A suspension of bacteria (final concentration 5×10^5 CFU/mL) and each compound (**25** and **31**) was prepared at a concentration double the MBC of each test compound.

Untreated control cultures were initiated using TSC only. Following incubation at 37°C for 16–24 h, the suspensions were centrifuged at 3000 x *g* and bacteria pellets were fixed by incubation with 2.5% glutaraldehyde for 2 h at 21°C. Following another centrifugation step, the cell pellets were washed in an ascending series of ethanol concentrations (from 10% to 100%), with 5 min incubation at each step and centrifugation between each wash. The entire 2 mL volume of the final suspension in 100% ethanol was filtered through a 0.2 μm polycarbonate membrane (Whatman™, Cytiva, UK) using a manifold system. The filtered membranes were transferred to Petri dishes and placed in a bell jar overnight to remove residual moisture.

Membranes were subsequently fixed to aluminium stubs using carbon adhesive tabs (Fisher Scientific, Loughborough, UK) and sputter-coated with 20 nm gold/palladium (Au/Pd). Scanning electron microscopy (SEM) images were acquired using a beam energy of 5 kV and an in-lens detector on a Sigma HD field gun Scanning Electron Microscope (Carl Zeiss Ltd, UK). Three representative fields of view were captured for each treatment at magnifications between 10,000 and 50,000.

Results and Discussion

Determination of in Vitro Antibacterial Activity Against ATCC/NTCT Isolates (Phase 1)

We recently reported the identification of quinoxaline derivatives as part of a high-throughput *ex vivo* screening campaign to identify potent anti-schistosomal compounds (Padalino et al. 2021). Medicinal chemistry optimisation resulted in the generation of 5 *N*-aryl analogues (compounds **25**, **30-32** and **35**) and two *N*-phenyl-alkyl analogues (compounds **36-37**) created via a one-step reaction using 2,3-Dichloro-6-nitroquinoxaline as the starting material (referred here as 2Cl-Q—Figure 1). The 6-acyl derivatives were obtained from classical catalytic hydrogenation of the 6-nitro-substituted quinoxaline **22** into the amino derivative, before a final acylation to assemble the analogues **22c-22g** (Figure 1). Due to solubility restrictions, only 13 compounds (amongst the originally 21 synthesised in (Padalino et al. 2021)) were selected for antibacterial screening (compounds **25, 30-32**, **35-37, 22b-22g** as well as the 2,3-Dichloro-6-nitroquinoxaline (**2Cl-Q**) and its 6-nitroquinoxaline-2,3-diol derivative starting materials, Figure 1—adapted from (Padalino et al. 2021)).

The minimum inhibitory concentration (MIC) of this library was initially assessed by the broth microdilution method (Wiegand, Hilpert, and Hancock 2008a). Representative species derived from Gram-positive and Gram-negative strains as well as *M. smegmatis* were first screened with all compounds at two concentrations (125.00 and 62.50 mg/L). Compounds showing inhibition of bacterial growth at 62.50 mg/L were further tested in a secondary dose–response assay. From these screens, the minimal concentrations of 15 quinoxaline analogues that reduced visible growth were calculated for each bacteria strain (Table 1).

Of the six bacteria species tested, *Escherichia coli*, *Pseudomonas aeruginosa, Bacillus cereus* and *Staphylococcus epidermidis* were minimally affected (MIC > 62.5–125 mg/L) by the compounds. Two compounds (**31** and **37**) demonstrated some

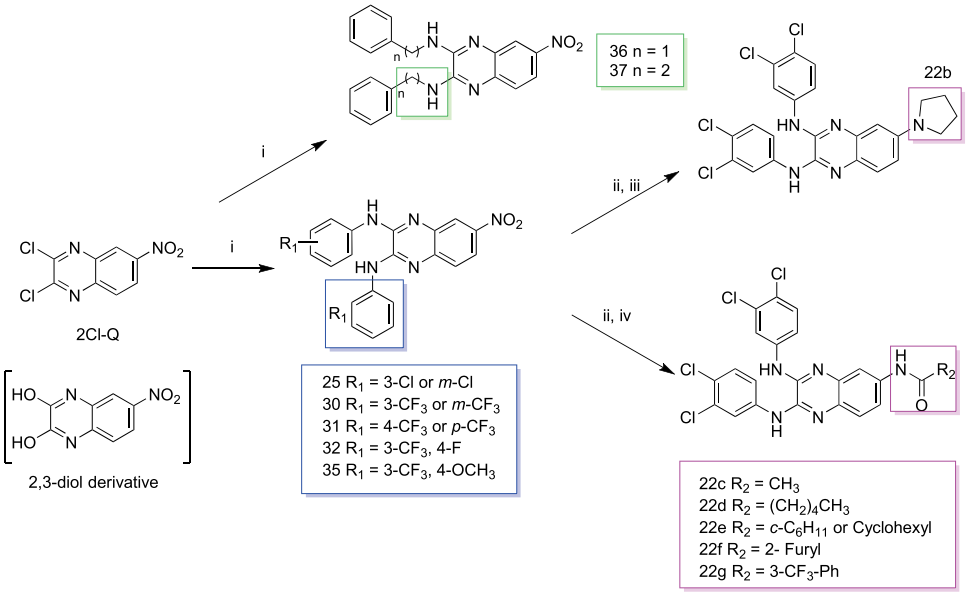


FIGURE 1 | Synthesis of the 2,3-bis(phenylamino)-quinoxaline series. The synthesised derivatives are grouped by structural similarity: the *N*-aryl analogues are highlighted with a blue box, the *N*-phenyl-alkyl analogues are contained in the green box and the 6-acyl derivatives are included in a magenta box. Reagents and conditions: (i) different substituted anilines (compounds **22**–**35**) or phenyl-alkyl amines (**36** and **37**), anhydrous DMSO, 130°C, 30 min; (ii) H_2, cat. Pd/C, AcOEt, rt, 2 h; (iii) $Br(CH_2)_2Br$, K_2CO_3, CH_3CN, MWI (300 W), 150°C, 15 min; (iv) R_2COCl, anhydrous Pyr, anhydrous DCM, 0°C → rt, 1 h. This figure is adapted from (Padalino et al. 2021) with particular focus only on the quinoxaline derivatives (Table S1A) and bacterial species (Table S1B) investigated in this study.

TABLE 1 | Minimum inhibitory concentrations (MICs, mg/L) of 15 quinoxaline derivatives.

	MIC (mg/L)					
	Gram-positive			Gram-negative		Mycobacteria[a]
Entry	*S. aureus*	*S. epidermidis*	*B. cereus*	*E. coli*	*P. aeruginosa*	*M. smegmatis* mc^{2}15
2,3-dichloro-6-nitroquinoxaline/2Cl-Q	< 3.91	< 62.50	< 62.50	62.50	125	1.95
6-nitroquinoxaline-2,3-diol	125	—	—	> 125	—	> 125
25	< 3.91	< 62.50	125	> 125	> 125	> 125
30	7.81	> 125	> 125	> 125	> 125	> 125
31	0.98	< 62.50	< 62.50	> 125	> 125	31.25
32	1.95	< 62.50	125	62.5	> 125	62.50
35	62.50	< 62.50	< 62.50	> 125	> 125	> 125
36	> 125	—	—	> 125	—	> 125
37	> 125	—	—	> 125	—	31.25
22b	15.63	> 125	> 125	> 125	> 125	> 125
22c	62.50	< 62.50	< 62.50	> 125	> 125	> 125
22 d	62.50	> 125	125	> 125	> 125	> 125
22e	> 125	—	—	> 125	—	125
22 f	31.25	125	< 62.50	> 125	> 125	> 125
22 g	> 125	—	—	> 125	—	125

Note: Strains used: *S. aureus* - ATCC 29213; *Staphylococcus epidermidis* - NTCT11077; *Bacillus cereus* - ATCC 14579; *Escherichia coli* - ATCC 25922; *Pseudomonas aeruginosa* ATCC 27853, *M. smegmatis mc^{2}15* ATCC 700084. Concentration tested: 125, 62.50, 31.25, 15.63, 7.81, 3.91, 1.95, 0.98 μg/mL; Green cells indicate MIC values of 31.25 mg/L or lower.

Cells containing the value '> 125' indicate that bacterial growth was still observed at the highest concentration tested (125 mg/L). Cells containing the value '< 62.5' or '< 3.91' indicate that there was no visible bacterial growth at that concentration (62.5 and 3.91 mg/L, respectively). A further titration (below 62.50 or 3.91 mg/L, respectively) would be needed to define the exact value of MIC. Cells containing '-' indicated that the MIC of that compound was not determined.

[a]48 h incubation with the drug. Other bacteria strains were incubated for 24 h.

antimycobacterial activity, with the initial building block (2,3-Dichloro-6-nitroquinoxaline) being the most active (MIC 1.95 mg/L). In contrast, *Staphylococcus aureus* showed the highest susceptibility to these compounds with **31** and **32** producing MICs of 0.98 and 1.95 mg/L, respectively. Only 5 of the 15 compounds tested (6-nitroquinoxaline-2,3-diol, **36**, **37**, **22e** and **22 g**) were not effective against this bacterium (MIC ≥ 125 mg/L). Compound **25** and 2,3-dichloro-6-nitroquinoxaline showed some activity with a MIC below 3.91 mg/L.

The antibacterial properties of 7 of the most active compounds against *S. aureus* prompted further studies on three methicillin-resistant *S. aureus* (MRSA) strains (Table 2).

Here, some compounds (e.g., 2,3-dichloro-6-nitroquinoxaline and compounds **30, 22b** and **22 g**) lost activity against some or all of the MRSA strains tested. Compounds **25**, **31** and **32** retained some activity on MRSA strains, but they were not as potent when compared to the *S. aureus* reference strain. Compound **35** was the only compound that showed an increased potency against the MRSA strains (15.53–31.25 mg/L compared to a MIC of 62.50 mg/L for the standard *S. aureus* strain).

Determination of in Vitro Antibacterial Activity Against Clinically Relevant Bacteria Strains (Phases 2 and 3)

Based on compounds **25**, **31**, **32**, **22 f**, **35** and 2,3-dichloro-6-nitroquinoxaline/**2Cl-Q**'s activities against MRSA strains, a wider antibacterial screen (Figure 2) was conducted on 19 clinically relevant bacterial species/strains (Table S2A).

Amongst the compounds tested, **2Cl-Q** showed minimal activities (MICs ≥ 8 mg/L) against the selected strains. Compound **22 f** displayed activities against *Neisseria gonorrhoeae* (ATCC 49226; MIC of 1 mg/l), one flucloxacillin-resistant *S. aureus* strain (*S. aureus* NCTC 12493; MIC = 1 mg/L) and two vancomycin-resistant *Enterococcus faecalis* (VRE) strains (NCTC 12201 - MIC = 0.250 mg/L and ATCC 51299 - MIC = 2 mg/L). Compounds **25**, **31**, **32** and **35** were active against *S. aureus* and *E. faecalis* wild-type strains. However, these compounds were even more active against the drug-resistant *S. aureus* and *E. faecalis* strains with compounds **25** and **31** generally being more active than the other two (Figure 2).

Exploring the wider panel of Gram-positive bacteria strains, compound **31** was the only compound with activity (MIC = 2 mg/L) against *Streptococcus pneumoniae* (ATCC 49619), the most common cause of community-acquired pneumonia and one of nine bacteria of international concern (World Health Organisation; WHO (Organization WH 2014)). This compound also retained its activity against the MLS-resistant *S. pneumoniae* strain (21395, MIC = 2 mg/L), but was less active against 18778 and 21394 (MIC = 4 mg/L).

Focusing on the Gram-negative species, compounds **25** and **31** were also the only two compounds active against *Neisseria* species and *Haemophilus influenzae*. In terms of *H. influenzae*, compound **25** demonstrated greater activity (compared to **31**) against both standard- (ATCC 49766, MIC = 4 and 16 mg/L for compounds **25**

TABLE 2 | Minimum inhibitory concentrations (MICs, mg/l) against three selected MRSA strains compared to a reference *S. aureus* strain.

	MIC (mg/L)			
		MRSA		
Entry	**Reference *S. aureus*[a] *ATCC 29213***	***USA 300***	***ATCC 33591***	***EM RSA***
2,3-dichloro-6-nitroquinoxaline/2Cl-Q	< 3.91	15.63	15.63	15.63
6-nitroquinoxaline-2,3-diol	125	—	—	—
25	< 3.91	3.91	3.91	3.91
30	7.81	> 125	> 125	> 125
31	0.98	< 3.91	< 3.91	3.91
32	1.95	< 3.91	< 3.91	7.81
35	62.50	15.63	31.25	31.25
36	> 125	—	—	—
37	> 125	—	—	—
22b	15.63	62.50	31.25	62.50
22c	62.50	—	—	—
22 d	62.50	—	—	—
22e	> 125	—	—	—
22 f	31.25	62.50	15.63	62.50
22 g	> 125	—	—	—

[a]MICs values against *S. aureus* reported here as a reference (from Table 1). Green cells indicate MIC values of 31.25 mg/l or lower. Those compounds were selected for further screening against MRSA strains. Strains used: standard *S. aureus* - *ATCC 29213*; Methicillin-resistant *S. aureus* (MRSA) isolates - *USA300, ATCC 33591, EM RSA*.

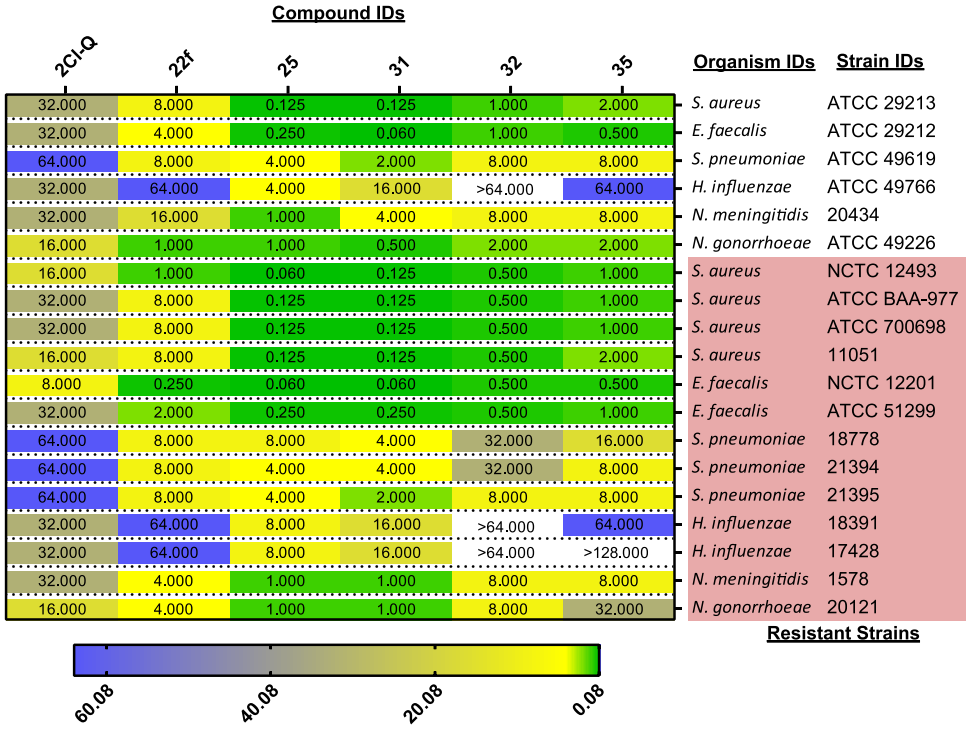


FIGURE 2 | Minimum Inhibitory Concentrations (MICs, mg/L) against 19 clinically relevant strains. More information about the strains can be found in Table S2A. MICs (expressed in mg/L) are indicated. A colour code was used based on the highest (64 mg/L – in blue) to the lowest value (0.06 mg/L – in green) contained within the data set (the coloured bar, in mg/l, is at the bottom of the picture). Relevant strains with resistance mechanisms are highlighted in pink – more details on the specific resistance and mechanism can be found in Table S2A. The complete antibacterial data set is available in Table S2B. 2Cl-Q = 2,3-dichloro-6-nitroquinoxaline.

TABLE 3 | Minimum inhibitory concentrations (MICs, mg/L) against drug-resistant *S. aureus* and *E. faecalis* strains.

				Compound IDs	
Organism ID	**Reference strain**	**Resistance**	**Mechanism**	**25 (MICs, mg/L)**	**31 (MICs, mg/L)**
E. faecalis	ATCC 29212	—	—	0.250	0.060
E. faecalis	NCTC 12201	Vancomycin	*vanA*	0.060	0.060
E. faecalis	ATCC 51299	Vancomycin	*vanB*	0.250	0.250
S. aureus	ATCC 29213	—	—	0.125	0.125
S. aureus	NCTC 12493	Flucloxacillin	*mecA*	0.060	0.125
S. aureus	ATCC BAA-977	ERY/CLIND	MLSB	0.125	0.125
S. aureus	ATCC 700698	Vancomycin	hVISA	0.125	0.125
S. aureus	11051	Tetracycline	—	0.125	0.125

and **31**, respectively) and resistant- strains (18391, 17428 with MIC = 8 and 16 mg/L for compounds **25** and **31**, respectively).

Compounds **31** and **25** showed the broadest and most potent antibacterial activities against all species/strains investigated, including the thirteen drug-resistant strains (Figure 2). Therefore, these two quinoxalines were selected for further investigations against additional drug-resistant strains of both *S. aureus* and *E. faecalis* (Table 3).

Both compounds were particularly active against vancomycin-resistant strains of *E. faecalis*, especially against the NCTC 12201 strain (MIC = 0.60 mg/L). They additionally demonstrated equivalent potencies against the flucloxacillin-, vancomycin-, tetracycline- and erythromycin/clindamycin-resistant *S. aureus* strains (Table 3).

Activity of Compounds 25 and 31 on Additional Clinically Relevant Bacterial Strains (Phase 3)

Based on the promising antibacterial effects of compounds **25** and **31** on representative Gram-positive bacteria of clinical importance, a more expansive panel of Gram-positive bacterial strains was next subjected to MIC investigations (Figure 3 and

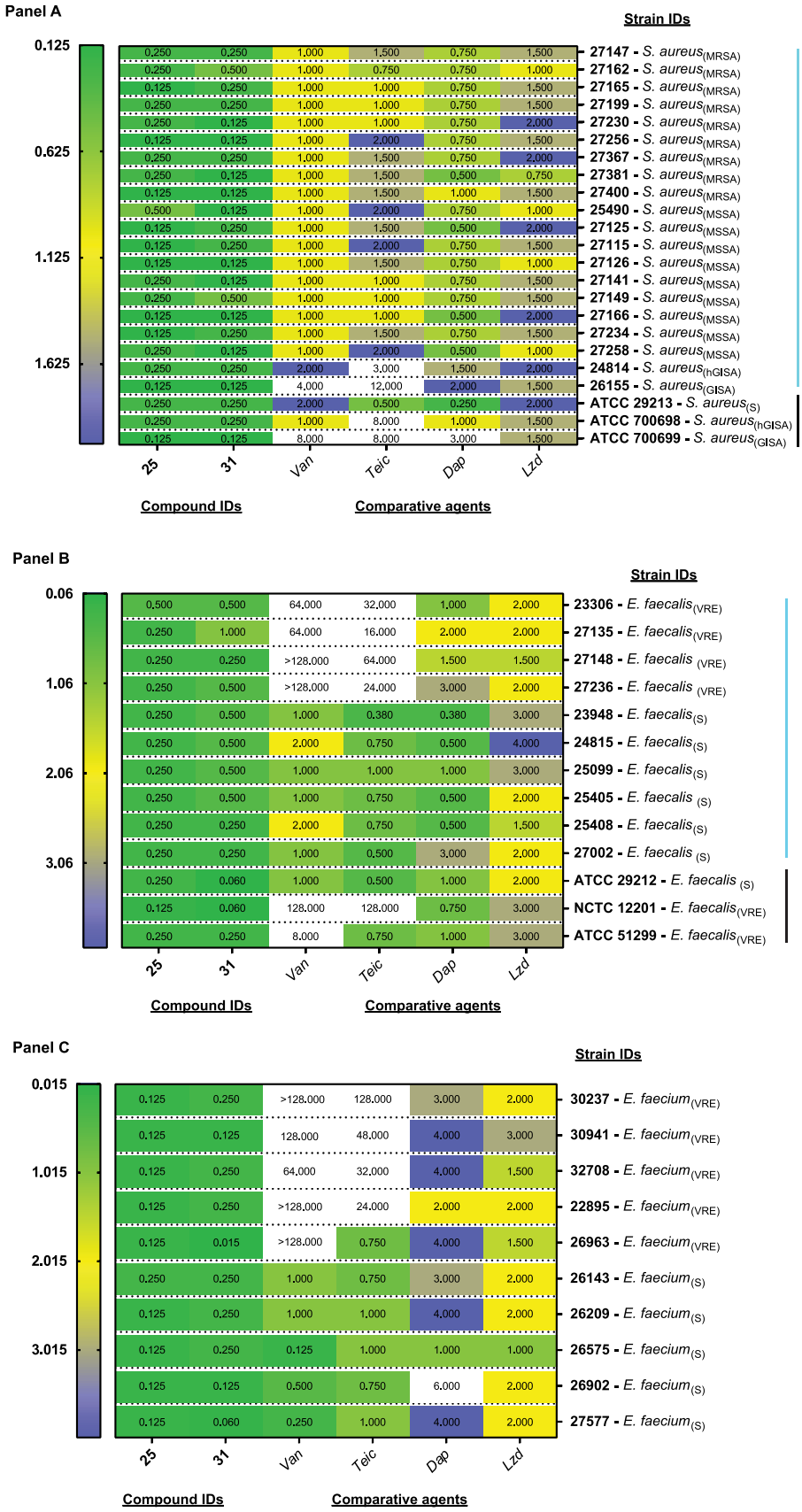


FIGURE 3 | Legend on next page.

Table S3). In addition to *S. aureus* (Figure 3A) and E. *faecalis* (Figure 3B), *Enterococcus faecium* (Figure 3C) was also included in these assays due to broader resistance and higher virulence than *E. faecalis* (Kramer et al. 2018).

Vancomycin, teicoplanin, linezolid and daptomycin MICs were first compared to the two quinoxaline analogues (Table S3A and Table S3B). Regarding *S. aureus*, three control strains (methicillin-sensitive *Staphylococcus aureus* strain ATCC 29213 - MSSA - and two MRSA strains ATCC 700698 and 700699) were evaluated along with 20 clinical isolates. Compounds **25** and **31** showed a good antibacterial profile across all *S. aureus* isolates under analysis with MICs ranging from 0.125 to 0.500 mg/L (Figure 3A).

A heterogeneous MIC spectrum for the four clinical antibiotics was identified for the *S. aureus* standard stains and clinical isolates with linezolid being the least potent. Both quinoxaline analogues demonstrated greater potencies than vancomycin, teicoplanin and linezolid with comparable activity to daptomycin in some cases. When looking at the GISA strains, the two test compounds outperformed the first-line treatment (vancomycin and teicoplanin—Figure 3A).

Compounds **25** and **31** were next tested against a panel of 13 *E. faecalis* strains (Figure 3B); these included three standard strains (sensitive strain ATCC 29212 and two vancomycin-resistant enterococci (VRE) strains (NTCC 12201 and ATCC 51299)) and ten clinically relevant strains (Table S3A). Both compounds **25** and **31** showed antibacterial activity against all *Enterococci* tested. While compound **25** appeared equally active across the strains under analysis, compound **31** had a much wider range of MICs from 0.060 mg/L against ATCC 29212 and NCTC 12201 to 1 mg/L against clinical isolate 27135.

Lastly, the antibacterial activities of compounds **25** and **31** were investigated against a selection of clinically relevant *E. faecium* strains (vancomycin-sensitive or resistant exemplars; Figure 3C). Both compounds had MIC values lower than 0.250 mg/mL against all strains examined (both sensitive and resistant strains of standard and clinical isolates of *E. faecium*). This represents a better antimicrobial profile over the second-line agents (daptomycin and linezolid) and, in some cases, an equal (or even better activity) when compared to first-line agents (vancomycin and teicoplanin).

Determination of MIC Following Pre-Exposure to Quinoxaline Analogues 25 and 31 (Phase 4)

To determine if pre-exposure to compounds **25** and **31** altered antimicrobial susceptibility, *S. aureus* and *E. faecalis* reference strains were exposed to sub-MIC levels (50% of the MIC for 16–24 h) of these quinoxaline analogues. MICs of pre-exposed (Spe) strains to controls without pre-exposure (Sc) were subsequently compared (Table 4). Following pre-exposure of bacteria to sub-MIC levels of compound **25**, MICs increased between two and eightfold. Pre-exposure of bacteria to sub-MIC levels of compound **31** increased MICs to between 64- and 256-fold.

Since pre-exposure of bacteria to the two quinoxaline analogues decreased their susceptibility, MICs of commonly used antibiotics (ampicillin, imipenem, vancomycin, levofloxacin, ciprofloxacin, trimethoprim/sulfamethoxazole, cefotixin, gentamicin, erythromycin, tetracycline, rifampicin and benzylpenicillin) were subsequently determined for *S. aureus* and *E. faecalis* with and without pre-exposure to compounds **25** and **31** (Table 5).

Pre-exposure of *S. aureus* ATTC 29213 or *E. faecalis* ATCC 29212 to sub-inhibitory concentrations of either compound did not alter these strains' susceptibility to any of the antibiotics tested. In contrast, pre-exposure of *E. faecalis* NCTC 12201 to compound **31** (but not compound **25**) led to vancomycin susceptibility with a zone of inhibition greater than the clinical breakpoint. Additionally, *E. faecalis* NCTC 12201 pre-exposed to a sub-inhibitory concentration of compound **31** (but not compound **25**) altered trimethoprim-susceptibility to a trimethoprim-resistant phenotype (Table 5).

Minimum Biocidal Concentration (MBC) Determination and Scanning Electron Microscopy (SEM) Analyses

To complement the extensive MIC testing of compounds **25** and **31**, minimum biocidal concentrations of these quinoxaline analogues were also determined for both *S. aureus* and *E. faecalis* (Table 6). Compound **25** demonstrated greater bactericidal activity against both *S. aureus* and *E. faecalis* when compared to compound **31** (Table 6).

SEM images of both *S. aureus* and *E. faecalis* exposed to double the MBC of compounds **25** and **31** revealed gross structural damage in comparison to untreated cells (Figure 4, Panels **A**, **B**, **G**, **H**). The morphology of *S. aureus* exposed to compound **25** changed from round, well-defined cells in the untreated cultures (**Panels A** and **B**) to dimpled and irregular cells (**Panels C** and **D**). The damage to *S. aureus* cells treated with compound **31** appeared even more pronounced, with greater levels of structural damage and possible loss of intracellular contents (**Panels E** and **F**). Similarly, *E. faecalis* exposed to compound

FIGURE 3 | Minimum Inhibitory Concentrations (MICs, mg/l) of two selected compounds and four comparative agents against a panel of *S. aureus* (Panel A), *E. faecalis* (Panel B) and *E. faecium* (Panel C). Panel A - All clinical strains of *S. aureus* (blue line), except for the final three standard *S. aureus* strains (ATCC 29213, ATCC 700698 and ATCC 700699, black line). MSSA = methicillin-sensitive *Staphylococcus aureus*; S = sensitive; GISA = glycopeptide-intermediate *S. aureus*; hGISA = hetero-GISA; Panel B - All clinical strains of *E. faecalis* (blue line), except for the final three ATCC/NTC strains (black line). VRE = vancomycin-resistant enterococci; S = sensitive short for MSSA - methicillin-sensitive *S. aureus*; Panel C - VRE = vancomycin-resistant enterococci; S = sensitive. Comparative agents: *Van* – Vancomycin; *Lzd* – Linezolid; *Teic* - Teicoplanin; *Dap* – Daptomycin. A colour code was used based on the highest (in blue) to the lowest MIC value (in green) of the data set (the coloured bar, mg/l, is included on the left-hand side of the picture). The highest range of the colour mapping was set to 2.00 (Panel A) or 4.00 (Panels B and C) for visualisation purposes.

TABLE 4 | Pre-exposure of *S. aureus* and *E. faecalis* to quinoxaline analogues decreases their sensitivities.

		MIC (mg/l)				Fold increase	
		Sc		Spe		Spe/Sc	
Organism ID	Ref. strain	Comp 25	Comp 31	Comp 25	Comp 31	Comp 25	Comp 31
S. aureus	ATCC 29213	0.250	0.125	2	8	8	64
E. faecalis	ATCC 29212	0.125	0.125	1	32	8	256
E. faecalis	NCTC 12201	0.125	0.063	0.250	4	2	63.5

Note: Values represent the mode of three independent experiments. Sc: strain without pre-exposure – control strain; Spe: strain with pre-exposure. Spe/Sc: ratio of compound MICs values in pre-exposed strain to control strain.

TABLE 5 | Pre-exposure of *S. aureus* and *E. faecalis* to compounds 25 and 31 and impact on cross resistance to clinically available antibiotics.

	S. aureus ATTC 29213			*E. faecalis* NCTC 12201			*E. faecalis* ATCC 29212		
	Sc	Spe_{25}	Spe_{31}	Sc	Spe_{25}	Spe_{31}	Sc	Spe_{25}	Spe_{31}
Ampicillin	ND	ND	ND	19.8	19.0	20.0	17.2	17.8	17.9
Imipenem	ND	ND	ND	26.4	26.5	26.5	27.1	27.8	27.8
Vancomycin	ND	ND	ND	10.7	10.9	15.3	15.9	16.6	15.6
Levofloxacin	ND	ND	ND	20.4	21.2	21.5	22.6	22.0	23.0
Ciprofloxacin	22.0	21.9	22.0	21.6	21.9	22.3	24.0	24.0	24.
Tri/Sulf	29.9	31.9	30.0	28.6	28.7	0	32.0	31.5	32.8
Cefotixin	28	28.2	28.3	ND	ND	ND	ND	ND	ND
Gentamicin	21.5	22.4	22.2	ND	ND	ND	ND	ND	ND
Erythromycin	25.7	27.2	26.6	ND	ND	ND	ND	ND	ND
Tetracycline	24.8	24.8	25.8	ND	ND	ND	ND	ND	ND
Rifampicin	31.6	32.2	33.0	ND	ND	ND	ND	ND	ND
Benzylpenicillin	14.8	14.2	13.0	ND	ND	ND	ND	ND	ND

Note: Zones of inhibition (mm) produced by antibiotic-loaded discs against *S. aureus* and *E. faecalis* species. Sc: strain without exposure; Spe_{25}: strain with exposure to compound **25**; Spe_{31}: strain with exposure to compound **31**; Tri/Sulf: Trimethoprim/sulfamethoxazole; ND: Not Determined; Bacteria were deemed susceptible (in green) or resistant (in blue) according to EUCAST clinical breakpoints.

TABLE 6 | MBC values of compounds 25 and 31 against *S. aureus* and *E. faecalis*.

	Reference strain	MBC (mg/L)	
Organism ID		Comp 25	Comp 31
S. aureus	ATCC 29213	1	8
E. faecalis	ATCC 29212	4	8
E. faecalis	NCTC 12201	4	8

Note: Values represent the mode of three independent experiments.

25 (**Panels I** and **J**) and **31** (**Panels K** and **L**) showed severe structural damage, including wrinkled cell surfaces and cells that appear to have collapsed.

Estimation of Minimum Biofilm Eradication Concentration (MBEC)

The efficacy of compounds **25** and **31** to eradicate pre-formed biofilms of *S. aureus* and *E. faecalis* was next determined and compared to a group of known antibiotics (vancomycin, rifampicin, linezolid, teicoplanin and sparfloxacin) (Table 7). An MBEC of 256 mg/L was found for both quinoxaline analogues against *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212 strains. While compound **25** also yielded an MBEC of 256 mg/L against *E. faecalis* ATCC 12201, compound **31** was less effective in eradicating biofilms caused by this bacterium. Except for rifampicin against *S. aureus* (MBEC < 128 mg/L), the other four antibiotics (vancomycin, linezolid, teicoplanin and sparfloxacin) all had higher MBECs (> 512 or > 1024 mg/L) than compounds **25** and **31** (Table 7).

Structure–Activity Relationship (SAR) Studies Based on Phase 1 Results

The antibacterial data collected for a small number of quinoxaline analogues in Phase 1 of this study highlighted that the central quinoxaline scaffold was responsible for some activity, particularly against *S. aureus* (2,3-dichloro-6-nitroquinoxaline, Table 1). The hydroxy derivative (6-nitroquinoxaline-2,3-diol), in contrast, had poor activity against *S. aureus* and was ineffective against Gram-negative bacteria and mycobacteria.

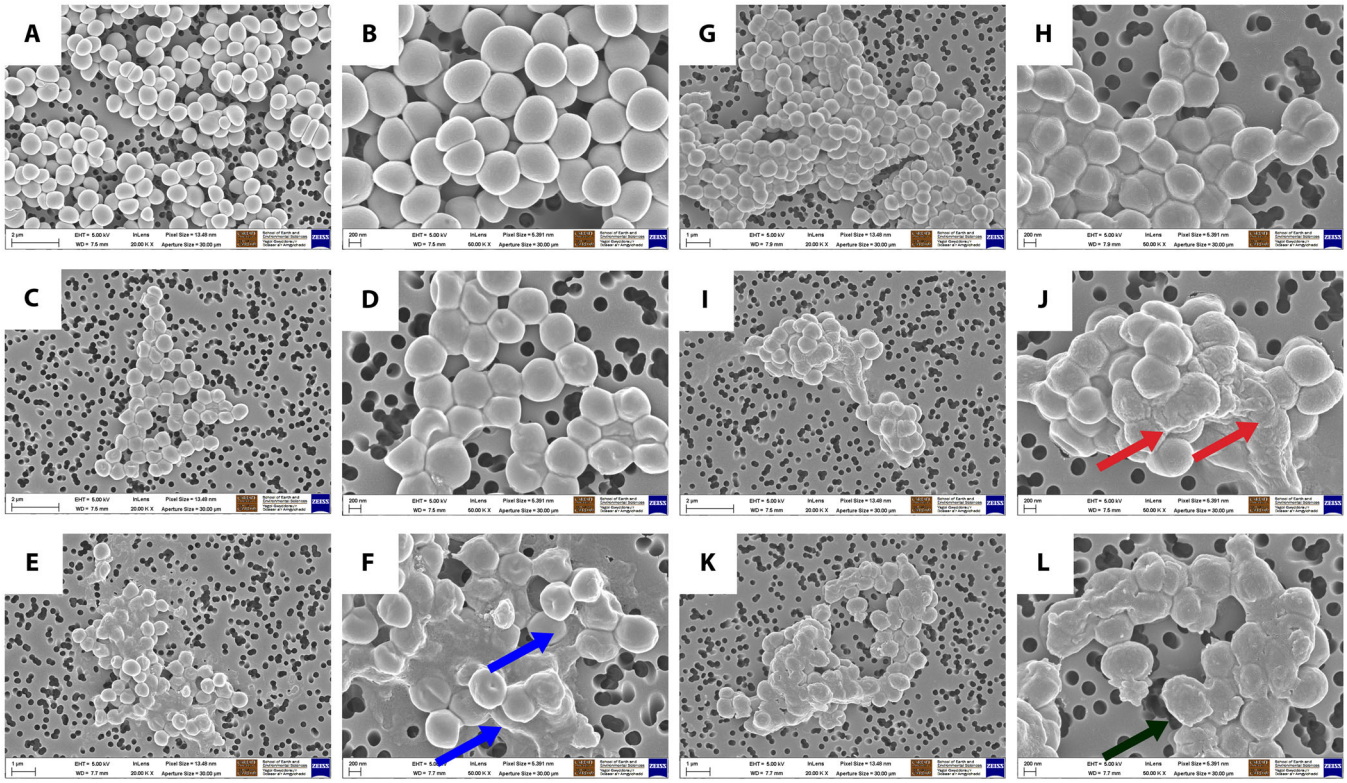


FIGURE 4 | Quinoxaline analogues induce phenotypic alterations to the surface of both *S. aureus* and *E. faecalis*. Scanning electron micrographs of *S. aureus* ATCC 29213 (A–F) and *E. faecalis* NCTC 12201 (G–L). Panels A and G and Panels B and H represent untreated cells captured at 20,000- and 50,000-times magnification, respectively. Bacteria treated with 2x MBC concentration of compound **25** are depicted in Panels C and **I** as well as Panels D and **J** at 20,000- and 50,000-times magnification, respectively. Bacteria treated with 2 x MBC concentration of compound **31** are shown in Panels E and K as well as Panels F and L at 20,000- and 50,000-times magnification, respectively. Blue arrows: loss of cellular content; red arrows: wrinkled cell surfaces; black arrow: cells that appear to have collapsed.

TABLE 7 | Quinoxaline analogues showed better anti-biofilm activities (MBEC) compared to five selected comparative antibiotics.

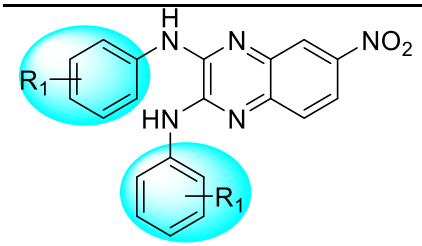
	MBEC (mg/L)		
	S. aureus **ATCC 29213**	***E. faecalis*** **ATCC 29212**	***E. faecalis*** **NCTC 12201**
Compound 25	256	256	256
Compound 31	256	256	> 256
Vancomycin	> 512	> 512	> 512
Rifampicin	< 128	> 512	> 512
Linezolid	> 1024	> 1024	> 1024
Teicoplanin	> 1024	> 1024	> 1024
Sparfloxacin	> 1024	> 1024	> 1024

Note: Values represent the mode of three independent experiments.

Although a limited number of compounds were screened, preliminary structural activity relationship (SAR) analyses highlighted the importance of aromatic ring substitutions and the functionalisation of position 6 of the central scaffold (highlighted in blue and magenta, respectively in Figure 1) for the antimicrobial activity. The *N*-aromatic derivatives of the central quinoxaline scaffold showed antibacterial activity primarily against Gram-positive bacteria with compounds **25**, **31** and **32** being particularly potent (Table 8). Regarding the effect of *para* or *meta* substitution, the para-trifluoromethyl (compound **31**) resulted in increased activity against both *S. aureus* and *M. smegmatis* when compared to the meta isomer (compound **30**). The introduction of fluorine (compound **32**) led to increased antibacterial activity when compared to the parent compound (the meta-trifluoromethyl derivative **30**) as previously observed with other antibacterial small molecules (Limban and Chifiriuc 2011). However, the combination of a methoxy substituent with a trifluoromethyl group (compound **35**) led to a decrease in activity (against *S. aureus*) when compared to compound **30**.

One of the original medicinal chemistry optimisations performed on these quinoxaline derivatives involved modifying the C-6 nitro group to mitigate cytotoxicity (Padalino et al. 2021). Therefore, the antibacterial activity of these C-6 derivatives was next explored to gain preliminary information about their SARs (Table 9). Overall, these analogues showed low activity on the selected bacterial strains suggesting that the nitro group on the C-6 position might be essential for their antibacterial effects, a finding similar to what has previously been reported (El-Hossary et al. 2018; Matos et al. 2013). The preliminary investigation of these quinoxaline derivatives showed low or no activity at all against *E. coli* and mycobacteria supporting the idea that a lipid-rich barrier (outer lipopolysaccharide membrane in Gram-negative or free lipid and mycolate layer in mycobacteria) is an impediment to the uptake of these compounds by these bacteria (Ebbensgaard et al. 2018; Mamelli et al. 2009). While *S. aureus*

TABLE 8 | Antibacterial activity of the N-aromatic quinoxaline analogues. (R1: residue of aromatic ring).



		Antibacterial activity MIC (mg/l)		
Cps	**R_1**	***E. coli***	***S. aureus***	***M. smegmatis***
25	*m*-Cl	> 125	< 3.91	> 125
30	*m*-CF_3	> 125	7.81	> 125
31	*p*-CF_3	> 125	0.98	31.25
32	3-CF_3, 4-F	62.50	1.95	62.50
35	3-CF_3, 4-OCH_3	> 125	62.50	> 125

was somewhat affected by the C-6 nitro-containing compound **22b** (MIC = 15.63 mg/L), the other three compounds lacking a C-6 nitro group (**22c**, **22 d** and **22 f**) were much less active. These findings broadly suggest that the nitro group offers less versatility for the antibacterial activity compared to the *N*-aromatic rings with further SAR conclusions related to the antibacterial activity of quinoxaline-containing analogues of this study summarised in Figure 5.

Discussion Around Phase 2 and 4 of This Study

A sub-selection of these quinoxaline derivatives (namely compound **22 f**, **25**, **31**, **32**, **35** and 2,3-dichloro-6-nitroquinoxaline/**2Cl-Q**) was selected for additional testing (Phase 2) against 19 clinically relevant strains as they showed initial activity against MRSA (Figure 2).

Here, we confirmed the importance of the *N*-aromatic ring's functionalisation for the antibacterial activity against *S. aureus* due to negligible activity of the central scaffold (**2Cl-Q**) and of the C6 derivative (**22 f**). Compounds **25**, **31**, **32** and **35** retained activities against the *S. aureus* clinical isolates (confirming Phase 1's results) and were even more active against the documented drug-resistant strains (Table S2B). This was particularly noticeable for compounds **25** and **31**.

We also obtained data about the antibacterial activity of the quinoxaline derivatives against *E. faecalis*. Similarly to *S. aureus*, compounds **25**, **31**, **32** and **35** were very active against both clinical and drug-resistant isolates including the most important *vanA* and *vanB* genotypes (Table 3). More specifically, *S. aureus* and *E. faecalis* expressing clinical resistance to systemic antibiotics (flucloxacillin, vancomycin, tetracycline and erythromycin/clindamycin) were both found to be susceptible to compounds **25** and **31** at low concentrations (0.06–0.25 mg/L—Table 3).

We extended our testing against a larger spectrum of Gram-positive bacteria (Figure 3) including *S. aureus*, *E. faecalis* and *E. faecium* to ensure our selected quinoxaline derivatives (namely compounds **25** and **31**) retained their activity against a broad range of clinical isolates including some having specific and documented antibiotic resistance mechanisms (Table S3). *E. faecium* was of particular interest due to its broader resistance and higher virulence compared to *E. faecalis* (Kramer et al. 2018).

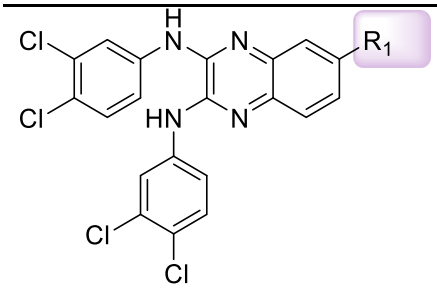
Encouragingly, compounds **25** and **31** maintained potent antibacterial activity against a wide panel of MRSA strains (Table S3), including clinically important pathogens with reduced clindamycin susceptibility (via the D-zone test (Lewis and Jorgensen 2005)) and containing Panton-Valentine Leukocidin (PVL) genes (*pvl* genes detected by PCR (McClure et al. 2006)). They demonstrated better antibacterial profiles than vancomycin and teicoplanin (first-line treatment) and linezolid (second-line treatment) with MICs ranging from 0.125 to 0.500 mg/L and comparable activity to the cyclic lipopeptide antibiotic daptomycin (Figure 3). In terms of *E. faecalis* and *E. faecium*, compounds **25** and **31** showed equal to or better activity with lower concentrations than most of the antibiotics tested except for teicoplanin and daptomycin against *E. faecalis* 23948 and vancomycin against *E. faecium* 26575. These results are particularly promising when considering the impact that both *Enterococci* have on urinary tract infections and hospital length of stay (i.e., *E. faecium*) (Nichol et al. 2006) as well as sepsis, endocarditis and meningitis in immunocompromised patients (i.e., *E. faecalis*) (Bolocan et al. 2019; Giucă et al. 2010).

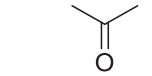
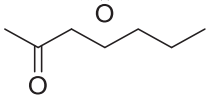
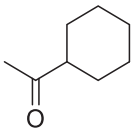
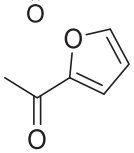
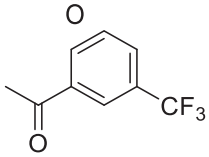
One of the main challenges in developing new antimicrobials relates to how long the targeted bacterial species would need to develop resistance (Salam et al. 2023; O'Neill 2016). Therefore, we decided to assess how pre-exposure to our quinoxaline derivatives (namely compounds **25** and **31**) affects bacterial susceptibility. Pre-exposure of all tested bacterial strains to sub-inhibitory doses of compounds **25** and **31** led to increases in MIC levels (Table 4). The increases were more profound for pre-exposure to compound **31**. Yet pre-exposure to compounds **25** and **31** did not alter the clinical susceptibility profile of commonly used antibiotics, with the expectation of trimethoprim/sulfamethoxazole and vancomycin in one of the two *E. faecalis* strain tested (*E. faecalis* NCTC 12201 - Table 5).

The pretreatment of vancomycin-resistant *E. faecalis* (NCTC 12201) with compound **31** (but not compound **25**) made this strain sensitive to vancomycin. At this stage, we can only speculate about the potential mechanism of resistance involved. Efflux, which is a common and efficient mechanism leading to multidrug resistance (Maillard and Pascoe 2024), is likely to be involved here (no change in tetracycline or quinolones (levofloxacin and ciprofloxacin) MIC—Table 5). Compound **31** might inhibit efflux pumps critical for expelling a wide range of structurally diverse compounds (Hernando-Amado et al. 2016) in vancomycin-resistant *E. faecalis* (NCTC 12201). A recent study also demonstrated that structurally similar quinoxaline-containing compounds inhibited efflux pump activity and restored drug susceptibility in drug-resistant non-tuberculous mycobacteria (Corona et al. 2022).

In contrast, pretreatment of *E. faecalis* (NCTC 12201) with compound **31** induced sensitivity to resistant transition to

TABLE 9 | Antibacterial activities of the C-6 derivatives. (R_1: residue on C-6 position of quinoxaline core).



Cps	R_1	Antibacterial activity MIC (mg/l)		
		E. coli	*S. aureus*	*M. smegmatis*
22b		> 125	15.63	> 125
22c		> 125	62.50	> 125
22 d		> 125	62.5	> 125
22e		> 125	> 125	125
22 f		> 125	31.25	> 125
22 g		> 125	> 125	125

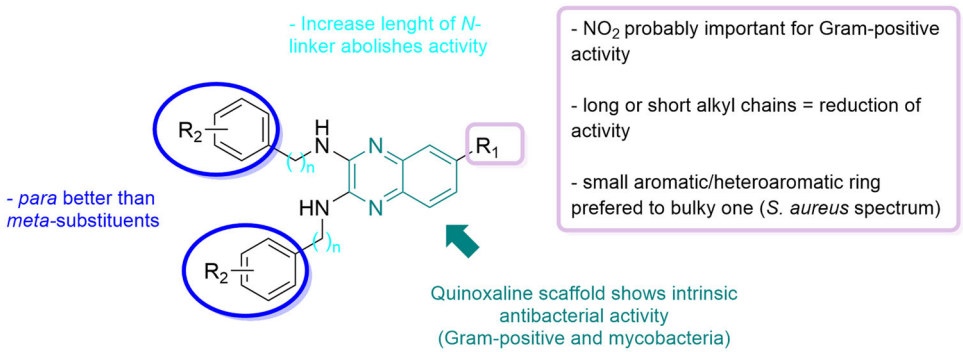


FIGURE 5 | Summary of the SAR studies performed on different regions of the synthesised derivatives. A total of 15 similarly structured compounds were analysed to generate this map. All the biological results regarding their antibacterial activity were included in Tables 1–2.

trimethoprim/sulfamethoxazole (Table 5). Once again, the mechanism behind this transition is currently unknown. Despite this, our lead compounds **25** and **31** were quite active against multidrug-resistant pathogens (Figure 2 and Table 3).

While MIC values represent a measure of antibacterial susceptibility (Tables 3 and 4), they do not reveal whether the antibiotic (or test compound) is bacteriostatic or bactericidal (Wiegand, Hilpert, and Hancock 2008b). Recent studies support the combined use of MIC and MBC to provide a more detailed understanding of the bacteria's susceptibility to compounds (Kłodzińska et al. 2018) and correlate in vitro data with possible outcomes of in vivo treatments (Pankey and Sabath 2004). In our investigations, the MBC of compounds **25** and **32** (Table 6) were higher than their MIC values (Tables 3 and 4) confirming the general trend of MBC being higher or equal to MIC (Abedon 2011). Moreover, we can conclude that compound **25** is bactericidal since the MBC is no more than four times the MIC value (French 2006; Cushnie et al. 2020).

SEM images of both *S. aureus* and *E. faecalis* exposed to double the MBC of compounds **25** and **31** revealed gross structural damage in comparison to untreated cells (Figure 4, Panels **A**, **B**, **G**, **H**). However, these findings cannot provide significant insights into the possible mechanism of action and/or final target of these compounds.

In addition to quantifying compounds **25** and **31**'s MICs and MBCs on *S. aureus* and *E. faecalis*, we also measured these quinoxaline-containing compounds' ability to affect biofilms. Biofilms have been recognised as a potential source of recurring infection and high levels of antibiotic tolerance are prevalent in bacterial biofilms (Zhao, Sun, and Liu 2023b). Equally, the formation of biofilms on implant surfaces is a major cause of implant-associated infection difficult to treat (Okae et al. 2022). Biofilm-producing bacteria show different behaviour when compared to planktonic (free-floating) bacteria that are typically used in the testing of traditional antibiotics; this behaviour often limits compound penetration through biofilms (Choi et al. 2023). Reassuringly, both compounds **25** and **31** retained their antibacterial against pre-formed *S. aureus* and *E. faecalis* biofilms and, more importantly, they performed better than all other antibiotics tested (except for rifampicin for *S. aureus* biofilm—Table 7). This result is particularly promising for the use of these compounds in antibiofilm products like implants and wound dressings, although further testing would be required against biofilms in vivo, which are notably more resistant to antibiotics than in vitro ones (Okae et al. 2022).

Conclusions

This study identified quinoxaline derivatives, particularly compounds **25** and **31**, with promising antibacterial activities against Gram-positive bacteria such as *S. aureus*, *E. faecalis* and *E. faecium*. These compounds showed activity comparable to or better than vancomycin, teicoplanin, linezolid, and daptomycin. They were also effective against bacterial biofilms, a property rarely tested at this stage. Further medicinal chemistry optimisation will examine the structure–activity relationships, aiming to develop more potent antibacterial candidates with biocidal and biofilm-inhibiting properties. Due to their poor aqueous solubility, these compounds are likely to be used in topical formulations for skin and soft-tissue infections or for coating implants and dressings.

Author Contributions

Gilda Padalino: conceptualisation, investigation, formal analysis, methodology, writing–review and editing, writing–original draft; visualization, validation, project administration. **Katrina Duggan:** investigation; data curation; formal analysis, methodology, writing–original draft; writing–review and editing; validation. **Luis A J Mur:** resources, writing–review and editing. **Jean-Yves Maillard:** resources, writing–review and editing. **Andrea Brancale:** resources, writing–review and editing, supervision. **Karl F Hoffmann:** conceptualisation, resources, funding acquisition, supervision, writing–original draft, writing–review and editing; project administration.

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Ethics Statement

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data generated and analysed during this study are included in this published article and its supporting information.

References

Abedon, S. 2011. "Chapter 1 - Phage Therapy Pharmacology: Calculating Phage Dosing." In *Advances in Applied Microbiology. 77*, edited by A. I. Laskin, S. Sariaslani and G. M. Gadd, 1–40). Academic Press.

Ajani, O. O. 2014. "Present Status of Quinoxaline Motifs: Excellent Pathfinders in Therapeutic Medicine." *European Journal of Medicinal Chemistry* 85: 688–715. https://doi.org/10.1016/j.ejmech.2014.08.034.

Baptista, R., D. M. Fazakerley, M. Beckmann, L. Baillie, and L. A. J. Mur. 2018. "Untargeted Metabolomics Reveals a New Mode of Action of Pretomanid (Pa-824)." *Scientific Reports* 8: 5084.

Bhowmick, S., M. Beckmann, J. Shen, and L. A. J. Mur. 2023. "Identification and Metabolomic Characterization of Potent Anti-MRSA Phloroglucinol Derivatives From Dryopteris Crassirhizoma Nakai (Polypodiaceae)." *Frontiers in Pharmacology* 13: 961087. https://doi.org/10.3389/fphar.2022.961087.

Bolocan, A. S., A. Upadrasta, and P. H. de Almeida Bettio, et al. 2019. "Evaluation of Phage Therapy in the Context of Enterococcus Faecalis and Its Associated Diseases." *Viruses* 11, no. 4: 366. https://doi.org/10.3390/v11040366.

Brauner, A., O. Fridman, O. Gefen, and N. Q. Balaban. 2016. "Distinguishing Between Resistance, Tolerance and Persistence to Antibiotic Treatment." *Nature Reviews Microbiology* 14, no. 5: 320–330. https://doi.org/10.1038/nrmicro.2016.34.

British Standards Institution. 2020. Available from: https://www.iso.org/obp/ui/en/#iso:std:iso:20776:-1:ed-2:v2:en.

Chait, R., K. Vetsigian, and R. Kishony. 2012. "What Counters Antibiotic Resistance in Nature?" *Nature Chemical Biology* 8, no. 1: 2–5. https://doi.org/10.1038/nchembio.745.

Choi, V., J. L. Rohn, P. Stoodley, D. Carugo, and E. Stride. 2023. "Drug Delivery Strategies for Antibiofilm Therapy." *Nature Reviews Microbiology* 21, no. 9: 555–572. https://doi.org/10.1038/s41579-023-00905-2.

Corona, P., R. Ibba, S. Piras, P. Molicotti, A. Bua, and A. Carta. 2022. "Quinoxaline-Based Efflux Pump Inhibitors Restore Drug Susceptibility in Drug-Resistant Nontuberculous Mycobacteria." *Archiv der Pharmazie* 355, no. 8: 2100492. https://doi.org/10.1002/ardp.202100492.

Courtenay, M., E. Castro-Sanchez, M. Fitzpatrick, R. Gallagher, R. Lim, and G. Morris. 2019. "Tackling Antimicrobial Resistance 2019-2024–the Uk's Five-Year National Action Plan." *Journal of Hospital Infection* 101, no. 4: 426–427.

Cushnie, T. P. T., B. Cushnie, J. Echeverría, et al. 2020. "Bioprospecting for Antibacterial Drugs: A Multidisciplinary Perspective on Natural Product

Source Material, Bioassay Selection and Avoidable Pitfalls." *Pharmaceutical Research* 37, no. 7: 125. https://doi.org/10.1007/s11095-020-02849-1.

Donlan, R. M. 2001. "Biofilm Formation: A Clinically Relevant Microbiological Process." *Clinical Infectious Diseases* 33, no. 8: 1387–1392.

Ebbensgaard, A., H. Mordhorst, F. M. Aarestrup, and E. B. Hansen. 2018. "The Role of Outer Membrane Proteins and Lipopolysaccharides for the Sensitivity of *Escherichia coli* to Antimicrobial Peptides." *Frontiers in Microbiology* 9: 2153. https://doi.org/10.3389/fmicb.2018.02153.

Ekkelenkamp, M. B., M. Díez-Aguilar, M. M. Tunney, J. S. Elborn, A. C. Fluit, and R. Cantón. 2022. "editors. Establishing Antimicrobial Susceptibility Testing Methods and Clinical Breakpoints for Inhaled Antibiotic Therapy." In *Open Forum Infectious Diseases*. Oxford University Press US.

El-Atawy, M. A., E. A. Hamed, M. Alhadi, and A. Z. Omar. 2019. "Synthesis and Antimicrobial Activity of Some New Substituted Quinoxalines." *Molecules* 24, no. 22: 4198. https://doi.org/10.3390/molecules24224198.

El-Hossary, E. M., K. U. Förstner, P. François, et al. 2018. "A Novel Mechanism of Inactivating Antibacterial Nitro Compounds in the Human Pathogen *Staphylococcus Aureus* by Overexpression of a Nadh-Dependent Flavin Nitroreductase." *Antimicrobial Agents and Chemotherapy* 62, no. 2: e01510-17. https://doi.org/10.1128/aac.01510-17.

French, G. L. 2006. "Bactericidal Agents in the Treatment of MRSA Infections—The Potential Role of Daptomycin." *Journal of Antimicrobial Chemotherapy* 58, no. 6: 1107–1117. https://doi.org/10.1093/jac/dkl393.

Giucă, M. C., A. Petrini, T. Ceornea, and V. Ungureanu. 2010. "[Study of Antibiotic Resistance of Enterococci Strains Received in 2009 in the National Reference Centre for Enterococci]." *Bacteriologia, virusologia, parazitologia, epidemiologia (Bucharest, Romania: 1990)* 55, no. 2: 77–82.

Hegemann, J. D., J. Birkelbach, S. Walesch, and R. Müller. 2023. "Current Developments in Antibiotic Discovery." *EMBO Reports* 24, no. 1: e56184. https://doi.org/10.15252/embr.202256184.

Hernando-Amado, S., P. Blanco, and M. Alcalde-Rico, et al. 2016. "Multidrug Efflux Pumps As Main Players in Intrinsic and Acquired Resistance to Antimicrobials." *Drug Resistance Updates* 28: 13–27. https://doi.org/10.1016/j.drup.2016.06.007.

Ingram-Sieber, K., N. Cowan, G. Panic, et al. 2014. "Orally Active Antischistosomal Early Leads Identified From the Open Access Malaria Box." *PLoS Neglected Tropical Diseases* 8, no. 1: e2610. https://doi.org/10.1371/journal.pntd.0002610.

International A. 2012. *ASTM E2799-12 Standard Test Method for Testing Disinfectant Efficacy Against Pseudomonas Aeruginosa Biofilm Using the MBEC Assay*. Conshohocken, PA: ASTM International West.

Keri, R. S., S. S. Pandule, S. Budagumpi, and B. M. Nagaraja. 2018. "Quinoxaline and Quinoxaline-1,4-di-N-oxides: An Emerging Class of Antimycobacterials." *Archiv der Pharmazie* 351, no. 5: 1700325. https://doi.org/10.1002/ardp.201700325.

Kester, J. C., and S. M. Fortune. 2014. "Persisters and Beyond: Mechanisms of Phenotypic Drug Resistance and Drug Tolerance in Bacteria." *Critical Reviews in Biochemistry and Molecular Biology* 49, no. 2: 91–101. https://doi.org/10.3109/10409238.2013.869543.

Kramer, T. S., C. Remschmidt, S. Werner, et al. 2018. "The Importance of Adjusting for Enterococcus Species When Assessing the Burden of Vancomycin Resistance: A Cohort Study Including over 1000 Cases of Enterococcal Bloodstream Infections." *Antimicrobial resistance & Infection control* 7: 1–9.

Kümpornsin, K., T. Kochakarn, and T. Yeo, et al. 2023. "Generation of a Mutator Parasite to Drive Resistome Discovery in Plasmodium Falciparum." *Nature Communications* 14, no. 1: 3059. https://doi.org/10.1038/s41467-023-38774-1.

Kłodzińska, S. N., P. A. Priemel, T. Rades, and H. M. Nielsen. 2018. "Combining Diagnostic Methods for Antimicrobial Susceptibility Testing – A Comparative Approach." *Journal of Microbiological Methods* 144: 177–185. https://doi.org/10.1016/j.mimet.2017.11.010.

Lewis, J. S., and J. H. Jorgensen. 2005. "Inducible Clindamycin Resistance in Staphylococci: Should Clinicians and Microbiologists be Concerned." *Clinical Infectious Diseases* 40, no. 2: 280–285. https://doi.org/10.1086/426894.

Limban, C., and M. C. Chifiriuc. 2011. "Antibacterial Activity of New Dibenzoxepinone Oximes With Fluorine and Trifluoromethyl Group Substituents." *International Journal of Molecular Sciences* 12, no. 10: 6432–6444. https://doi.org/10.3390/ijms12106432.

Maillard, J.-Y., and M. Pascoe. 2024. "Disinfectants and Antiseptics: Mechanisms of Action and Resistance." *Nature Reviews Microbiology* 22, no. 1: 4–17. https://doi.org/10.1038/s41579-023-00958-3.

Mamelli, L., S. Petit, J. Chevalier, et al. 2009. "New Antibiotic Molecules: Bypassing the Membrane Barrier of Gram Negative Bacteria Increases the Activity of Peptide Deformylase Inhibitors." *PLoS One* 4, no. 7: e6443. https://doi.org/10.1371/journal.pone.0006443.

Matos, M. J., S. Vazquez-Rodriguez, L. Santana, et al. 2013. "Synthesis and Structure-Activity Relationships of Novel Amino/Nitro Substituted 3-Arylcoumarins as Antibacterial Agents." *Molecules (Basel, Switzerland)* 18: 1394–1404.

McClure, J. A., J. M. Conly, and V. Lau, et al. 2006. "Novel Multiplex PCR Assay for Detection of the Staphylococcal Virulence Marker Panton-Valentine Leukocidin Genes and Simultaneous Discrimination of Methicillin-Susceptible From -Resistant Staphylococci." *Journal of Clinical Microbiology* 44, no. 3: 1141–1144. https://doi.org/10.1128/jcm.44.3.1141-1144.2006.

Montana, M., V. Montero, O. Khoumeri, and P. Vanelle. 2021. "Quinoxaline Moiety: A Potential Scaffold Against Mycobacterium Tuberculosis." *Molecules* 26, no. 16: 4742. https://doi.org/10.3390/molecules26164742.

Murray, C. J. L., K. S. Ikuta, F. Sharara, et al. 2022. "Global Burden of Bacterial Antimicrobial Resistance in 2019: a Systematic Analysis." *The lancet* 399, no. 10325: 629–655.

Nichol, K., M. Sill, N. Laing, J. Johnson, D. Hoban, and G. Zhanel. 2006. "Molecular Epidemiology of Urinary Tract Isolates of Vancomycin-Resistant Enterococcus Faecium From North America." *International Journal of Antimicrobial Agents* 27, no. 5: 392–396. https://doi.org/10.1016/j.ijantimicag.2005.12.006.

Okae, Y., K. Nishitani, and A. Sakamoto, et al. 2022. "Estimation of Minimum Biofilm Eradication Concentration (MBEC) on In Vivo Biofilm on Orthopedic Implants in a Rodent Femoral Infection Model." *Frontiers in Cellular and Infection Microbiology* 12: 896978. https://doi.org/10.3389/fcimb.2022.896978.

O'Neill, J. 2016. *Tackling Drug-resistant Infections Globally: Final Report and Recommendations*. https://amr-review.org/sites/default/files/160518_Final%20paper_with%20cover.pdf.

Organization WH. 2014. *Antimicrobial Resistance: Global Report on Surveillance*. World Health Organization.

Padalino, G., N. El-Sakkary, L. J. Liu, et al. 2021. "Anti-Schistosomal Activities of Quinoxaline-Containing Compounds: From Hit Identification to Lead Optimisation." *European Journal of Medicinal Chemistry* 226: 113823. https://doi.org/10.1016/j.ejmech.2021.113823.

Pankey, G. A., and L. D. Sabath. 2004. "Clinical Relevance of Bacteriostatic Versus Bactericidal Mechanisms of Action in the Treatment of Gram-Positive Bacterial Infections." *Clinical Infectious Diseases* 38, no. 6: 864–870. https://doi.org/10.1086/381972.

Pereira, J. A., A. M. Pessoa, and M. N. D. S. Cordeiro, et al. 2015. "Quinoxaline, Its Derivatives and Applications: A State of the Art Review." *European Journal of Medicinal Chemistry* 97: 664–672. https://doi.org/10.1016/j.ejmech.2014.06.058.

Rodrigues, J. H. S., T. Ueda-Nakamura, A. G. Corrêa, D. P. Sangi, and C. V. Nakamura. 2014. "A Quinoxaline Derivative as a Potent Chemotherapeutic Agent, Alone or in Combination with Benznidazole,

Against Trypanosoma Cruzi." *PLoS One* 9, no. 1: e85706. https://doi.org/10.1371/journal.pone.0085706.

Salam, M. A., M. Y. Al-Amin, and M. T. Salam, et al. 2023. "Antimicrobial Resistance: A Growing Serious Threat for Global Public Health." *Healthcare* 11, no. 13: 1946. https://doi.org/10.3390/healthcare11131946.

Silva de Jesus Passaes, A. C., J. Arantes Dantas, F. Landim Lopes, et al. 2023. "Quinoxalines Against Leishmania Amazonensis: Sar Study, Proposition of a New Derivative, Qsar Prediction, Synthesis, and Biological Evaluation." *Scientific Reports* 13, no. 1: 18136. https://doi.org/10.1038/s41598-023-45436-1.

Standardization IOf. ISO 20776-1: 2019. Susceptibility Testing of Infectious Agents and Evaluation of Performance of Antimicrobial Susceptibility Test Devices—Part 1: Broth Micro-dilution Reference Method for Testing the in Vitro Activity of Antimicrobial Agents Against Rapidly Growing Aerobic Bacteria Involved in Infectious Diseases. ISO; 2019.

Wiegand, I., K. Hilpert, and R. E. Hancock. 2008b. "Agar and Broth Dilution Methods to Determine the Minimal Inhibitory Concentration (MIC) of Antimicrobial Substances." *Nature Protocols* 3, no. 2: 163–175. https://doi.org/10.1038/nprot.2007.521.

Wiegand, I., K. Hilpert, and R. E. W. Hancock. 2008a. "Agar and Broth Dilution Methods to Determine the Minimal Inhibitory Concentration (MIC) of Antimicrobial Substances." *Nature Protocols* 3: 163. https://doi.org/10.1038/nprot.2007.521.

Yadav, S., and A. Kapley. 2021. "Antibiotic Resistance: Global Health Crisis and Metagenomics." *Biotechnology Reports* 29: e00604. https://doi.org/10.1016/j.btre.2021.e00604.

Zhao, A., J. Sun, and Y. Liu. 2023a. "Understanding Bacterial Biofilms: From Definition to Treatment Strategies." *Frontiers in Cellular and Infection Microbiology* 13: 1137947.

Zhao, A., J. Sun, and Y. Liu. 2023b. "Understanding Bacterial Biofilms: From Definition to Treatment Strategies." *Frontiers in Cellular and Infection Microbiology* 13: 1137947. https://doi.org/10.3389/fcimb.2023.1137947.

Supporting Information

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