FULL PAPER

Design, synthesis and biological evaluation of *N*-sulfonyl-phenylglyoxamide-based antimicrobial peptide mimics as novel antimicrobial agents

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Abstract: Antibiotic resistance is a major global health concern. There is a significant and urgent need for the development of novel antimicrobials to tackle the increasing incidence of antibiotic resistance. Recently, phenylglyoxamide-based small molecular antimicrobial peptide mimics have been identified as potential new leads to treat bacterial infection. Here, we describe the synthesis of novel phenylglyoxamide derivatives *via* the ring-opening reaction of *N*-sulfonylisatins with primary amines, followed by conversion into hydrochloride (HCl), quaternary ammonium iodide or guanidine salts. The antibacterial activity of the compounds against *Staphylococcus aureus* was evaluated *in vitro* by a disk diffusion assay and a minimum inhibitory concentration (MIC) assay. Structure-activity relationship studies revealed that a 5-bromo-substituent at the phenyl ring, octyl group appended to the *ortho* sulfonamide group or a guanidine hydrochloride salt as the terminal group significantly contributed to potency. The most potent compound in this study, the guanidine salt **35d**, exhibited an MIC value of 12 µM. The most active compounds induced an increase in membrane conduction, suggesting that they are potential antimicrobial pore-forming agents. The compounds had therapeutic indices ranging from 0.8 to 15. Overall, the results identified **35d** as a new lead antimicrobial compound that possesses an acceptable therapeutic window.

Introduction

Infectious diseases caused by bacteria are one of the leading causes of death worldwide.[1] The discovery of the powerful bactericidal agent, beta-lactam (penicillin), in the 1930s provided an effective strategy to treat bacterial infection.[2] Different classes of antibiotics target different metabolic processes in bacteria, such as inhibition of bacterial cell wall synthesis, protein synthesis, DNA and RNA synthesis, fatty acids biosynthesis, folate synthesis, or depolarizing bacterial cell membrane potentials.[3]

However, the use of conventional antibiotics can exert selective survival pressure on bacteria, leading to the development of drug resistance. The most common antibiotic resistance mechanism is the enzymatic inactivation of antibiotics, whereby metabolic enzymes produced by bacteria metabolise antibiotics into their inactive form. For instance, beta-lactam antibiotics are inactivated by beta-lactamase enzymes, which hydrolyse the amide group and cleave the beta-lactam ring.[4] Other antibiotic resistance mechanisms involve mutations at the binding sites so that the antibiotics has reduced affinity, or physical removal of the antibiotic from the bacterial cell by efflux pumps.[5]

The misuse and overuse of antibiotics has accelerated the development of antibiotic resistance.[6] Moreover, with typical antibiotic development strategies relying heavily on merely modifying the periphery of existing antibiotic scaffolds, resistance to one antibiotic can rapidly spread to other antibiotics within the same class as they share a common core.[7]

Antibiotic-resistant bacteria cause more than 23,000 deaths in the US every year.[8] Alarmingly, multi-drug resistant bacterial strains have emerged that are resistant to all known antibiotics.[9] Therefore, there is an urgent need to develop novel and efficacious therapies to treat bacterial infection, particularly those caused by drug-resistance strains.

Antimicrobial peptides (AMPs) are naturally occurring amphipathic peptides found in most living organisms, including humans.[10] They play an important role as the first line of defence

in our immune system and protect us against a wide variety of pathogenic organisms, such as bacteria, fungi, protozoa, yeast and viruses.[11] AMPs are typically 12 to 50 amino acids in length and contain both hydrophilic (cationic) and hydrophobic residues.[10a] They possess rigid secondary alpha-helix or beta-sheet structures with the hydrophilic cationic residues located on one face of the molecule and hydrophobic residues on the opposite face.[1, 10a, 11a, 12] Unlike traditional antibiotics, AMPs do not act *via* enzyme or receptor interactions. Instead, they kill pathogens by disrupting their membrane integrity, causing the leakage of cellular components or the loss of membrane potential, both resulting in cell death.[13] The ability of AMPs to disrupt membranes is predominately attributed to their facially amphiphilic structure.[11a] The hydrophilic cationic residues on one face of the AMP initially bind electrostatically to the negatively-charged bacterial cell membrane. Once the AMP is bound, the hydrophobic residues on the opposite face assist in the insertion of the molecule into the bacterial cell membrane, causing the formation of pores in the bacterial cell membrane.[11a, 12, 14] As AMPs kill bacteria *via* disrupting bacterial cell membranes, a complete restructuring of the bacterial outer cell membrane would be required for bacteria to develop resistance to AMPs.[11a, 13c] More importantly, AMPs have a high degree of selectivity towards bacterial cells over human cells, which can be attributed to the difference in the lipid composition between the two types of cell membranes.[15] While the extracellular side of the human lipid bilayer is mainly composed of zwitterionic phospholipids such as phosphatidylcholine with only a minute amount of negatively-charged lipids, the bacterial cell membrane contains a significant portion of negatively-charged lipids, increasing their affinity towards AMPs.[13b, 15-16] AMPs have thus attracted attention as potential therapeutic agents to combat bacterial infection that are resistant to conventional antibiotics.

Although AMPs can exhibit potent and selective antibacterial activity, they have several drawbacks. Firstly, the synthesis of AMPs involves a high manufacturing cost. Due to the antibacterial nature of AMPs, it is difficult to use bacterial fermentation methods for synthesis. Current production methodologies are based on solid-phase peptide synthesis, which requires expensive precursor components and extensive purification, but only produces AMPs in low yield.[17] Furthermore, AMPs are susceptible to protease hydrolysis. Proteases in the serum can cleave the peptide bonds in AMPs, reducing the metabolic stability of AMPs *in vivo*.[18] The drawbacks of AMPs have stimulated the development of AMP mimics, such as α-peptides and β-peptides.[19] In addition, several small molecule AMP mimics (*e.g.* 1 and 2) have shown high antimicrobial activities against a variety of bacterial strains (Figure 1).[15, 20] LTX-109 3 is now in Phase-II clinical trials for the topical treatment of multi-drug resistant Gram-positive and Gram-negative bacteria.[21]

N-Aryl, *N*-acyl or *N*-sulfonylisatins **4** (Figure 2) can undergo ring-opening reaction with alcohols and amines to yield the corresponding phenylglyoxamide.[22] Recently, our research group has reported that *N*-naphthoyl-phenylglyoxamide derivatives **5** (Figure 2), possess moderate to high antibacterial activities against the common bacterial pathogens *Staphylococcus aureus* and *Pseudomonas aeruginosa*.[22b] These phenylglyoxamides can be synthesised by the ring-opening reaction of *N*-sulfonylisatin with amines. As the effect of varying the *N*-substituent appended to *ortho* amino group on the activity of these compounds has not been explored, it is of great interest to synthesise novel *N*-sulfonyl-phenylglyoxamide derivatives as potential antibacterial agents.

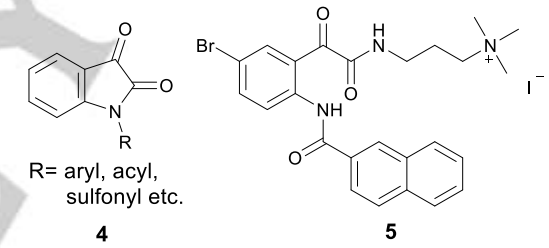


Figure 2. Structures of *N*-substituted isatins **4** and *N*-naphthoyl-phenylglyoxamide derivatives **5**.

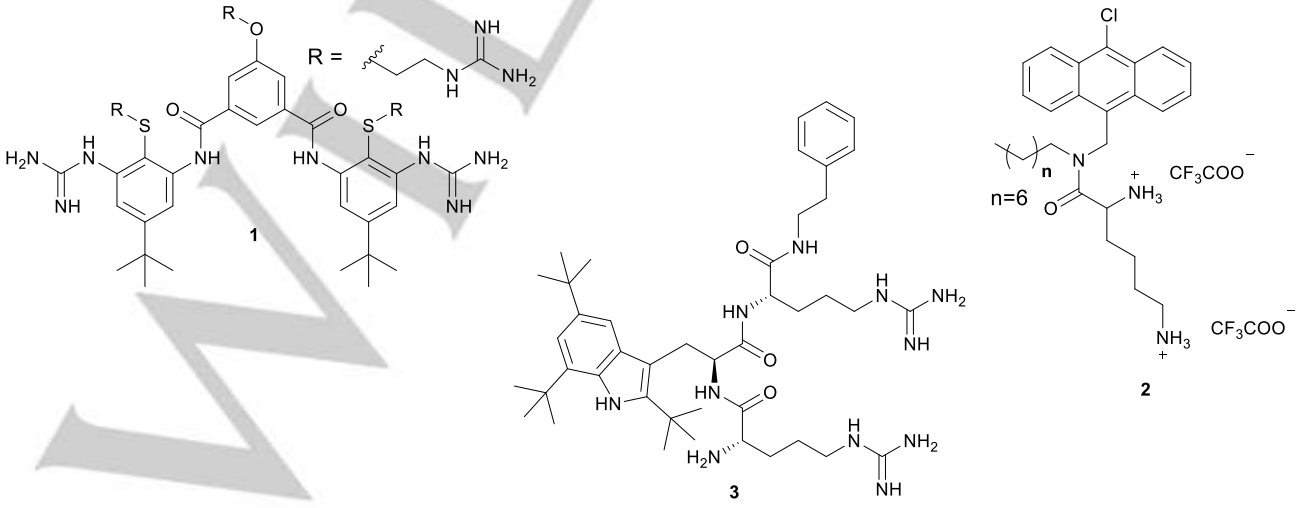


Figure 1. Chemical structures of AMP mimics **1-2** and LTX-109 **3**.[15, 20a, 21b].

In this study, we report the synthesis of novel *N*-sulfonyl-phenylglyoxamide derivatives as small molecular AMP mimics. First, a panel of *N*-sulfonylisatins were synthesized via a simple and time-efficient method. Subsequently, the *N*-sulfonylisatins were ring-opened with diamines (*N*,*N*-dimethylethane-1,2-diamine or *N*,*N*-dimethylpropane-1,3-diamine) to give the corresponding phenylglyoxamide derivatives. Finally, these were converted into their hydrochloride or iodide salts to give the desired AMP mimics. In addition, *N*-octanesulfonylisatin and *N*-naphthalenesulfonylisatin were also ring-opened with *tert*-butyl *N*-(3-aminopropyl)carbamate followed by installation of the guanidine moiety to furnish AMP mimics with an alternative cationic group.

The *N*-sulfonyl-phenylglyoxamide derivatives are amphipathic in nature due to the presence of both the hydrophobic groups at the *N*-sulfonyl position, and the hydrophilic ammonium or guanidine hydrochloride salts at the end of the glyoxamide chain (Figure 3). The synthesised compounds were evaluated for their antimicrobial potency against two common bacterial pathogens *S. aureus* and *P. aeruginosa*. Selected analogues with high antibacterial activity were tested for their ability to disrupt bacterial membrane integrity using tethered bilayer lipid membranes. Finally, their cytotoxicity against normal mammalian cells was also evaluated by using the Alamar Blue assay.

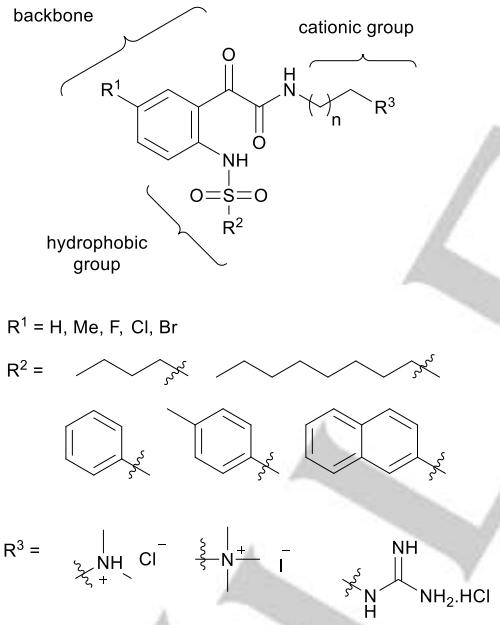


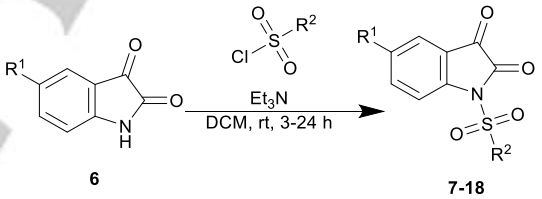
Figure 3. General structure of *N*-sulfonyl-phenylglyoxamide-based small molecular AMP mimics.

Results and Discussion

Synthesis

Initially, the synthesis of *N*-sulfonylisatins followed a modified version of the procedure described by Chin *et al.* in 2002,[23] in which isatin **6** was converted into its sodium salt by stirring with 1.2 equivalents of sodium hydride in dimethylformamide (DMF) at 0 °C for 20 min under a nitrogen atmosphere. An appropriate sulfonyl chloride was then added dropwise with stirring to the purple reaction mixture. After 18 h of stirring at room temperature, thin layer chromatography (TLC) confirmed the formation of product. The orange reaction mixture was poured into an ice-water mixture and the precipitate was filtered to give an orange solid. However, after washing the crude solid with methanol, only poor yields (0-17%) of the *N*-sulfonylisatin products were obtained.

A modified version of the synthetic scheme described by Chung *et al.* in 2003 was then attempted in the synthesis of *N*-sulfonylisatins.[24] This method utilises milder reaction condition, and was reported to produce a moderate to high yield of the product in a time-efficient manner. In this method, a mixture of the appropriate isatin **6** and an appropriate sulfonyl chloride was stirred with triethylamine in dichloromethane (DCM) at room temperature for 3-24 h under a nitrogen atmosphere. This afforded *N*-sulfonylisatins **7-18** in 23-86% yields (Scheme 1, Table 1). Although this method gave moderate to high yield of the *N*-sulfonylisatin products, some of the compounds were lost when washing the crude product with methanol as evidenced by TLC. In an attempt to improve upon this, flash column chromatography on silica (ethyl acetate/n-hexane = 1:4) was used to purify the crude product **8** instead. However, this gave only a 3% yield of the pure product **8**, and some of the product also co-eluted with isatin despite the good separation of the two species on the TLC plate. This could be explained by the poor solubility of isatin and *N*-sulfonylisatin **8** in the ethyl acetate/n-hexane mixture. Therefore, washing the crude with methanol was deemed to be the most suitable purification method for the synthesis of *N*-sulfonylisatins in terms of both time and yield.[22a]

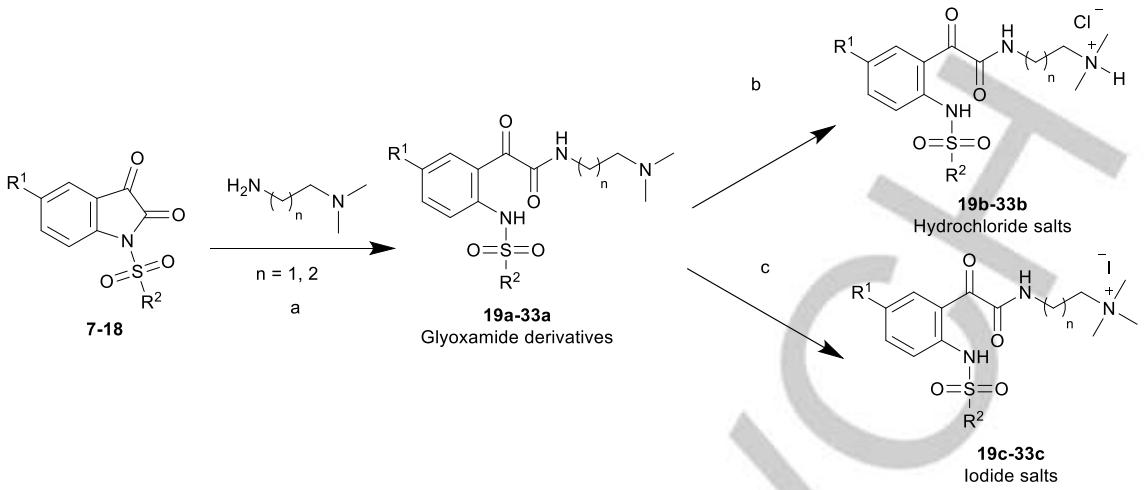


Scheme 1. Synthesis of *N*-sulfonylisatins **7-18**.

Table 1. Yields of synthesised *N*-sulfonylisatins.

Entry	R^1	R^2	*N*-sulfonylisatin (yield)
1	H	Phenyl	**7** (86%)
2	H	4-Tolyl	**8** (78%)
3	H	Octyl	**9** (50%)
4	H	Butyl	**10** (50%)
5	Br	Phenyl	**11** (52%)
6	Br	4-Tolyl	**12** (48%)
7	Br	Octyl	**13** (51%)
8	Br	Butyl	**14** (32%)
9	Br	2-Naphthalenyl	**15** (26%)
10	Cl	Octyl	**16** (50%)
11	F	Octyl	**17** (23%)
12	Me	Octyl	**18** (64%)

After the successful synthesis of *N*-sulfonylisatins **7-18**, the ring-opening reactions were performed by reacting *N*-sulfonylisatins **7-18** with either *N*,*N*-dimethylethane-1,2-diamine or *N*,*N*-dimethylpropane-1,3-diamine in dichloromethane at room temperature for 1-24 h to afford the novel phenylglyoxamide derivatives **19a-33a** in 95-99% yields (Scheme 2**,** Table 2).



Scheme 2. Synthesis of hydrochloride salts **19b-33b** and iodide salts **19c-33c** from *N*-sulfonylisatins **7-18**. Reaction conditions: (a) DCM, rt, 1-24 h; (b) 4 M HCl/dioxane, DCM/diethyl ether, rt, 20 min-6 h; (c) CH_3I, DCM/THF, rt, 14-48 h.

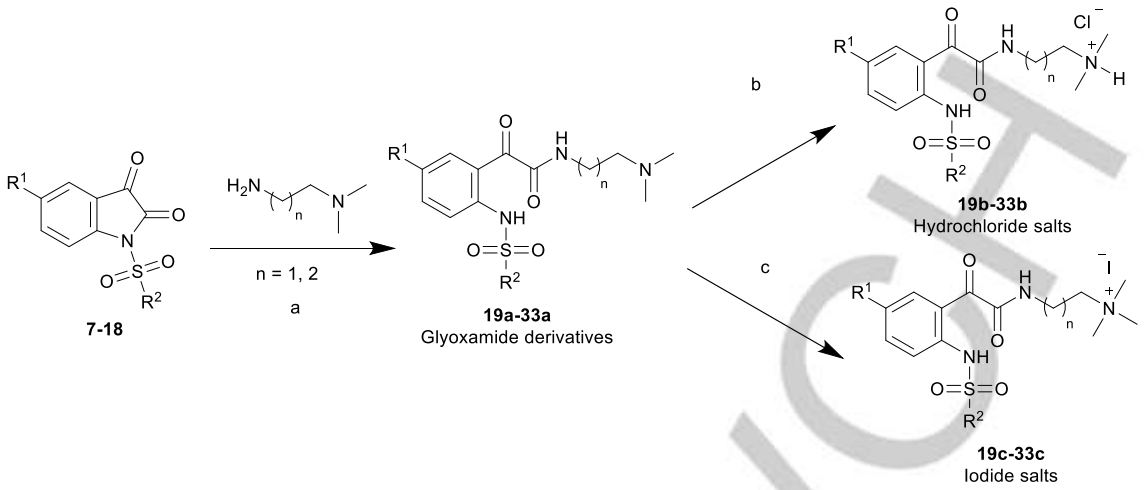
Table 2. Yields of synthesised glyoxamide derivatives**,** hydrochloride salts and iodide salts.

Entry	R^1	R^2	n	Glyoxamide derivative (yield)	Hydrochloride salt (yield)	Iodide salt (yield)
1	H	Phenyl	2	**19a** (99%)	**19b** (97%)	**19c** (99%)
2	H	4-Totyl	2	**20a** (97%)	**20b** (100%)	**20c** (94%)
3	H	Octyl	1	**21a** (98%)	**21b** (90%)	**21c** (82%)
4	H	Octyl	2	**22a** (96%)	**22b** (70%)	**22c** (81%)
5	H	Butyl	2	**23a** (99%)	**23b** (100%)	**23c** (99%)
6	Br	Phenyl	2	**24a** (95%)	**24b** (99%)	**24c** (100%)
7	Br	4-Totyl	2	**25a** (99%)	**25b** (85%)	**25c** (87%)
8	Br	Octyl	1	**26a** (97%)	**26b** (88%)	**26c** (97%)
9	Br	Octyl	2	**27a** (97%)	**27b** (94%)	**27c** (100%)
10	Br	Butyl	2	**28a** (99%)	**28b** (100%)	**28c** (72%)
11	Br	2-Naphthalenyl	1	**29a** (97%)	**29b** (67%)	**29c** (98%)
12	Br	2-Naphthalenyl	2	**30a** (97%)	**30b** (85%)	**30c** (83%)
13	Cl	Octyl	2	**31a** (97%)	**31b** (93%)	**31c** (86%)
14	F	Octyl	2	**32a** (95%)	**32b** (60%)	**32c** (79%)
15	Me	Octyl	2	**33a** (97%)	**33b** (80%)	**33c** (63%)

The phenylglyoxamide compounds were then converted to the corresponding ammonium salts by treatment with either HCl or methyl iodide. For the formation of hydrochloride salts **19b-33b**, phenylglyoxamide derivatives **19a-33a** were treated with 4 M hydrogen chloride/dioxane in dichloromethane or diethyl ether at room temperature for 20 min to 6 h. The yellow reaction mixture was then concentrated *in vacuo* to remove the solvent and excess hydrogen chloride/dioxane solution, and the precipitated crude product was washed with diethyl ether to remove unreacted phenylglyoxamide starting material, affording the hydrochloride salts **19b-33b** in 60-100% yields (Scheme 2, Table 2).

Similarly, the synthesis of the iodide salts **19c-33c** involved the reaction of phenylglyoxamide derivatives **19a-33a** with iodomethane in either dichloromethane or tetrahydrofuran (THF) at room temperature for 14-48 h. The reaction mixture was then concentrated *in vacuo* to remove the solvent and excess iodomethane, and the precipitated crude product was washed with diethyl ether to remove phenylglyoxamide starting material to afford iodide salts **19c-33c** in 63-100% yields (Scheme 2, Table 2).

We also investigated the installation of the guanidine group at the end of the glyoxamide chain of analogues, in place of the tertiary or quaternary ammonium group. The first step of the reaction sequence involved the ring-opening of 1-octanesulfonylisatins **9** and **13** and 2-naphthalenesulfonylisatin **15** with *tert*-butyl *N*-(3-aminopropyl)carbamate in dichloromethane at room temperature for 15-21 h, which afforded glyoxamide derivatives **34a-36a** in 97-98% yields (Scheme 3, Table 3). Solutions of the glyoxamide derivatives **34a-36a** in dichloromethane were then treated with 4 M hydrogen chloride/dioxane at room temperature for 18 h to cleave the Boc group and liberate the amine functionality. After full conversion of starting material to product, as evidenced by TLC, the solvent and unreacted reagents were removed *in vacuo* and the residue was washed with diethyl ether to remove traces of starting material and impurities to afford aminoglyoxamide hydrochloride salts **34b-36b** in 82-87% yields.



Scheme 3. Synthetic route for the synthesis of guanidine hydrochloride salts **34d-36d**.

Table 3. Yields of synthesised guanidine hydrochloride salts and their corresponding intermediates.

Entry	R^1	R^2	Boc-protected glyoxamide (yield)	Amino- glyoxamide hydrochloride salt (yield)	Boc-protected guanidine glyoxamide (yield)	Guanidine hydrochloride salt (yield)
1	H	Octyl	**34a** (98%)	**34b** (82%)	**34c** (33%)	**34d** (71%)
2	Br	Octyl	**35a** (98%)	**35b** (83%)	**35c** (51%)	**35d** (72%)
3	Br	2-Naphthalenyl	**36a** (97%)	**36b** (87%)	**36c** (36%)	**36d** (67%)

The aminoglyoxamide hydrochloride salts **34b-36b** were converted to the free amine by treatment with 2.5 equivalents of triethylamine, and then reacted with 1.0 equivalent of *N,N'*-di-Boc-1*H*-pyrazole-1-carboxamidine in dichloromethane at room temperature for 15-18 h to give the Boc-protected guanidine glyoxamides **34c-36c**. The Boc-protected guanidine glyoxamide **34c** could be isolated in 33% yield after purification by flash column chromatography on silica, however, analogues **35c-36c** were isolated in a very poor yield of 3% using the same procedure (Scheme 4, Table 4). We observed that during the synthesis of glyoxamide **35c**, a significant amount of white precipitate was found in the reaction mixture, which was removed by filtration before column chromatography. This white precipitate was not observed during the synthesis of analogue **34c**. NMR spectroscopy study revealed that the identity of the white precipitate was not the aminoglyoxamide **35b** starting material, as evidenced by the absence of a broad amine singlet peak integrating to 2 protons. Moreover, the spectrum of the white precipitate lacked hydrochloride signals. This led to the hypothesis that instead of reacting with *N,N'*-di-Boc-1*H*-pyrazole-1-carboxamidine, the terminal amine group of aminoglyoxamide **35b** reacted instead with the carbonyl group of the glyoxamide moiety of another molecule of aminoglyoxamide **35b,** to form the dimeric imine product **37** (Figure 4). The formation of dimer was confirmed by the high-resolution mass spectrum of the white precipitate, which showed a molecular peak at *m/z* 939.1939 that was consistent with the molecular formula $C_{38}H_{56}Br_2N_6O_6S_2Na$ ($[M+Na]^+$ required 939.1941). The corresponding dimeric product could have also been formed in the synthesis of analogue **34c**, however, due to its higher solubility in dichloromethane, it may not have precipitated out of the reaction mixture. With the identity of the dimeric side product determined, the reaction conditions (such as solvent, different amount and type of base and amount of *N,N'*-di-Boc-1*H*-pyrazole-1-carboxamidine) for the synthesis of **35c** were optimised (Table 4). The results showed that the use of acetonitrile as a solvent gave the highest yield of product **35c** in this reaction. The optimised conditions (Entry 6) were then also applied to aminoglyoxamide **36b**, producing Boc-protected guanidine glyoxamide **36c** in 36% yield.

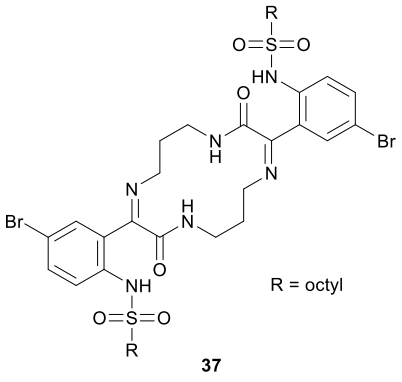
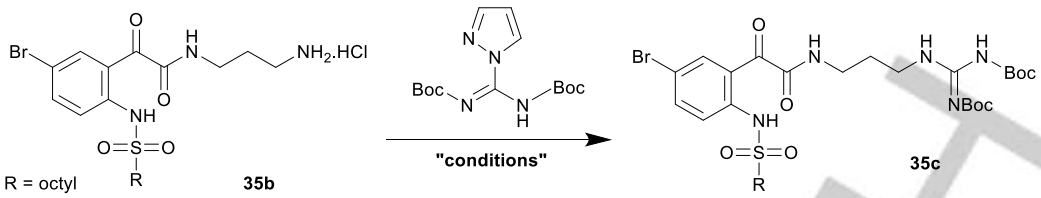


Figure 4. Chemical structure of compound **37**.



Scheme 4. Synthesis of **35c** (conditions are listed in **Table 4**)

Table 4. Conditions tested for optimising the synthesis of **35c**.

Entry	Base	Solvent	*N,N'*-di-Boc-1*H*-pyrazole-1-carboxamidine	Time (h)	Yield (%)
1	2.5 eq. Et_3N	DCM	1.0 eq.	15	3
2	2.5 eq. Et_3N	DCM	1.0 eq.	30	16
3	1.0 eq. Et_3N	DCM	1.0 eq.	15	6
4	2.5 eq. Et_3N	DCM	1.2 eq.	15	11
5	2.5 eq. Et_3N	ACN	1.2 eq.	15	51
6	2.5 eq. DIPEA	ACN	1.2 eq.	15	51
7	2.5 eq. DIPEA	DMF	1.2 eq.	15	31

Finally, Boc-protected guanidine glyoxamides **34c-36c** were deprotected by reacting with trifluoroacetic acid in dichloromethane (1:1) at room temperature for 2 h. After the completion of reaction as monitored by TLC, the dichloromethane and excess trifluoroacetic acid were removed *in vacuo* and impurities were removed by washing the precipitated product with diethyl ether. An ion exchange reaction was then carried out by treating the trifluoroacetate salt with 4 M hydrogen chloride/dioxane in dichloromethane at room temperature for 30 min. Dichloromethane and excess hydrogen chloride/dioxane solution were removed *in vacuo* and the impurities were again removed by washing the crude solid with diethyl ether to afford the guanidine hydrochloride salts **34d-36d** in 67-72% yields.

The overall synthesis of the hydrochloride and iodide salts was achieved in only three steps from isatin and did not require any chromatographic purification. In the first step, *N*-sulfonylisatins **7-18** were synthesised in moderate to high yields (48-86%) except compounds **14** (32%), **15** (26%) and **17** (23%). These compounds were isolated *via* washing with methanol and could be used in the next step without further purification. The ring-opening reactions of the *N*-sulfonylisatins produced glyoxamide derivatives **19a-33a** that could be isolated in sufficient purity using simple aqueous work-up. Finally, the salt compounds **19b-33c** were also isolated as pure compounds *via* washing with diethyl ether. For the guanidine series, the intermediates **34-36a** and **34b-36b** as well as the targeted **34d-36d** guanidine hydrochloride salts were all isolated either by filtration with washing or *via* aqueous work-up. The only compounds that were purified by column chromatography were the Boc-protected guanidine glyoxamides **34c-36c**. ^{1}H NMR was used to confirm the structure of all synthesised compounds, with the presence of the sulfonamide NH proton (δ 10-11) and the glyoxamide NH proton (δ 8-9) being characteristic for compounds of these series. This novel synthetic strategy therefore provides a new and efficient pathway for the synthesis of *N*-sulfonylisatins and their glyoxamide derivatives, allowing for the development and elucidation of structure-activity relationship (SAR) of glyoxamide-based antibacterial agents.

Antibacterial activity

The antibacterial activity of the synthesised salt compounds **19b-33c** and **34d-36d** was evaluated by the determination of zone-of-inhibition diameter using the disk diffusion assay and minimum inhibitory concentration (MIC) values from the MIC assay.

Disk diffusion assay

The synthesized compounds **19b-33c** and **34d-36d** were first screened by using the disk diffusion assays against *Staphylococcus aureus* (SA38) and *Pseudomonas aeruginosa* (PAO1). In this assay, bacterial cultures were incubated overnight in tryptone soya broth (TSB) at 37 °C and the resulting bacterial cultures were spread onto nutrient agar plates. The compounds to be tested were administered to sterile 6 mm paper disks at 80 nmol per disk as solutions in DMSO and dried. A well-known antibiotic, gentamicin (0.01 mg per disk) was used as positive control. The diameter of zone of inhibition was measured after incubation at 37 °C for 24 h (Table 5).

In this assay, active analogues showed zones of inhibition ranging from 7 to 16 mm against *S. aureus*. The most active compounds against *S. aureus* in this assay were the guanidine hydrochloride salts **34d** and **35d** which showed zone of inhibition values of 16 and 15 mm against *S. aureus* respectively. However, none of the compounds synthesised showed antibacterial activity against the *P. aeruginosa* strain in the disk diffusion assay.

The antibacterial potency of compounds cannot be determined quantitatively by the disk diffusion assay due to the different diffusion rates of compounds in agar. Therefore, compounds which showed activities in the disk diffusion assay were further analysed by determining their minimum inhibitory concentration (MIC) values.

Minimum inhibitory concentration (MIC) assay

Compounds that showed antibacterial activities in the disk diffusion assay were then tested in the minimum inhibitory concentration assay to quantitatively determine the concentration of compound required to inhibit bacterial growth in liquid media. In this assay, *S. aureus* SA38 in TSB was incubated with the compounds with final concentration ranging from 8-250 μM at 37 °C for 24 h. The optical density (OD) value at 600 nm was then measured at the end of the 24 h period. The MIC value of each compound was determined as the lowest

Table 5. Zone of inhibition (in mm) and minimum inhibitory concentration (in μM) of compounds against *S. aureus* (SA38).

Compound	Zone of inhibition (mm)	MIC (μM)	Compound	Zone of inhibition (mm)	MIC (μM)	Compound	Zone of inhibition (mm)	MIC (μM)
19a	–	ND	**19b**	–	ND	**19c**	–	ND
20a	–	ND	**20b**	–	ND	**20c**	–	ND
21a	7	63	**21b**	7	250	**21c**	9	125
22a	7	250	**22b**	–	ND	**22c**	9	250
23a	–	ND	**23b**	–	ND	**23c**	–	ND
24a	–	ND	**24b**	–	ND	**24c**	–	ND
25a	–	ND	**25b**	7	>250	**25c**	–	ND
26a	7	94	**26b**	10	63	**26c**	11	63
27a	9	47	**27b**	11	94	**27c**	13	63
28a	–	ND	**28b**	–	ND	**28c**	–	ND
29a	8	>250	**29b**	8	>250	**29c**	7	>250
30a	8	250	**30b**	10	>250	**30c**	8	>250
31a	10	47	**31b**	12	94	**31c**	11	94
32a	8	63	**32b**	9	94	**32c**	9	94
33a	8	125	**33b**	9	250	**33c**	11	250
34d	16	47	**35d**	15	12	**36d**	12	94
Gentamicin	23	ND	**MSI-78**[a]	ND	8-16			

ND = Not determined, [a] Literature value[25]

concentration that completely inhibited bacterial growth as indicated by an OD value of equal or less than the control with no bacteria (Table 5). Among the tested compounds, the guanidine hydrochloride salt **35d** showed the lowest MIC value of 12 μM against *S. aureus*. The antibacterial activity of **35d** was comparable with the positive control compound **MSI-78**, which is a 22-amino acid AMP that is under phase III clinical trials as a topical antibiotic.[26]

Structure-activity relationship studies

The results of the biological assays were used to investigate the structural-activity relationships of these *N*-sulfonyl-phenylglyoxamide-based compounds. Modifying the substituents appended to the *ortho* sulfonamide group had the greatest influence on the antibacterial activity of a compound. Among all synthesised compounds, only compounds with an octyl group (**22a, 22c** and **27a-27c**) or 2-naphthalenyl group (**29a-30c**) appended to the *ortho* sulfonamide group showed antibacterial activity in the disk diffusion assay, whereas compounds with phenyl or tosyl group attached to the sulfonamide group (*e.g.* **19a-20c** and **23a-24c**) showed no antibacterial activity. Compounds **32a-33c** bearing a butyl chain instead of an octyl chain attached to the sulfonamide group also showed no antibacterial activity in the disk diffusion assay, indicating that a longer alkyl group is preferred Furthermore, the MIC data showed that octanesulfonamide compounds had better antibacterial activity (MIC = 47-250 μM) than the naphthalenesulfonyl compounds (MIC ≥ 250 μM).

Moreover, compounds bearing a bromine substituent at the 5-position of the phenyl ring showed significantly higher antibacterial activities. For instance, 5-Br octyl compounds **27a** and **27c** showed MIC values of 47 and 63 μM against S. aureus, respectively, meaning that they were at least 4 times more potent than the parent 5-unsubstituted compounds **22a** and **22c** which both had MIC values of 250 μM (**22b** was not tested in the MIC assay). Further comparison between compounds indicated that antibacterial ability was diminished once the halogen was removed from the 5-position of the phenyl ring. Specifically, octyl compounds **27a-27c** (5-Br), **31a-31c** (5-Cl) and **32a-32c** (5-F) showed MIC values of 47-94 μM, whereas compounds **22a**,**c** (5-H) and **33a-33c** (5-Me) showed MIC values of 125-250 μM. Among the three types of halogen analogues, the bromo-substituted compounds showed higher antibacterial activity compared to the fluoro or chloro compounds.

From the disk diffusion assay (Table 5), the propylene-linked compounds (**22a-c, 27a-c, 30a-30c**) showed similar or higher potencies compared to the corresponding ethylene-linked compounds (**21a-c, 26a-c, 29a-29c**). However, in the MIC assay, the relationship between linker length and antibacterial activity was less clear (Table 5). For example, while the 3-carbon propylene glyoxamide derivative **27a** (MIC = 47 μM) was more potent than its corresponding 2-carbon ethylene glyoxamide derivative **26a** (MIC = 94 μM), the opposite trend was observed when comparing the 3-carbon propylene glyoxamide derivative **22a** (MIC = 250 μM) with the corresponding 2-carbon ethylene glyoxamide derivative **21a** (MIC = 63 μM).

From the MIC assay, the glyoxamide derivatives and their corresponding hydrochloride salts and iodide salts showed similar antibacterial activities, with the glyoxamide derivatives having slightly higher potencies than the ionic salts. Interestingly, when a guanidine group was installed at the end of the glyoxamide chain, the antibacterial activities of compounds increased significantly. Compounds **34d** and **35d** with a guanidine hydrochloride group showed a remarkable improvement (4-5 fold) in antibacterial activity compared to their corresponding glyoxamide derivatives **22a** and **27a** respectively.

In summary, the SAR analysis for these *N*-sulfonyl-phenylglyoxamides suggested that having an octyl chain on the sulfonamide group and a 5-bromo substituent at the phenyl ring were the most desirable for antibacterial activity. Moreover, while the glyoxamide amine derivatives showed only slightly higher potencies than the corresponding hydrochloride or methyl iodide salts, compounds with a terminal guanidine hydrochloride group possessed markedly enhanced antibacterial activity. Finally, no significant difference in potency was found between compounds with the 3-carbon propylene linker and those with the 2-carbon ethylene linker.

Membrane conduction

To test how selected potent phenylglyoxamide compounds (**27a**, **26b**, **34d**, **35d**) might interact with cell membranes, tethered bilayer lipid membrane (tBLM) technology was used in conjunction with electrical impedance spectroscopy.[22b, 27] By anchoring a lipid membrane onto a gold substrate, it is possible to measure changes in intrinsic membrane conduction in real time upon addition of the compounds. The addition of 30% palmitoyl-oleoyl-phosphatidylglycerol (POPG) lipids gives the membrane an overall negative charge as would be found in many bacterial cell membranes.

Conduction versus concentration graph for the compounds is depicted in Figure 5. The changes in membrane conduction as a result of adding the molecules were compared to the response of a known pore-forming antimicrobial LL-37.[28]

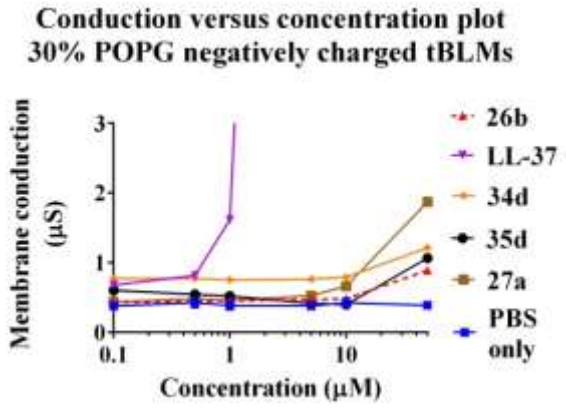


Figure 5. Conduction versus concentration graphs of selected potent compounds **27a**, **26b**, **34d**, **35d** and **LL-37** for tethered bilayer lipid membranes (tBLMs).

The results showed that high concentrations of phenylglyoxamide compounds could induce an increase in membrane conduction in the tBLM assay. We hypothesize that the compounds might bind to lipid head groups and alter their relative packing density, which induces a change of curvature in lipid morphology into a more curved structure. This could induce lipids to relocate into membrane toroidal pore defects within lipid bilayers, increasing the pore surface area and thereby overall membrane conduction.[29]. However, although the phenylglyoxamide compounds had similar MIC values to LL-37 in the antibacterial assay (MIC = 49 µM), they showed much less activity than LL-37 in the tBLM assay. The increase in membrane conduction was observed only at relatively high concentrations of phenylglyoxamide (≥ 50 µM), whereas for LL-37, an effect on membrane conduction was evident at 1 µM. One reason for the lower activity of the phenylglyoxamides could be that they require a clustering arrangement of compounds into a certain multimeric state in order for pore to form. Regardless of the mechanism, the demonstrated increase in membrane conduction demonstrates the potential of these compounds to act as antimicrobial pore-forming agents.

Toxicity assay

The *in vitro* toxicity of selected potent compounds (**26b-c**, **27a**, **34d**, **35d**) was determined against MRC-5 normal human lung fibroblasts using the Alamar blue assay.[30] A dose-response curve for each compound was generated to determine the IC_{50} values (concentration of compound which reduced cell proliferation by 50% of the vehicle control). The IC_{50} values which were then used to determine the therapeutic window (IC_{50} value divided by MIC value), allowing the specificity of the compounds to be ranked (Figure 6, Table 6).

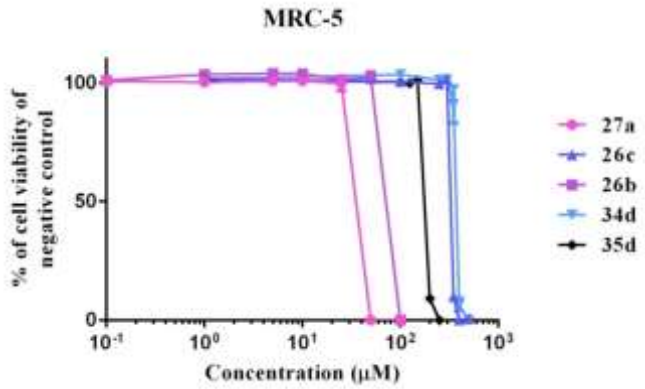


Figure 6. *In vitro* anti-proliferative properties of selected potent compounds (**26b-c**, **27a**, **34d**, **35d**) against MRC-5 normal human lung fibroblast cells after 72 h incubation, relative to a DMSO control.

The glyoxamide compound **27a** showed a high level of toxicity (IC_{50} = 37.4 µM) to human cells. This can be explained by the lack of cationic charge at the terminal group of compound **27a**. As a consequence, compound **27a** has higher hydrophobicity and can more easily bind to the uncharged human cell membrane. Compound **27a** showed a therapeutic window of less than 1, meaning that it would be unlikely to be useful as an antibacterial drug.

While the hydrochloride salt **26b** and iodide salt **26c** had the same values, hydrochloride salt **26b** (IC_{50} = 75.5 µM) showed a higher toxicity towards human cells than the iodide salt **26c** (IC_{50} = 328 µM). Additionally, comparison between the two guanidine hydrochloride salts **34d-35d** showed that compound **35d** (IC_{50} = 178 µM) containing the more hydrophobic bromine substituent at the 5-position of the phenyl ring was more toxic than compound **34d** (IC_{50} = 373 µM) which has a hydrogen atom. This suggested that there might be a positive correlation between the hydrophobicity and the toxicity of the compounds towards human cells.

Although the guanidine hydrochloride salt **35d** had a relatively low IC_{50} value compared to compounds **26c** and **34d**, it also had a significantly lower MIC value which resulted in a higher therapeutic window value. Overall, these data suggest that guanidine hydrochloride salts of glyoxamides could potentially be useful scaffolds for the further development of antimicrobial drugs to treat human bacterial infection.

Conclusions

This study has described an efficient synthetic and purification method for the preparation of *N*-sulfonylisatins. Twelve *N*-sulfonylisatins were synthesised, and these were then ring-opened with *N*,*N*-dimethylethane-1,2-diamine or *N*,*N*-dimethylpropane-1,3-diamine, and subsequently converted to hydrochloride or iodide salts to generate a library of 30 novel small molecular AMP mimics. Furthermore, three guanidine hydrochloride mimics were also generated *via* ring-opening reaction of *N*-sulfonylisatins with Boc-protected amine, followed by Boc-deprotection and installation of the guanidine moiety. *In vitro* biological studies of analogues identified that an octyl group appended to *ortho* sulfonamide group was essential for high antibacterial potency. Moreover, the presence of a halogen at the 5-position of phenyl ring and a terminal guanidine hydrochloride group also enhanced the antibacterial potency of the compounds. Guanidine hydrochloride salt **35d**, possessing all three favourable elements, possessed the highest antibacterial potency with an acceptable therapeutic window. Guanidine hydrochloride salt **34d** and iodide salt **26c** also showed an acceptable therapeutic window despite having lower antibacterial potency. Mechanistically, these compounds could act as antimicrobial pore-forming agents as evidenced by an increase in membrane conduction using a tBLM assay. Thus, this novel class of phenylglyoxamide-based small molecular AMP mimics represents an innovative pathway for the development of novel and cost-effective antimicrobial agents.

Experimental Section

Chemistry

All commercially available reagents were purchased from standard suppliers (Sigma Aldrich and Alfa-Aesar) and used without further purification. All reactions were performed under anhydrous condition with an atmosphere of nitrogen and anhydrous solvent unless otherwise specified and anhydrous solvents were obtained using PureSolv MD Solvent Purification System. Reactions were monitored by thin layer chromatography precoated with Merck silica gel 60 F_{254} and visualization was performed by using short or long wavelength of ultraviolet light. Flash chromatography was carried out using Grace Davisil LC60A silica. Melting points were measured using an OptiMelt melting point apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were obtained in the specified solvents on a Bruker Avance III HD 400 or Bruker Avance III 600 Cryo spectrometer. Chemical shift (δ) are in parts per million (ppm) internally referenced to the solvent nuclei. Multiplicities are assigned as singlet (s), broad singlet (bs), doublet (d), triplet (t), quartet (q), quintet (quint), sextet (sext), septet (sept), multiplet (m) or a combination of these (*e.g.* dd, dt, td), and coupling constants (*J*) are described in Hertz (Hz). Infrared (IR) spectra were recorded using a Cary 630 FTIR spectrometer fitted with a diamond attenuated total reflectance (ATR) sample interface. UV-Vis spectra were recorded using a Cary 100 Bio double beam UV-Vis spectrometer in the specified solvents and data are reported as wavelength (λ) in nm and absorption coefficient (ε) in $M^{-1}cm^{-1}$. High-resolution mass spectrometry (HRMS) was performed by the UNSW Bioanalytical Mass Spectrometry Facility using a Thermo LTQ Orbitrap XL instrument.

General synthetic procedure A for *N*-sulfonylisatin compounds (7-18). To a solution of appropriate isatin (1.0 equivalent) in dichloromethane (10 mL) was added triethylamine (1.1 equivalents) at 0 °C under nitrogen atmosphere. The reaction mixture was stirred at 0 °C for 20 min. An appropriate sulfonyl chloride (1.0 equivalent) was added slowly to the reaction mixture at 0 °C. The reaction mixture was then stirred at room temperature for 3-24 h. The resulting mixture was concentrated *in vacuo* and washed with methanol to afford the product.

General synthetic procedure B for glyoxamide derivatives and Boc-protected glyoxamides (19a-36a). To a solution of sulfonylisatin or arylisatin (1.0 equivalent) in dichloromethane (5 mL) was added an appropriate amine (1.0 equivalent) at 0 °C under nitrogen atmosphere. The reaction mixture was stirred at room temperature for 1-24 h. After completion of the reaction, water was added to the reaction mixture and the product was extracted into dichloromethane (3 × 30 mL), washed with brine, dried over anhydrous sodium sulfate and concentrated *in vacuo* to afford the product.

General synthetic procedure C for hydrochloride and iodide salts (19b-33c). To a solution of glyoxamide derivative (1.0 equivalent) in dichloromethane, tetrahydrofuran or diethyl ether was added 4 M HCl/dioxane (5.0 equivalents) or iodomethane (2.5 equivalents). The reaction mixture was stirred at room temperature for 20 min to 48 h. After completion of reaction, the reaction mixture was concentrated *in vacuo*, washed with diethyl ether and freeze-dried to afford the product.

General synthetic procedure D for aminoglyoxamides (34b-36b). To a solution of Boc-protected glyoxamide (1.0 equivalent) in dichloromethane (10 mL) was added 4 M HCl/dioxane (3 mL). The reaction mixture was stirred at room temperature for 18 h. After completion of reaction, the reaction mixture was concentrated *in vacuo* and washed with diethyl ether to afford the product.

General synthetic procedure E for Boc-protected guanidine glyoxamides (34c-36c). To a solution of aminoglyoxamides (1.0 equivalent) and *N,N'*-di-Boc-1*H*-pyrazole-1- carboxamidine (1.2 equivalents) in dichloromethane or acetonitrile (10 mL) was added triethylamine (2.5 equivalents) at 0 °C under nitrogen atmosphere. The reaction mixture was stirred at room temperature for 15-18 h. After completion of the reaction, the mixture was filtered, followed by concentrating *in vacuo*. The product was purified by flash chromatography on silica using ethyl acetate/*n*-hexane (1:4) as eluent to afford the product.

General synthetic procedure F for guanidine hydrogen chloride salts (34d-36d). To a solution of Boc-protected guanidine glyoxamide (1 equivalent) in dichloromethane (1 mL) was added trifluoroacetic acid (1 mL). The reaction mixture was stirred at room temperature for 2 h. After completion of the reaction, the reaction mixture was concentrated *in vacuo* and washed with diethyl ether. To the residue in dichloromethane (1 mL) was added 4 M HCl/dioxane (1 mL). The reaction mixture was stirred at room temperature for 30 min. After completion of reaction, the reaction mixture was concentrated *in vacuo* and washed with diethyl ether to afford the product.

5-Bromo-1-(octylsulfonyl)indoline-2,3-dione (13). The title compound **13** was synthesised from 5-bromoisatin (1.06 g, 4.22 mmol), triethylamine (0.65 mL, 4.66 mmol) and octane-1-sulfonyl chloride (0.85 mL, 4.21 mmol) following **general synthetic procedure A**. Reaction stirred for 4 h. The product **13** was obtained as a yellow solid (0.96 g, 51%); mp 138.0-140.1 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.96-7.85 (m, 2H, ArH), 7.70-7.60 (m, 1H, ArH), 3.66-3.53 (m, 2H, CH_2), 1.83-1.72 (m, 2H, CH_2), 1.43-1.16 (m, 10H, CH_2), 0.89-0.79 (m, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6): δ 177.5 (CO), 156.0 (CO), 145.7 (ArC), 139.6 (ArCH), 127.1 (ArCH), 121.4 (ArC), 117.2 (ArC), 116.1 (ArCH), 53.7 (CH_2), 31.1 (CH_2), 28.4 (CH_2), 28.3 (CH_2), 27.3 (CH_2), 22.1 (CH_2), 22.0 (CH_2), 13.9 (CH_3); IR (ATR): υ_{max} 3063, 2914, 2849, 2341, 2112, 1740, 1594, 1458, 1370, 1174, 1137, 1268, 1114, 1061, 936, 844, 720, 783 cm^{-1}; UV-Vis (ACN): λ_{max} 223 nm (ε 18,600 cm^{-1} M^{-1}), 244 (20,400), 291 (4,800), 402 (748).

2-(5-Bromo-2-(octylsulfonamido)phenyl)-*N*-(2-(dimethylamino)ethyl)-2-oxoacetamide (26a) The title compound **38a** was synthesised from 5-bromo-1-(octylsulfonyl)indoline-2,3-dione **13** (0.16 g, 0.39 mmol) and *N*,*N*-dimethylethane-1,2-diamine (43 μL, 0.39 mmol) following **general synthetic procedure B**. Reaction stirred for 24 h. The product **26a** was obtained as a yellow oil (0.19 g, 97%); ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.68-8.65 (m, 1H, ArH), 7.70-7.66 (m, 2H, ArH), 7.53 (bs, 1H, NH), 3.50 (q, *J* = 5.4 Hz, 2H, CH_2), 3.17-3.10 (m, 2H, CH_2), 2.57 (t, *J* = 5.9 Hz, 2H, CH_2), 2.32 (s, 6H, CH_3), 1.82-1.72 (m, 2H, CH_2), 1.40-1.18 (m, 10H, CH_2), 0.86 (t, *J* = 6.6 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 190.3 (CO), 162.0 (CO), 141.0 (ArC), 139.3 (ArCH), 137.5 (ArCH), 120.5 (ArC), 119.8 (ArCH), 115.3 (ArC), 57.3 (CH_2), 52.9 (CH_2), 45.2 (CH_3), 37.0 (CH_2), 31.8 (CH_2), 29.0 (CH_2), 29.0 (CH_2), 28.2 (CH_2), 23.5 (CH_2), 22.7 (CH_2), 14.2 (CH_3); IR (ATR): υ_{max} 3054, 2292, 2853, 2386, 2102, 1649, 1525, 1460, 1388, 1331, 1285, 1193, 1146, 1099, 971, 913, 824, 711 cm^{-1}; UV-Vis (MeOH): λ_{max} 232 nm (ε 17,000 cm^{-1} M^{-1}), 344 (1,500); HRMS (+ESI): Found *m/z* 490.1373 $[M+H]^+$, $C_{20}H_{33}BrN_3O_4S$ required 490.1370.

2-(2-(5-Bromo-2-(octylsulfonamido)phenyl)-2-oxoacetamido)-*N*,*N*-dimethylethan-1-aminium chloride (26b). The title compound **26b** was synthesised from 2-(5-bromo-2-(octylsulfonamido)phenyl)-*N*-(2-(dimethylamino)ethyl)-2-oxoacetamide **26a** (20 mg, 0.041 mmol) and 4 M HCl/dioxane (0.10 mL, 0.40 mmol) in diethyl ether (10 mL) following **general synthetic procedure C**. Reaction stirred for 20 min. The product **26b** was obtained as a white sticky solid (19 mg, 88%); ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.24 (bs, 1H, NH), 9.99 (bs, 1H, NH), 9.09 (t, *J* = 5.8 Hz, 1H, NH), 7.99 (d, *J* = 2.4 Hz, 1H, ArH), 7.84 (dd, *J* = 8.8, 2.5 Hz, 1H, ArH), 7.44 (d, *J* = 8.8 Hz, 1H, ArH), 3.58 (q, *J* = 6.0 Hz, 2H, CH_2), 3.29-3.23 (m, 2H, CH_2), 3.20-3.13 (m, 2H, CH_2), 2.81 (s, 6H, CH_3), 1.69-1.59 (m, 2H, CH_2), 1.37-1.16 (m, 10H, CH_2), 0.84 (t, *J* = 6.6 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6): δ 189.0 (CO), 162.4 (CO), 137.2 (ArC), 136.7 (ArCH), 134.2 (ArCH), 128.4 (ArC), 124.6 (ArCH), 116.2 (ArC), 55.1 (CH_2), 51.6 (CH_2), 42.3 (CH_3), 34.2 (CH_2), 31.1 (CH_2), 28.4 (CH_2), 28.3 (CH_2), 27.3 (CH_2), 22.8 (CH_2), 22.0 (CH_2), 13.9 (CH_3); IR (ATR): υ_{max} 3258, 2922, 2852, 2695, 2106, 1671, 1529, 1488, 1389, 1331, 1199, 1140, 1093, 1019, 914, 830, 764, 700 cm^{-1}; UV-Vis (H_2O): λ_{max} 231 nm (ε 15,000 cm^{-1} M^{-1}); HRMS (+ESI): Found *m/z* 490.1373 $[M+H]^+$, $C_{20}H_{33}BrN_3O_4S$ required 490.1370.

2-(2-(5-Bromo-2-(octylsulfonamido)phenyl)-2-oxoacetamido)-*N*,*N*,*N*-trimethylethan-1-aminium iodide (26c). The title compound **26c** was synthesised from 2-(5-bromo-2-(octylsulfonamido)phenyl)-*N*-(2-(dimethylamino) ethyl)-2-oxoacetamide **26a** (31 mg, 0.063 mmol) and iodomethane (12 μL, 0.19 mmol) in tetrahydrofuran (3 mL) following **general synthetic procedure C**. Reaction stirred for 18 h. The product **26c** was obtained as a yellow sticky solid (39 mg, 97%); ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.97 (bs, 1H, NH), 9.13 (t, *J* = 5.7 Hz, 1H, NH), 7.93-7.82 (m, 2H, ArH), 7.41 (d, *J* = 8.6 Hz, 1H, ArH), 7.36-7.31 (m, 1H, ArH), 3.65 (q, *J* = 6.0 Hz, 2H, CH_2), 3.50 (t, *J* = 6.2 Hz, 2H, CH_2), 3.20-3.07 (m, 11H, CH_2, CH_3), 1.68-1.58 (m, 2H, CH_2), 1.36-1.14 (m, 10H, CH_2), 0.84 (t, *J* = 6.7 Hz, 3H, CH_3); ^{13}C NMR (150 MHz, DMSO-d_6): δ 189.3 (CO), 162.8 (CO), 137.2 (ArC), 136.9 (ArCH), 133.9 (ArCH), 129.1 (ArC), 125.2 (ArCH), 116.8 (ArC), 63.5 (CH_2), 52.7 (CH_3), 51.3 (CH_2), 33.5 (CH_2), 31.1 (CH_2), 28.4 (CH_2), 28.3 (CH_2), 27.3 (CH_2), 22.8 (CH_2), 22.0 (CH_2), 14.0 (CH_3); IR (ATR): υ_{max} 3366, 3276, 2921, 2852, 2321, 2100, 1647, 1478, 1386, 1334, 1316, 1195, 1146, 1040, 976, 916, 818, 722 cm^{-1}; UV-Vis (H_2O): λ_{max} 228 nm (ε 30,000 cm^{-1} M^{-1}); HRMS (+ESI): Found *m/z* 504.1526 $[M]^+$, $C_{21}H_{35}BrN_3O_4S$ required 504.1526.

2-(5-Bromo-2-(octylsulfonamido)phenyl)-*N*-(3-(dimethylamino) propyl)-2-oxoacetamide (27a). The title compound **27a** was synthesised from 5-bromo-1-(octylsulfonyl)indoline-2,3-dione **13** (0.15 g, 0.39 mmol) and *N*,*N*-dimethylpropane-1,3-diamine (49 μL, 0.39 mmol) following **general synthetic procedure B**. Reaction stirred for 18 h. The product **27a** was obtained as a yellow oil (0.37 g, 97%); ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.89 (bs, 1H, NH), 8.62 (t, *J* = 1.3 Hz, 1H, ArH), 7.69-7.66 (m, 2H, ArH), 3.52 (t, *J* = 6.0 Hz, 2H, CH_2), 3.15-3.10 (m, 2H, CH_2), 2.56 (t, *J* = 6.1 Hz, 2H, CH_2), 2.34 (s, 6H, CH_3), 1.84-1.73 (m, 4H, CH_2), 1.40-1.18 (m, 10H, CH_2), 0.86 (t, *J* = 6.6 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 190.8 (CO), 162.2 (CO), 140.8 (ArC), 139.1 (ArCH), 137.4 (ArCH), 120.9 (ArC), 120.0 (ArCH), 115.4 (ArC), 58.8 (CH_2), 52.8 (CH_2), 45.2 (CH_3), 40.0 (CH_2), 31.8 (CH_2), 29.1 (CH_2), 29.0 (CH_2), 28.2 (CH_2), 25.1 (CH_2), 23.5 (CH_2), 22.7 (CH_2), 14.2 (CH_3); IR (ATR): υ_{max} 3258, 3049, 2922, 2853, 2521, 2112, 1897, 1650, 1529, 1460, 1388, 1287, 1146, 1194, 1099, 972, 915, 823, 710 cm^{-1}; UV-Vis (MeOH): λ_{max} 231 nm (ε 24,100 cm^{-1} M^{-1}), 343 (1,610); HRMS (+ESI): Found *m/z* 504.1528 $[M+H]^+$, $C_{21}H_{35}BrN_3O_4S$ required 504.1526.

***tert*-Butyl(3-(2-(5-bromo-2-(octylsulfonamido)phenyl)-2-oxoacetamido)propyl) carbamate (35a)**. The title compound **35a** was synthesised from 5-bromo-1-(octylsulfonyl)indoline-2,3-dione **13** (0.21 g, 0.51 mmol) and *tert*-butyl (3-aminopropyl)carbamate (92 mg, 0.51 mmol) following **general synthetic procedure B**. Reaction stirred for 21 h. The product **35a** was obtained as a yellow oil (0.29 g, 98%); ^{1}H NMR (400 MHz, $CDCl_3$): δ 10.39 (bs, 1H, NH), 8.70-8.63 (m, 1H, ArH), 7.76 (bs, 1H, NH), 7.72-7.66 (m, 2H, ArH), 4.80 (bs, 1H, NH), 3.46 (q, *J* = 6.4 Hz, 2H, CH_2), 3.24 (q, *J* = 5.8 Hz, 2H, CH_2), 3.16-3.11 (m, 2H, CH_2), 1.83-1.70 (m, 4H, CH_2), 1.45 (s, 9H, CH_3), 1.40-1.19 (m, 10H, CH_2), 0.86 (t, *J* = 6.7 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 190.4 (CO), 162.2 (CO), 157.0 (CO), 141.0 (ArC), 139.3 (ArCH), 137.5 (ArCH), 120.4 (ArC), 119.8 (ArCH), 115.2 (ArC), 80.0 (C), 52.9 (CH_2), 37.2 (CH_2), 36.4 (CH_2), 31.8 (CH_2), 30.2 (CH_2), 29.0 (CH_2), 29.0 (CH_2), 28.5 (CH_3), 28.2 (CH_2), 23.5 (CH_2), 22.7 (CH_2), 14.2 (CH_3); IR (ATR): υ_{max} 3226, 3176, 2922, 2852, 2318, 2117, 1684, 1631, 1519, 1479, 1390, 1334, 1247, 1136, 1010, 914, 835, 723 cm^{-1}; UV-Vis (MeOH): λ_{max} 231 nm (ε 19,000 cm^{-1} M^{-1}), 347 (950); HRMS (+ESI): Found *m/z* 598.1559 $[M+Na]^+$, $C_{24}H_{38}BrN_3O_6SNa$ required 598.1557.

***N*-(3-Aminopropyl)-2-(5-bromo-2-(octylsulfonamido)phenyl)-2-oxoacetamide hydrochloride (35b)**. The title compound **35b** was synthesised from *tert*-butyl (3-(2-(5-bromo-2-(octylsulfonamido)phenyl)-2-oxoacetamido)propyl)carbamate **35a** (0.26 g, 0.45 mmol) following **general synthetic procedure D**. The product **35b** was obtained as a

yellow sticky solid (0.19 g, 83%); ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.08 (bs, 1H, NH), 8.99 (t, *J* = 5.8 Hz, 1H, NH), 8.12 (bs, 1H, NH), 7.96 (bs, 2H, NH), 7.90-7.83 (m, 2H, ArH), 7.46 (d, *J* = 8.7 Hz, 1H, ArH), 3.32-3.26 (m, 2H, CH_2), 3.20-3.15 (m, 2H, CH_2), 2.91-2.80 (m, 2H, CH_2), 1.87-1.78 (m, 2H, CH_2), 1.68-1.59 (m, 2H, CH_2), 1.36-1.16 (m, 10H, CH_2), 0.84 (t, *J* = 6.8 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6): δ 190.3 (CO), 162.9 (CO), 137.6 (ArC), 137.0 (ArCH), 134.2 (ArCH), 127.7 (ArC), 124.3 (ArCH), 116.2 (ArC), 51.4 (CH_2), 36.6 (CH_2), 35.9 (CH_2), 31.1 (CH_2), 28.3 (CH_2), 28.3 (CH_2), 27.3 (CH_2), 26.8 (CH_2), 22.8 (CH_2), 22.0 (CH_2), 13.9 (CH_3); IR (ATR): υ_{max} 3329, 3252, 2920, 2047, 1640, 1523, 1475, 1385, 1334, 1256, 1195, 1141, 1019, 916, 818, 762, 715 cm^{-1}; UV-Vis (H_2O): λ_{max} 231 nm (ε 19,000 cm^{-1} M^{-1}), 368 (790); HRMS (+ESI): Found *m/z* 476.1215 $[M+H]^+$, $C_{19}H_{31}BrN_3O_4S$ required 476.1213.

(*E*)-*N*-(*N*-(3-(2-(5-Bromo-2-(octylsulfonamido)phenyl)-2-oxoacetamido)propyl)-*N'*-((*tert*-butyloxidanyl)carbonyl) carbamimidoyl)-1-(*tert*-butyl)-oxidanecarboxamide (35c). The title compound **35c** was synthesised from *N*-(3-aminopropyl)-2-(5-bromo-2-(octylsulfonamido)phenyl)-2-oxoacetamide hydrochloride **35b** (0.11 g, 0.20 mmol), triethylamine (70 μL, 0.50 mmol) and *N,N'*-di-Boc-1*H*-pyrazole-1- carboxamidine (72 mg, 0.23 mmol) in acetonitrile (10 mL) following **general synthetic procedure E**. Reaction stirred for 15 h. The product **35c** was obtained as a yellow oil (74 mg, 51%); ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.47 (bs, 1H, NH), 10.52 (bs, 1H, NH), 8.68 (t, *J* = 5.9 Hz, 1H, NH), 8.52 (t, *J* = 6.2 Hz, 1H, NH), 8.46 (d, *J* = 2.0 Hz, 1H, ArH), 7.72-7.65 (m, 2H, ArH), 3.54 (q, *J* = 6.4 Hz, 2H, CH_2), 3.44 (q, *J* = 6.0 Hz, 2H, CH_2), 3.15-3.09 (m, 2H, CH_2), 1.83-1.72 (m, 4H, CH_2), 1.50 (s, 9H, CH_3), 1.40-1.19 (m, 19H, CH_2, CH_3), 0.86 (t, *J* = 6.7 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 191.4 (CO), 163.1 (CO), 163.0 (CN), 157.5 (CO), 153.4 (CO), 141.1 (ArC), 139.2 (ArCH), 137.3 (ArCH), 120.0 (ArC), 119.6 (ArCH), 115.1 (ArC), 83.8 (C), 79.7 (C), 52.9 (CH_2), 37.1 (CH_2), 35.8 (CH_2), 31.8 (CH_2), 30.1 (CH_2), 29.0 (CH_2), 29.0 (CH_2), 28.3 (CH_3), 28.2 (CH_2), 28.2 (CH_3), 23.5 (CH_2), 22.7 (CH_2), 14.2 (CH_3); IR (ATR): υ_{max} 3328, 3190, 2925, 2091, 1722, 1620, 1571, 1477, 1412, 1328, 1284, 1130, 1050, 1025, 903, 815, 767, 711 cm^{-1}; UV-Vis (MeOH): λ_{max} 233 nm (ε 64,000 cm^{-1} M^{-1}), 368 (1,200); HRMS (+ESI): Found *m/z* 718.2480 $[M+H]^+$, $C_{30}H_{49}BrN_5O_8S$ required 718.2480.

2-(5-Bromo-2-(octylsulfonamido)phenyl)-*N*-(3-guanidinopropyl)-2-oxoacetamide hydrochloride (35d). The title compound **35d** was synthesised from (*E*)-*N*-(*N*-(3-(2-(5-bromo-2-(octylsulfonamido)phenyl)-2-oxoacetamido)propyl)-*N'*-((*tert*-butyloxidanyl)carbonyl)carbamimidoyl)-1-(*tert*-butyl)-oxidanecarboxamide **35c** (30 mg, 0.042 mmol) following **general synthetic procedure F**. The product **35d** was obtained as a yellow sticky solid (17 mg, 72%); ^{1}H NMR (600 MHz, DMSO-d_6): δ 10.07 (bs, 1H, NH), 8.94 (bs, 1H, NH), 7.91-7.82 (m, 2H, ArH), 7.64 (t, *J* = 5.3 Hz, 1H, NH), 7.45 (d, *J* = 8.7 Hz, 1H, ArH), 7.34 (bs, 2H, NH), 7.03 (bs, 2H, NH), 3.26 (q, *J* = 6.6 Hz, 2H, CH_2), 3.20-3.15 (m, 4H, CH_2), 1.75-1.69 (m, 2H, CH_2), 1.66-1.60 (m, 2H, CH_2), 1.35-1.17 (m, 10H, CH_2), 0.84 (t, *J* = 7.0 Hz, 3H, CH_3); ^{13}C NMR (150 MHz, DMSO-d_6): δ 190.5 (CO), 163.0 (CO), 156.9 (CN), 137.7 (ArC), 137.0 (ArCH), 134.2 (ArCH), 127.6 (ArC), 124.2 (ArCH), 116.2 (ArC), 51.4 (CH_2), 38.3 (CH_2), 36.1 (CH_2), 31.1 (CH_2), 28.4 (CH_2), 28.3 (CH_2), 28.3 (CH_2), 27.3 (CH_2), 22.8 (CH_2), 22.0 (CH_2), 13.9 (CH_3); IR (ATR): υ_{max} 3337, 3178, 2923, 2852, 2101, 1640, 1523, 1477, 1384, 1326, 1135, 1051, 1026, 902, 817, 770, 711 cm^{-1}; UV-Vis (H_2O): λ_{max} 231 nm (ε 21,000 cm^{-1} M^{-1}), 368 (960); HRMS (+ESI): Found *m/z* 518.1425 $[M+H]^+$, $C_{20}H_{33}BrN_5O_4S$ required 518.1431.

Disk diffusion assay

The disk diffusion method was used to initially evaluate the antibacterial potential of the test compounds against *S. aureus* (strain 38) and *P. aeruginosa* (PAO1). Overnight cultures of bacteria were prepared in tryptone soya broth (TSB; Oxoid, UK) at 37 °C and the resulting bacterial cultures (200 μL) were spread onto nutrient agar plates. The compounds to be tested were dissolved in DMSO and dried on sterile 6 mm diameter paper disks (80 nmol per disk), and placed onto nutrient agar plates. Sterile disks loaded with gentamicin (0.01 mg per disk) were used as positive control. After incubation at 37 °C for 24 h, the diameter of the inhibition zone was measured to the nearest 1.0 mm.

Minimal inhibitory concentration (MIC) assay

Compounds that were shown active from disk diffusion assay were further assessed by determining their minimum inhibitory concentration (MIC) according to a previously published protocol.[31] A single colony of bacteria was cultured overnight in TSB at 37 °C. The resulting bacterial culture was collected by centrifugation and re-suspended in TSB twice. The optical density (OD) of the resulting culture was adjusted to OD_{600} = 0.1 in TSB (equivalent to 10^8 colony forming unit (CFU)/mL bacteria), and was further diluted to 10^5 CFU/mL in TSB. 100 μL of the bacterial solution was then added to wells of a 96-well plate containing 100 μL serially diluted compound, with final concentration ranging from 8-250 μM. The plates were then incubated at 37 ˚C for 24 h and the data was recorded by measuring the OD value at 600 nm using a Wallac Victor (Perkin-Elmer) microplate reader. The MIC value of each compound was determined as the lowest concentration that completely inhibited the growth of bacteria. Each experiment was performed in triplicate and was repeated in two independent experiments.

Tethered bilayer lipid membrane assay

Tethered bilayer lipid membranes (tBLMs) in association with alternating current electrical impedance spectroscopy techniques were employed to determine if the compounds are lytic to cell membranes. Sparsely tethered tBLMs were created using the solvent exchange technique described previously.[32] In short, pre-prepared tethered benzyl-disulfide (tetra-ethyleneglycol) n = 2 C20-phytanyl tethers benzyl-disulfide-tetra-ethyleneglycol-OH spacers in the ratio of 1:10 were coated onto a gold patterned polycarbonate slide (SDx Tethered Membranes Pty Ltd, Australia). Using a specialised cartridge chamber, a 3 mM solution of a standard mobile lipid phase [70% zwitterionic C20 Diphytanyl-Glycero-Phosphatidylcholine lipid: 30% C20 Diphytanyl-diglyceride-OH ether] supplemented with 30% palmitoyl-oleoyl-phosphatidylglycerol (POPG) (Avanti Lipids, USA) was added to the tethering chemistries to create negatively charged membranes like those present in bacterial species. All lipids were dissolved in 100% ethanol. Lipids were left for 2 minutes to associate with the tethering chemistries before being washed with 3 × 200 mL phosphate buffered saline (PBS). AC impedance spectrometry was then employed to verify the presence of the tethered lipid bilayers and to report on changes in membrane conduction as a result of adding the compounds.

Toxicity assay

Normal human lung fibroblasts MRC-5 were cultured in minimal essential medium (MEM, Invitrogen) supplemented with 10% foetal calf serum, 2% sodium bicarbonate, 1% L-glutamine-penicillin-streptomycin, 1% non-

essential amino acids (NEAA) and 1% sodium pyruvate. The cell line was maintained at 37 °C in 5% CO_2 as an adherent monolayer and was passaged upon reaching confluence by standard cell culture techniques. MRC-5 cells were then seeded at 2×10^4 cells per well in 96-well plates to ensure full confluence (quiescence). 24 h after seeding, cells were treated with 0.1 to 500 µM of compounds. After 72 h incubation, the treated media was replaced with fresh media containing 10% Alamar blue and the cells were incubated for another 6 h. The metabolic activity was detected by spectrophotometric analysis by assessing the absorbance of Alamar blue as previously described by O'Brien *et al*.[30] Cell proliferation was determined and expressed as a percentage of untreated control cells. The IC_{50} values were determined using GraphPad Prism 6.

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Keywords: antimicrobial peptide mimics • antibacterial activity • phenylglyoxamide • guanidine hydrochloride

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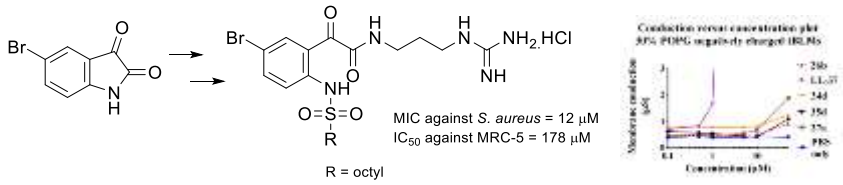

Potential new antibiotics: A series of novel *N*-sulfonyl-phenylglyoxamides antimicrobial peptide mimics have been synthesised starting from isatin *via* facile ring-opening reaction and evaluated for antibacterial efficacy. The lead compounds showed potent activity against *Staphylococcus aureus* at concentration non-toxic to human lung fibroblast cells.