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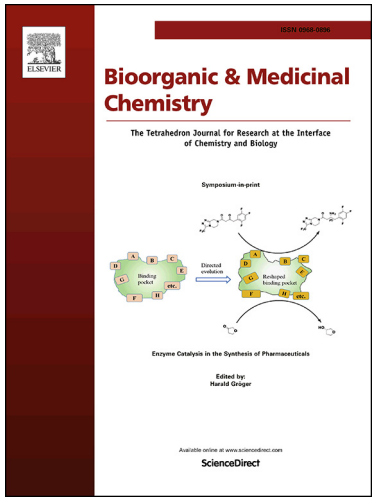

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Manuscript for submission

Amphipathic sulfonamidobenzamides mimicking small antimicrobial marine natural products; investigation of antibacterial and anti-biofilm activity against antibiotic resistant clinical isolates

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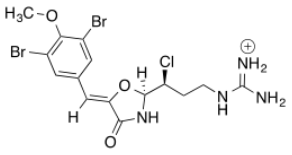
ABSTRACT

There is an urgent need for novel antimicrobial agents to address the threat of bacterial resistance to modern society. We have used a structural motif found in antimicrobial marine *hit compounds* as a basis for synthesizing a library of antimicrobial sulfonamidobenzamide *lead compound*s. Potent *in vitro* antimicrobial activity against clinically relevant bacterial strains was demonstrated for two compounds, **G6** and **J18**, with minimal inhibitory concentrations (MIC) of 4–16 μg/ml against clinical methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* (VRE). The two compounds **G6** and **J18**, together with several other compounds of this library, also caused ≥90% eradication of pre-established biofilm of methicillin-resistant *S. epidermidis* (MRSE) at 40 μg/ml. Using a luciferase assay, the mechanism of action of **G6** was shown to resemble the biocide chlorhexidine by targeting the bacterial cell membrane.

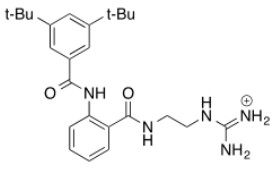
1.Introduction

Development of novel antimicrobial drugs is a high-risk business due to the general massive costs of any drug R&D program, potential limited sales volume, and restricted use of any innovative antimicrobial to avoid development of resistance.[1] Most antimicrobial drugs on the market interfere with highly specific targets in bacteria, and few novel unique targets have been identified. Design of antimicrobial agents killing bacteria through interactions with non-specific targets can be a valuable strategy to encounter the challenges of antimicrobial resistance. Cationic antimicrobial peptides (AMPs) have gained much attention in recent decades since they attack the cell membrane of bacteria.[2] The pharmacokinetics of AMPs may however disfavor them as drugs because of poor bioavailability and low proteolytic stability.

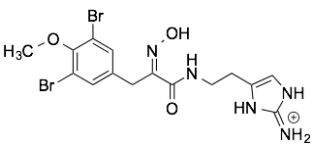
We have previously prepared a library of small cationic amphipathic aminobenzamides based on a common structural motif found in several isolated marine antimicrobials and that may also be considered as peptidomimetics of small AMPs (Figure 1).[3] The pharmacophore or active motif of this class of antimicrobial *marine natural product mimics* (MNPMs) was explored by investigating a central benzamide group linked to a lipophilic 3,5-di-*tert*-butyl-benzyl group and various cationic groups through amide bonds. Improved antimicrobial activity was achieved compared to the marine natural products that formed the motivating *hit molecules* for the design, such as ianthelline[4] and the synoxazolidinones.[5] This strategy has also been successfully explored in synthesis of amphipathic 1,2,3-triazole MNPM antimicrobials.[6,7]



Synoxazolidinone A



E23

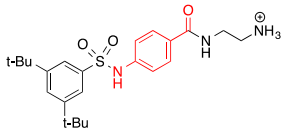


Ianthelline

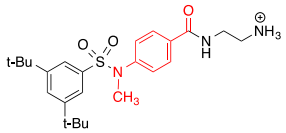
Figure 1. Isolated cationic amphipathic marine natural products *synoxazolidinone A*[5] and *ianthelline*,[4] and the synthetic aminobenzamide *marine natural product mimic* **E23**.[3]

In the present study we report a library of related molecules that contain a sulphonamide linker between the lipophilic and central spacer group, and we have further explored the importance of the central spacer group by varying the substitution pattern, and also including aliphatic spacer groups (Figure 2). The sulphonamide group is bioisosteric to an amide group but has also other characteristics, such as different conjugation properties and contains an acidic proton with possibility of ionization. An expanded panel of lipophilic groups were included together with the promising 3,5-di-*tert*-butyl-benzyl group. Primary amine and guanidine groups were used as cationic groups and varied in distance by two or three methylene groups in accordance to our previous results.[3] A structure-activity relationship (SAR) study was done by screening against bacterial reference strains of Gram-positive *Staphylococcus aureus*, methicillin resistant *Staphylococcus epidermidis* (MRSE), *Bacillus subtilis* and *Corynebacterium glutamicum*, and Gram-negative *Escherichia coli* and *Pseudomonas aeruginosa*. These studies were followed up by screening promising compounds from the **G**- to **L**-series against 275 clinical isolates deposited at the *Norwegian Organization for Surveillance of Resistant Microorganisms (NORM)* (2012-2014), and a panel of 30 multi-resistant clinical isolates from the strain collection at *The Norwegian National Advisory Unit on Detection of Antimicrobial Resistance*.[8-10] Promising compounds were also screened for their ability to eradicate preformed polysaccharide biofilm of MRSE and the *mode-of-action* was determined for two representative compounds.

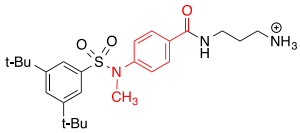
Amine derivatives:



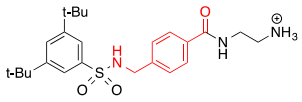
G1



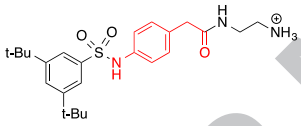
G3



G4



H7



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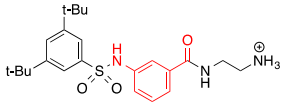
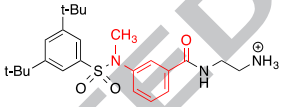
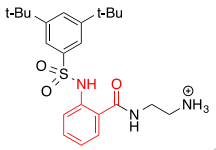


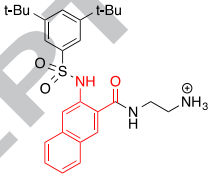
I11



I12

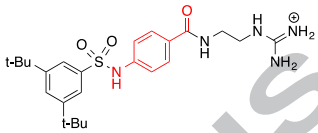


J15

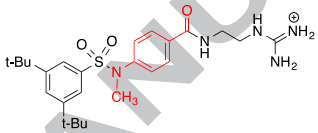


J16

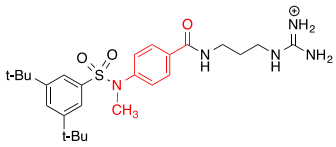
Guanidine derivatives:



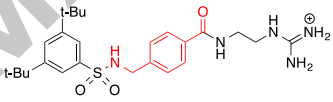
G2



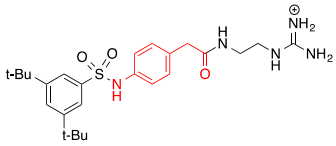
G5



G6



H9



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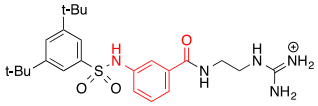

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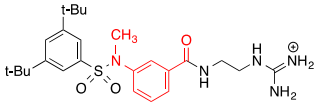

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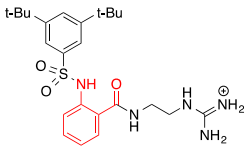
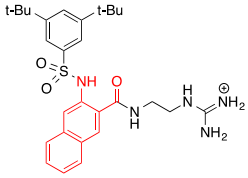
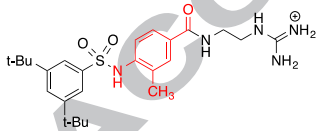


J17

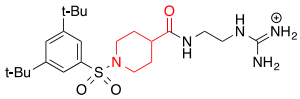


J18

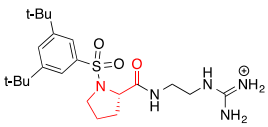
Variations in spacer group – guanidine derivatives:



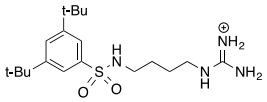
K19



K20

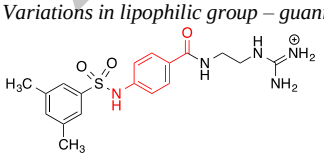


K21

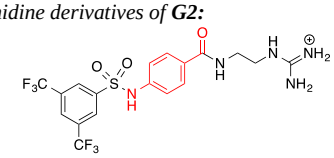


K22

*Variations in lipophilic group – guanidine derivatives of **G2:***



L23



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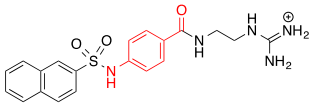

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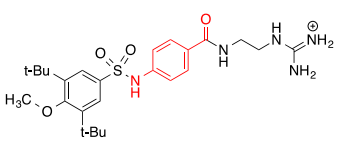


L26

Figure 2. Library of sulfonamidobenzamides synthesised and investigated for antimicrobial activity. All compounds were isolated and tested as HCl salts.

Results and discussion

2.1 Synthesis

An outline for synthesis of the library of sulfonamidobenzamides is shown in Scheme 1. For all compounds except **K22** the first step was formation of a sulphonamide bond linking the *lipophilic group* to the *spacer group* by reaction of the corresponding sulfonyl chlorides with the appropriate amines. The reactions were conducted without solvent at 150 °C and with 2 equivalents of the appropriate amines. In case of secondary amines; 1 equivalent of $NaHCO_3$ was added. The amide bond between the spacer group and cationic group was formed by amidation of the ester group with the appropriate aliphatic di-amines. Guanidine derivatives were prepared by reaction with 1-amidino-1H-1,2,4-triazole hydrochloride. In order to ensure optimal solubility in aqueous test media, the final compounds were isolated as HCl salts and purified by recrystallisation from MeOH and Et_2O.

2.2 Antimicrobial activity against bacterial reference strains and toxicity against human cells

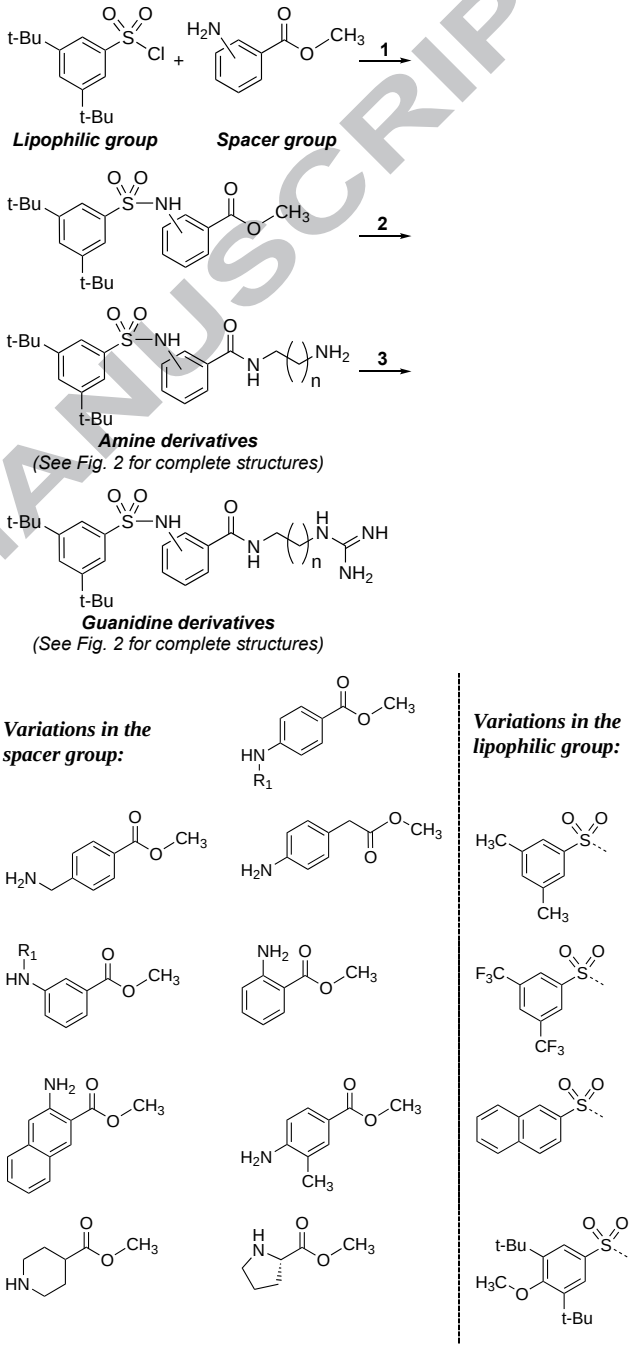
The library of compounds constituted six different structural series (**G** – **L**) as governed by the structures of the central spacer groups (Figure 2). Minimum inhibitory concentrations (MICs) were initially determined against a panel of Gram-positive and Gram-negative bacterial reference strains (Table 1). Hemolytic activity (EC_{50}) against human red blood cells (RBCs) and cytotoxicity (EC_{50}) against normal human lung fibroblasts (MRC-5) cells were used as a measurement of toxicity.[11] A selectivity index SI was calculated by comparing toxicity against RBC and MIC against MRSE ($EC_{50\ RBC}$ / MIC_{MRSE}).

As observed for the previously reported aminobenzamides,[3] the results showed in general higher antimicrobial activity against Gram-positive strains than against Gram-negative strains. Against Gram-positive strains the most potent compounds displayed MIC values of 1–4 µg/ml. Lower activity was obtained against Gram-negative strains with MIC values of 8–16 µg/ml for the most potent derivatives (Table 1).

A positive effect of guanylation on antimicrobial activity against Gram-positive strains was revealed by the **G**-series. The **G**-series contained a central 4-aminobenzamide spacer group and the guanidine derivatives **G2**, **G5** and **G6** clearly displayed a higher antimicrobial activity (MIC: 2–8 µg/ml) against Gram-positive strains than the corresponding amine derivatives **G1**, **G3** and **G4** (MIC: 8–32 µg/ml). The effect of guanylation was less obvious against Gram-negative strains and relatively small variations in antimicrobial potencies were observed (MIC: 16–64 µg/ml).

Another important implication of guanylation that was obvious throughout the **G**- to **L**-series was lower RBC toxicity and MRC-5 cell cytotoxicity of the guanylated compounds compared to the amine derivatives (Table 1). An exception was **G3** (amine), which was less hemolytic than its guanidine counterpart **G5**. For all other amine and guandidine analogues of the **G**- to **L**-series the guanidine derivatives were less toxic than the amines.

A structural variation within the **G**-series was methylation of the sulphonamide group that resulted in improved antimicrobial activity against Gram-negative strains but had less effect on improving activity against Gram-positive strains. All the compounds **G3**, **G4**, **G5**, and **G6** had a methylated sulphonamide group, whereas **G1** and **G2** were non-methylated. The positive effect of methylation of the sulphonamide group was evident when comparing **G1** (non-methylated amine derivative) with **G3** (methylated amine derivative) where the activity against *E. coli* was improved by methylation. Similarly, when comparing **G2** (non-methylated guanidine) with the more potent **G5** (methylated guanidine) against *P. aeruginosa*. The chemical implications of methylation of the sulphonamide group are first of all abolishing its ability to ionize. The sulphonamide proton of **G1** is weakly acidic (calculated pKa 7.71, *ChemBioDraw version 13.0.2*) and **G1** is likely ~30% ionized at physiological conditions (pH 7.4). Methylation increases overall lipophilicity and may also have an impact on overall spatial structure, i.e., by restricting rotation and forcing the two adjacent aromatic rings of the lipophilic and spacer groups out of plane.



Scheme 1. Reactants and conditions are shown in the scheme for synthesis of sulfonamidobenzamides investigated for antimicrobial and antibiofilm activity, and together with variations in spacer and lipophilic groups (R_1 = H or CH_3). *Step **1***. Sulfonyl chloride was reacted with 2 equivalents of the appropriate amines at 150 °C (thermal fusion) for 1 h. 1 equivalent of $NaHCO_3$ was added when using secondary amines. *Step **2***. Excess of $H_2N\text{-}CH_2(CH_2)_nNH_2$ (n = 1, 2, or 3) stirred at 100 °C for 18 h. *Step **3***. 1-Amidino-1*H*-1,2,4-triazole hydrochloride in DMF at room temperature for 18 h.

The non-methylated derivative **G1** was less toxic against RBC and MRC-5 cells than its methylated analogue **G3**, and similarly was **G2** (non-methylated) less toxic than **G5** (methylated analogue). The hemolytic activity within the **G**-series varied with EC_{50} values of 28–64 µg/ml and against MRC-5 with EC_{50} values of 5–>100 µg/ml. This resulted in the guanylated **G2** having the highest SI: 16 within the **G**-series. The two guanylated and methylated derivatives **G5** and **G6** displayed also high SI of 11–14 that was close to the SI: 16 obtained for **G2**.

Varying the length of the methylene chain between the spacer group and cationic group was ineffective with respect to antimicrobial activity. Almost identical MIC values were obtained for **G3** and **G4**, which differed in cationic side-chain length by a methylene group. This was also observed for **G5** and **G6**. A short ethylene chain was therefore selected for the following **H**- to **L**-series except for **K22**.

Increasing flexibility by introducing a methylene group in the **H**-series was of little benefit for improving antimicrobial potency compared to the **G**-series. The flexible **H**-series displayed however very low hemolytic activity with EC_{50} values of 73–113 µg/ml. The **H**-series differed structurally from the **G**-series by having an additional methylene group positioned either between the sulphonamide functionality and the spacer group (**H7** and **H9**), or between the spacer group and the carboxamide functionality (**H8** and **H10**). The MIC values varied from 4–32 µg/ml against the Gram-positive strains, and from 32–64 µg/ml against the Gram-negative strains. The guanidine derivatives **H9** and **H10** displayed highest antimicrobial activity with MIC values of 4–16 µg/ml against Gram-positive strains, and low toxicity against RBCs resulting in SI: 6–7. The guanylated derivatives **H9** and **H10** (EC_{50}: 55–>100 µg/ml) were also clearly less cytotoxic to MRC-5 cells than their amine analogues **H7** and **H8** (EC_{50}: 20 µg/ml). Introduction of a spatial methylene group in the **H**-series increases the flexibility of the compounds and may also disrupt π-bond interactions between the functional sulphonamide and carboxamide groups connected to the aromatic spacer group. Clearly, this was not beneficial for antimicrobial activity, but advantageous for reducing toxicity against RBC and MRC-5 cells.

Apparently 1,3-substitution of the aromatic spacer group in the **I**-series was more favorable than 1,4-substitution of the **G**-series, especially when evaluating toxicity (Figure 2). No increased hemolytic activity was observed following methylation of the sulphonamide group in **I13** and **I14** compared to what was observed for the previous **G**-series. Compounds of the **I**-series had a 3-aminobenzamide spacer group and displayed MIC: 2–16 µg/ml against Gram-positive strains and MIC: 16–64 µg/ml against Gram-negative strains. The most potent compound of the **I**-series was the methylated and guanylated derivative **I14** with MIC: 2–4 µg/ml against the Gram-positive strains. **I14** thereby displayed similar high antimicrobial activity against the Gram-positive strains as the most potent compound **G6** of the first series, but much lower toxicity against RBC and MRC-5 cells than **G6**. Structurally it's important to emphasize that **I14** had a methylated sulphonamide group, which in the **G**-series gave increased RBC and MRC-5 cell toxicity. Similar increased toxicity following methylation was thus not observed for **I14** and it thereby had the second highest SI: 24 of all studied compounds. A repulsive steric effect or less optimal conjugation between the methylated sulphonamide and amide functionalities in the case of 1,3-substitution could be a plausible reason for this beneficial shift in activity of **I14**. The second most potent derivative against the Gram-positive strains within the **I**-series was the guanylated **I13** (MIC: 4–8 µg/ml) that also displayed low toxicity against RBC and MRC-5 cells (EC_{50}: ≥92 and SI: 12). As observed for the G-series, the guanylated compounds **I13** and **I14** showed both higher overall antimicrobial activity and lower RBC and MRC-5 cell toxicity compared to the amine analogues **I11** and **I12**.

Exploring 1,2-substitution of the aromatic spacer in the **J**-series did not result in further improvement in antimicrobial potency for **J15** and **J17** compared to the previous 1,3-substitution of the **I**-series. Compounds **J15** and **J17** had a 2-aminobenzamide spacer group, in which the guanylated 2-aminobenzamide **J17** (MIC: 4–8 µg/ml) was clearly more potent than the corresponding amine derivative **J15** (MIC: 16–32 µg/ml) against Gram-positive strains. Furthermore, **J17** displayed lower hemolytic activity and MRC-5 cell cytotoxicity than **J15**, and the advantage of guanylation was thereby once more demonstrated by **J17** (guanidine) compared to **J15** (amine).

Increasing lipophilicity and size by having a 3-sulfonamido-2-naphthamide spacer group in **J16** and **J18** improved antimicrobial activity against the Gram-negative strains, and especially against *E. coli* (MIC: 8 µg/ml). High antimicrobial activity against Gram-positive strains was also maintained (MIC: 2–4 µg/ml). RBC toxicity was however high for **J16** and **J18** with EC_{50}: 21–25 µg/ml that resulted in low SI: 5–6. The 3-sulfonamido-2-naphthamide derivatives **J16** and **J18** were more potent than **J17** against the Gram-positive strains and *E. coli*. The positive effect of guanylation was however lost by the increased lipophilicity provided by the 3-sulfonamido-2-naphthamide spacer group. Further optimizations are needed for compounds with the naphthalenic spacer group to reduce RBC toxicity.

Toxicity against MRC-5 cells was less affected by the naphthalenic spacer group in **J16** and **J18**. This resulted in an important observation; for the whole **G** to **L**-series toxicity against MRC-5 cells of the amine derivatives was within a narrow range of EC_{50}: 5–25 µg/ml, whereas toxicity against MRC-5 cells for the guanidine derivatives was EC_{50}: 42–>100 µg/ml. In comparison, hemolytic activity of both the amine and guanidine derivatives varied more, displaying EC_{50}values of 21–92 µg/ml for the amine derivatives, and 25–>100 µg/ml for the guanidine derivatives. I.e. the differences in EC_{50} values between amine and guanidine derivatives were larger against the nucleated MRC-5 cells compared to the non-nucleated RBCs. Since compounds with an amine group in general diffuse more easily across cell membranes than compounds with a guanidine group, this observation may imply that the amine derivatives may reach additional intracellular targets in nucleated cells such as MRC-5 cells explaining the higher MRC-5 cell cytotoxicity of the amine derivatives. The differences in diffusion rate is attributed to the general lower average pKa value and lower ionization state of amines, favoring a higher proportion un-ionized amine, compared to guanidines being fully ionized at physiological conditions.

Alteration of the central spacer group showed a positive effect on reducing RBC toxicity. The **K**-series contained a variety of spacer groups and all compounds were guanylated. Compound **K19** had an additional methyl substituent on the aromatic spacer group. Compared to **G5** having a methylated sulphonamide group, **K19** was comparable potent against the Gram-positive strains (MIC: 4–8 µg/ml) and *E. coli* (MIC: 32 µg/ml). Compound **K19** showed also lower hemolytic activity and MRC-5 cell cytotoxicity than **G5**. Strangely **K19** was inactive against *P. aeruginosa* as opposed to **G5**. As for methylation of the sulphonamide group in **G5**, the methyl group in **K19** may similarly restrict rotation by introducing steric hindrance and increase overall lipophilicity.

Introduction of aliphatic cyclic spacer groups resulted in low antimicrobial activity. Compound **K20** contained an aliphatic cyclic piperidine-4-carboxamide spacer group and **K21** an *L*-proline spacer group (Figure 2). Both **K20** and **K21** showed low antimicrobial activity except against *C. glutamicum*. The low antimicrobial activity correlated also with no detectable RBC toxicity or MRC-5 cell cytotoxicity for **K20** and **K21**. The low antimicrobial activity following introduction of aliphatic cyclic spacer groups demonstrated the superiority of the previous aromatic spacer groups investigated.

To our surprise the highly flexible derivative **K22** having no spacer group displayed high antimicrobial activity against Gram-positive strains (MIC: 4–16 µg/ml) and low hemolytic activity (EC_{50}: 110 µg/ml and SI: 14). Although cytotoxicity against MRC-5 cells was observed (EC_{50}: 42 µg/ml), the highly flexible **K22** is an attractive model compound for synthesis of mimics of, e.g., the marine antimicrobial ianthelline[4], which has a flexible 2-(hydroxyimino)acetamide spacer group linking its lipophilic and cationic groups (Figure 1).

The **L**-series showed that reducing the lipophilicity and bulkiness of the lipophilic group decreased antimicrobial activity compared to the structurally related **G2** as reference. The **L**-series contained a 4-aminobenzamide spacer-group connected to four different lipophilic groups (Figure 2). The **L**-series showed highly variable MIC: 32–≥128 µg/ml for the least lipophilic members **L23**, **L24** and **L25**, and with no antimicrobial activity against Gram-negative strains. No cytotoxicity against MRC-5 cells was observed for any of the gyanylated derivatives in the **L**-series. Compound **L26** was most structural similar to **G2** and had an additional 4-methoxy-group that increased overall lipophilicity and bulkiness. The two compounds **L26** and **G2** showed similar high antimicrobial activity against Gram-positive strains (MIC: 4–8 µg/ml), but both encountered solubility challenges, as observed when evaluating RBC toxicity (precipitations above 64 µg/ml in the hemolytic assay).

In summary, the SAR study against bacterial reference strains showed that the guanylated analogues were both more potent and less toxic against RBC and MRC-5 cells than the amine analogues as first shown by the **G**-series (Figure 3). Methylation of the sulphonamide group had different effects on antimicrobial activity and toxicity depending on the substitution pattern of the spacer group, as shown when comparing the **G**- and **I**-series. Increased flexibility did not improve antimicrobial activity, although RBC toxicity was reduced in the **H**-series. Aromatic spacer groups were more efficient for high antimicrobial activity, whereas cyclic aliphatic spacers such as piperidine-4-carboxamide (**K20**) or *L*-proline (**K21**) were less efficient. Clearly, a large size of the lipophilic group mattered as follows from comparison of **G2** and the **L**-series. Of special interest was **K22**, which had no cyclic spacer at all, but to our surprise showed good antimicrobial activity and low RBC toxicity. Compared to the previous reported aminobenzamide **E23**,[3] both the novel promising compounds **I14** and **K19** showed antimicrobial activity in the same range against MRSE (Table 1). Importantly, both **I14** and **K19** were less hemolytic than **E23** and had a higher SI: 24 for **I14** and SI: 26 for **K19** than **E23** (SI: 19). We therefore consider the present sulfonamidobenzamides as a promising class of antimicrobials for further optimization studies.

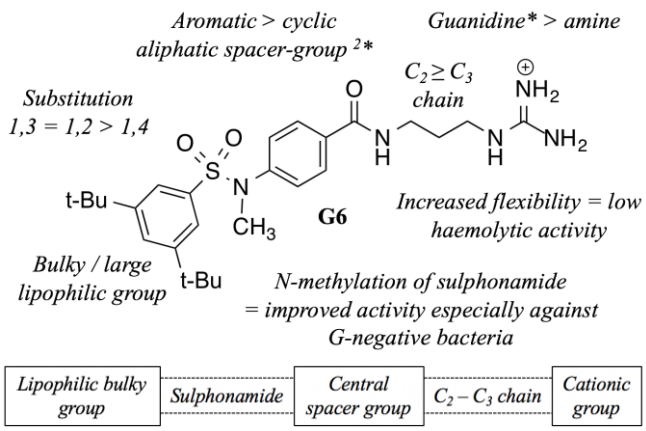


Figure 3. Summary of SAR resulting in high antimicrobial activity of synthesised sulfonamidobenzamides (exemplified by **G6**). *Lower toxicity against RBC and MRC-5 cells was observed for guanidine compared to amine derivatives. [2]*An exception to the low antimicrobial potency of compounds with an aliphatic spacer group was **K22**, which had no spacer group and still displayed high antimicrobial activity against Gram-positive bacteria and low hemolytic activity.

Table 1. Antimicrobial activity (MIC in μg/ml) against bacterial reference strains and hemolytic activity against human RBCs (EC_{50} in μg/ml) of synthesized amphipathic sulfonamidobenzamides.

		Cationic		Antimicrobial activity [a] - μg/ml						RBC	MRC-5	SI [c]
Series	Comp	group	Mw [b]	MRSE	*S. aureus*	*B. subtilis*	*C. glutamicum*	*E. coli*	*P. aeruginosa*	EC_{50}	EC_{50}	RBC/MRSE
G	**G1**	Amine	468.1	32	32	16	8	64	64	63	25	2
	G2	Guanidine	510.1	4	4	8	4	32	64	64[d]	>100	16
	G3	Amine	482.1	16	16	8	16	16	32	53	5	3
	G4		496.1	16	16	8	8	16	32	28	9	2
	G5	Guanidine	524.1	4	4	4	2	32	16	45	36	11
	G6		538.2	4	4	4	2	16	16	55	36	14
H	**H7**	Amine	482.1	16	32	8	8	32	32	92	20	6
	H8		482.1	16	32	16	16	64	64	73	20	5
	H9	Guanidine	524.1	8	16	8	4	32	32	113	>100	14
	H10		524.1	8	16	8	8	64	64	101	55	13
I	**I11**	Amine	468.1	8	16	8	8	32	64	58	16	7
	I12		482.1	16	16	8	8	16	32	26	10	2
	I13	Guanidine	510.1	8	8	8	4	32	32	92	>100	12
	I14		524.1	4	4	4	2	32	32	95	91	24
J	**J15**	Amine	468.1	32	32	32	16	64	128	56	10	2
	J16		518.1	4	4	4	2	8	32	21	22	5
	J17	Guanidine	510.1	8	4	8	4	32	64	77	42	10
	J18		560.2	4	4	4	2	8	32	25	55	6
K	**K19**	Guanidine	524.1	4	8	8	4	32	>128	104	56	26
	K20		502.1	16	16	16	4	64	128	124	>100	8
	K21		488.1	32	64	32	8	>128	128	>128	>100	>4
	K22		419.0	8	8	16	4	64	64	110	42	14
L	**L23**	Guanidine	425.9	64	128	64	64	>128	>128	>128	>100	>2
	L24		533.9	>128	>128	>128	>128	>128	>128	64[d]	>100	-
	L25		447.9	32	64	64	32	>128	>128	>128	>100	>4
	L26		540.1	4	4	4	4	32	64	64[d]	>100	16
	E23 [e]	Guanidine	474.1	2	1	1	0.5	8	16	37	22	19

[a] *Staphylococcus aureus* ATCC 9144; methicillin resistant *Staphylococcus epidermidis* RP62A (MRSE),[50] CCUG 31568 (ATCC 35984); *Bacillus subtilis* 168,[18] laboratory collection (ATCC 23857); *Corynebacterium glutamicum* ATCC 13032; *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* PA01, DSM 19880 (ATCC 15692). [b] Mw including 1 eq. of HCl. [c] Selectivity index (SI) calculated as the EC_{50} value against RBC divided by the MIC value against MRSE. [d] Precipitation observed in test buffer used for RBC determination at higher concentrations. [e] Antimicrobial activity and RBC toxicity previously reported for the synthetic cationic amphipathic aminobenzamide **E23** (Figure 1).[3]

2.3 Antimicrobial profile against clinical isolates

We continued addressing clinical relevance by screening 15 of the most potent sulfonamidobenzamides from all six series against 25 clinical isolates (Table 2). Gram-positive bacteria were represented by *S. aureus* and *Enterococcus* spp., and Gram-negative bacteria were *E. coli*, *P. aeruginosa*, and *Klebsiella pneumoniae*. Five isolates of each group of bacteria were used.

The antimicrobial activity against the clinical isolates was in general similar or a little lower (by one titer step) compared to the initial screening against the reference strains (Table 2 – top section). The overall most potent and broad-spectrum compounds against the 25 clinical isolates were **G6** and **J18**. The activity against the Gram-positive clinical isolates was MIC: 4–32 μg/ml, and MIC: 8–128 μg/ml against the Gram-negative clinical isolates. Relatively high antimicrobial activity against clinical isolates of *Enterococcus* spp. was also maintained for compounds **G6**, **I14**, **J16**, and **J18** (MIC: 8–16 μg/ml). The effect of the same compounds against clinical isolates of *K. pneumonia* were however variable (MIC: 16–128 μg/ml).

The two most broad-spectrum compounds **G6** and **J18** were furthermore tested against an expanded panel of additional 50 clinical isolates of each bacterial group (250 isolates; lower section Table 2). MIC_{90} values, i.e., the minimum concentrations that inhibited 90% of these isolates (45 out of 50 isolates), were in line with the preceding screening results (Table 2 – top section). Thus, MIC_{90} against the Gram-positive isolates did not exceed 8 μg/ml for **J18** and 16 μg/ml for **G6**. Against the Gram-negative isolates, only **J18** had MIC_{90} higher than 32 μg/ml against *P. aeruginosa*. Thus, the antimicrobial activity of **G6** and **J18** against the reference strains was confirmed when tested against the 275 clinical isolates.

Table 2. Antimicrobial activity (MIC in μg/ml) against 25 randomly chosen clinical isolates. MIC_{90} is the concentration of the compounds that inhibited ≥90% of all clinical isolates when screened against 50 additional isolates within each group of bacteria (in total 250 isolates).

	MIC - μg/ml				
Comp	*S. aureus*	*Enterococcus* spp.	*E. coli*	*P. aeruginosa*	*K. pneumoniae*
No. of isolates:	5	5	5	5	5
G2	8	32	>128	>128	>128
G5	4	32	32	16	64
G6	4	8	16	8	32
H9	16	64	128	64	128
H10	16	32	64	64	128
I13	8	32	64	64	128
I14	8	16	32	32	64
J15	32	32	128	128	128
J16	8	8	32	32	128
J17	8	32	64	32	128
J18	4	8	8	64	16
K19	8	32	64	64	>128
K22	16	32	128	64	128
L25	128	>128	>128	>128	>128
L26	8	64	>128	32	>128
	MIC_{90} - μg/ml				
No. of isolates:	50	50	50	50	50
G6	4	16	16	16	32
J18	4	8	8	>32	32

2.4 Antimicrobial activity against 30 multi-resistant isolates

The clinical relevance of **G6** and **J18** was finally tested by determination of their antimicrobial activity against a panel of 30 multi-resistant clinical isolates from the strain collection at *The Norwegian National Advisory Unit on Detection of Antimicrobial Resistance*.[8-10] (Table 3). Included in the screening were methicillin resistant *S. aureus* (MRSA), vancomycin resistant enterococci (VRE), and Gram-negative bacteria producing extended spectrum β-lactamases–carbapenemases (ESBL–CARBA). As shown in Table 3 compounds **G6** and **J18** were potent against MRSA and VRE (MIC: 4–16 µg/ml) but lost their activity against the majority of the Gram-negative isolates. The *P. aeruginosa* isolates were still comparatively susceptible to **G6** with MIC: 32 µg/ml and **J18** had similar MIC: 32 µg/ml against the included *Acinetobacter baumannii* isolates.

Table 3. Antimicrobial activity (MIC in µg/ml) of **G6** and **J18** against 30 multi-resistant isolates.

Multi-resistant isolate	**G6**	**J18**	ESBL-CARBA[a]
S. aureus N315	8	4	
S. aureus NCTC 10442	8	8	
S. aureus strain 85/2082	8	8	
S. aureus strain WIS	8	8	
S. aureus IHT 99040	8	8	
E. faecium 50673722	16	8	
E. faecium 50901530	16	8	
E. faecium K36-18	16	8	
E. faecium 50758899	8	8	
E. faecium TUH50-22	16	4	
E. coli 50579417	≥32	≥32	OXA[b]-48
E. coli 50639799	32	≥32	VIM[c]-29
E. coli 50676002	≥32	>32	NDM[d]-1
E. coli 50739822	≥32	>32	NDM-1
E. coli 50857972	32	>32	IMP[e]-26
P. aeruginosa K34-7	>32	>64	VIM-2
P. aeruginosa K34-73	32	≥32	VIM-4
P. aeruginosa K44-24	32	>64	IMP-14
P. aeruginosa 50692172	32	>64	NDM-1
P. aeruginosa 50692520	32	≥64	VIM
K. pneumoniae K47-25[g]	≥32	>32	KPC[f]-2
K. pneumoniae K66-45	32	32	NDM-1
K. pneumoniae 50531633[g]	≥32	>32	NDM-1+OXA-181
K. pneumoniae 50625602	≥32	≥32	OXA-245
K. pneumoniae 50667959	≥32	≥32	VIM-1
A. baumannii K12-21	≥32	32	OXA-58
A. baumannii K44-35	≥32	32	OXA-23
A. baumannii K47-42	32	32	OXA-23
A. baumannii K55-13	≥32	32	OXA-24
A. baumannii K63-58[g]	≥32	32	OXA-23

[a] ESBL-CARBA: Extended spectrum β-lactamase-carbapenemase-producing isolates. [b] OXA, oxacillinase; [c] VIM, Verona integron-encoded metallo-β-lactamase; [d] NDM, New Delhi metallo-β-lactamase; [e] IMP, imipenem-type carbapenemase; [f] KPC, *K. pneumoniae* carbapenemase; [g] Clinical isolates resistant to the antibiotic colistin.

2.5 Anti-biofilm activity

Serious human infections are frequently associated with formation of bacterial biofilms hampering antibiotic treatment.[19,20] Medical device-associated infections are often caused by coagulase-negative staphylococci and particularly MRSE presumably due to its prevalence for biofilm formation.[21-23] Mechanical properties, physiological heterogeneity and flexibility[24,25] make biofilm cultures considerably more tolerant to biocides and antibiotics than their planktonic counterparts. For example, the rate of killing MRSE in biofilm by the antibiotic rifampin was shown to be seven times slower and 157 times slower by vancomycin, compared to rate of killing MRSE in the planktonic state.[26]

In the present study, the most potent guanidine derivatives were also screened for effects on eradicating preformed polysaccharide biofilm of MRSE (Table 4). The threshold was set as ≥90% biofilm eradication, which considers the non-specific binding of growth medium components. The compounds were tested with main emphasis to determine whether this class of amphipathic sulfonamidobenzamides could eradicate MRSE biofilm on a general basis.

Overall, the results showed that this class of small amphipathic molecules was able to enter into the complex biofilm matrix, presumably kill the bacteria, and effectuate the disintegration of the biofilm. The results revealed that an overall 5–10 times higher concentration than the MIC value of individual derivatives against planktonic MRSE was required for achieving ≥90% biofilm eradication, which corresponded to concentrations of 40–80 µg/ml of the compounds tested (Table 4). However, due to variation between individual biofilm eradication experiments, it was challenging to differentiate between individual compounds with respect to SAR on biofilm eradication. The challenges with reproducibility of the results and complexity of eradicating biofilms produced by MRSE may be explained by the complex biofilm matrix and phenotypic variation reported for the MRSE strain.[27,28] However, when evaluating the combined effects of high antimicrobial activity against planktonic MRSE (i.e. MIC ≤4 µg/ml), a maximum concentration of 40 µg/ml for ≥90% biofilm eradication, and low RBC toxicity (EC_{50}: >90 µg/ml), the most promising compounds for biofilm treatment were **I14** and **K19**. If a higher MIC (8 µg/ml) against planktonic MRSE is tolerated, compounds **H10**, **I13** and **K22** can be added to this list of selected compounds for further optimizations.

Table 4: Antimicrobial activity (MIC, µg/ml) compared to anti-biofilm activity (concentration required for ≥90% eradication of biofilm, µg/ml) of selected compounds against MRSE.

	MIC [a]	≥90% Biofilm eradication [b]		RBC [a]
Comp	*S. epiderimidis*	µg/ml	x MIC	EC_{50}
G2	4	40	10	64
G5	4	40	10	45
G6	4	40	10	55
H9	8	80	10	113
H10	8	40	5	101
I13	8	40	5	92
I14	4	40	10	95
J18	4	40	10	25
K19	4	40	10	104
K20	16	80	5	124
K22	8	40	5	110
L25	32	160	5	>128
L26	4	40	10	64

[a] MIC planktonic MRSE and RBC values are from Table 1.
[b] Concentrations tested: 2x, 5x and 10x MIC.

2.6 Membrane integrity investigations

We tested also two representative compounds **G6** and **I14** for their ability to alter bacterial cell membrane integrity. A luciferase-expressing *Bacillus subtilis* strain was used that emits luminescence upon the entrance of the substrate D-luciferin into bacterial cells if membrane integrity is compromised.[29] Both compounds **G6** and **I14** displayed a dose-response effect against *B. subtilis* (Figure 4). A clear membranolytic effect was observed for **G6** at a concentration close to the MIC value against *B. subtilis* (MIC: 4 µg/ml, Table 1), whereas for **I14** a concentration of 12.5 µg/ml (i.e. 3x MIC: 4 µg/ml) was needed to induce luminescence (Figure 4). The different kinetics of luminescence emission shown for the two compounds could indicate differences in the way they interacted with the cell membrane (Figure 5). The membrane-disruptive activity of the compounds appeared to be comparable to that of chlorhexidine, a biocide known for its membranolytic activity.[30] The results were similar

to the effects observed for the amidobenzamide derivatives[3] and small marine natural antimicrobials reported previously.[4]

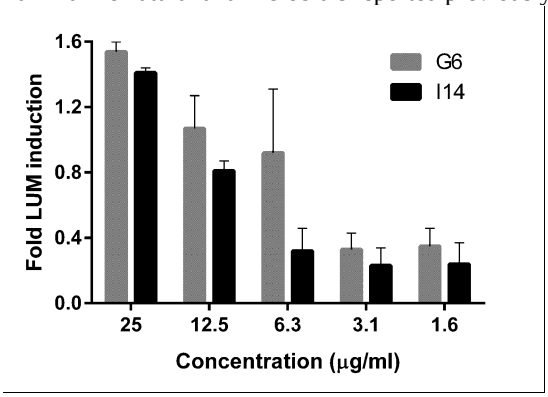


Figure 4. Luminescence (LUM) induction in *B. subtilis* 168/pCSS962 treated with **G6** and **I14** at concentrations 25–1.6 µg/ml. Data for the first 30 sec after injection of the bacterial suspension (containing D-luciferin) is presented. Chlorhexidine was used as a membrane-disruptive control. The *Fold LUM induction* was the ratio between the maximum luminescence in compound-treated samples compared to the chlorhexidine-treated control (31 µg/ml of chlorhexidine was added in case of **I14** and 20 µg/ml for **G6**). The mean of two experiments ± SD is displayed.

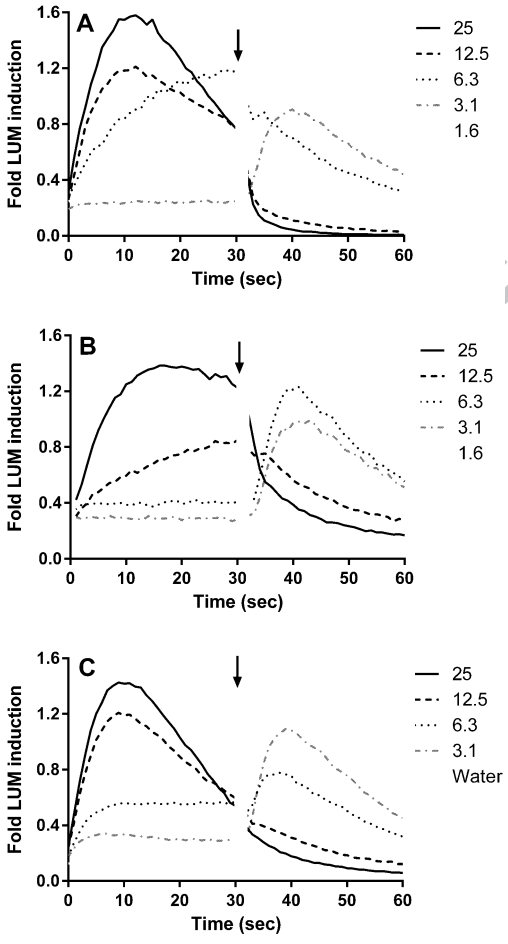


Figure 5. The figure shows kinetics of luminescence (LUM) induction in *B. subtilis* 168/pCSS962 in presence of D-luciferin after the addition of different concentrations (µg/ml) of the compounds; A) **G6**, B) **I14**, and C) chlorhexidine. Data for chlorhexidine are from reference.[3] LUM was monitored for up to 180 sec, but only the first 60 sec are presented. Water was added to an untreated control. Complete membrane disruption was achieved by addition of chlorhexidine (31 µg/ml) to the samples at the time point indicated by an arrow. The *Fold LUM induction* compared to the maximum LUM of the untreated control after chlorhexidine addition is shown. Data from a single experiment, representative of two independent experiments, are displayed.

3. Conclusions

To conclude, a promising class of amphipathic sulfonamidobenzamides have been designed and synthesized with potential for further drug development of antimicrobial and anti-biofilm efficient drugs. Since bacterial biofilms are known to be associated with wound infections,[31-33] topical wound healing is in that respect an area of interest, especially for treatment of chronic wound infections, which are of particular importance in aged and immobilized patients.[31,34] The broad-spectrum activity of the described class of compounds indicated by the potencies of selected derivatives **G6** and **J18** against clinical isolates, including multi-drug resistant isolates, makes them promising candidates for treatment of polymicrobial wound infections.[31,35] Although MRSE is not the leading causative agent in such infections,[23] it nevertheless served well as a pilot biofilm model. Apart from **G6** and **J18**, the guanidine derivatives, **G2** and **I14**, displayed both potent activity against MRSE biofilms and favorable selectivity for staphylococci. This combination of anti-staphylococcal properties makes **G2** and **I14** promising candidates for further investigation with clinically relevant biofilm-forming staphylococci, commonly associated with wounds and implant associated infections.

4.Experimental section

4.1 Chemicals and equipment

All chemicals used for synthesis were purchased from Sigma-Aldrich Inc., USA. The course of the reactions was monitored using a Waters Alliance 2695 Separation Module HPLC system (Waters Inc., USA) accompanied by Micromass Qattro LC (Micromass, UK) MS system. ^{1}H and ^{13}C NMR spectra were recorded on a 400 MHz NMR spectrometer (Varian, USA). Chemical shifts are expressed in ppm relative to methanol-d_4 (δH: 3.310 ppm and δC: 49.000 ppm). High-resolution MS spectra were acquired on a Waters LC-MS system (Milford, MA, USA) composed of an Acquity UPLC coupled to an LCT-Premier TOF MS with electrospray ionization (ESI). The MS data were obtained in positive ESI mode. Melting points were determined in open capillary tubes with a Büchi B-540 melting point apparatus.

4.2 Synthesis of starting materials

3,5-Di-tert-butylbenzene-1-sulfonyl chloride was synthesised according to Hall.[36] Briefly, 1,3-di-*tert*-butylbenzene (1 g, 5 mmol) was dissolved in anhydrous amylenes stabilised chloroform (20 ml). Chlorosulfonic acid (5.83 g, 50 mmol) was dissolved in anhydrous amylenes stabilised chloroform (40 ml) and added drop-wise to the reaction mixture with cooling and vigorous stirring for 3 h. The reaction mixture was stirred additionally at room temperature for 1 h before it was poured into crushed ice (300 g). The organic phase was dried with $CaCl_2$. Chloroform was removed *in vacuo* and 3,5-di-*tert*-butylbenzene-1-sulfonyl chloride was obtained in 86% yield and used without further purification. Spectroscopic data were in accordance to Ris et al.[37]

3,5-Di-tert-butyl-4-methoxybenzene-1-sulfonyl chloride: 1,3-Di-*tert*-butyl-2-methoxybenzene was synthesised according to Bai et al.[38] Briefly, 2,6-di-*tert*-butylphenol (5.0 g, 24 mmol), NaH (1.15 g, 29 mmol), and iodomethane (4.11 g, 29 mmol) were added to DMF (25 ml) at 0 °C for 2 h. The reaction mixture was poured into cold water, extracted with diethyl ether, and dried with Na_2SO_4. Diethyl ether was evaporated under reduced pressure. Spectroscopic data were in accordance to Bai et al.[38] 1,3-Di-*tert*-butyl-2-methoxybenzene (1 g, 4.5 mmol) was dissolved in anhydrous amylenes stabilised chloroform (20 ml). Chlorosulfonic acid (5.83 g, 50 mmol) was dissolved in anhydrous amylenes stabilised chloroform (40 ml) and added drop-wise to the reaction mixture with

cooling and vigorous stirring for 3 h. The reaction mixture was stirred additionally at room temperature for 1 h before it was poured into crushed ice (300 g). The organic phase was dried with $CaCl_2$. Chloroform was removed *in vacuo* and 3,5-di-*tert*-butyl-4-methoxybenzene-1-sulfonyl chloride was obtained in 80% yield. It was used in subsequent reactions without further purification. ^{1}H NMR ($CDCl_3$): 7.90 (s, 2H), 3.77 (s, 3H), 1.46 (s, 18H).

Methyl 2-(4-aminophenyl)acetate 4-methylbenzenesulfonate (or 4-(2-methoxy-2-oxoethyl)benzenaminium 4-methylbenzenesulfonate): 4-Aminophenylacetic acid (5 g, 33 mmol) was heated under reflux in an excess of methanol (100 ml) in the presence of *para*-toluene sulfonic acid (7.6 g, 40 mmol) for 18 h. Methanol was evaporated under reduced pressure. Methyl 2-(4-aminophenyl)acetate 4-methylbenzenesulfonate was obtained in approx. quantitative yield and was used in further reactions without purification.

Methyl 4-(methylamino)benzoate: 4-Methylaminobenzoic acid (5 g, 33 mmol) was refluxed in an excess of methanol (100 ml) in the presence of *para*-toluene sulfonic acid (7.6 g, 40 mmol) for 18 h[39]. Methanol was evaporated *in vacuo* and a saturated solution of $NaHCO_3$ was added to the residue. The product was isolated by extraction with ethyl acetate. The organic solvent was evaporated under reduced pressure and methyl 4-methylaminobenzoate was obtained in approx. quantitative yield. Spectroscopic data were in accordance to Jones et al.[39]

Methyl 3-(methylamino)benzoate and *methyl 4-amino-3-methylbenzoate* were synthesised by the same method and with approx. quantitative yield. Spectroscopic data for methyl 3-(methylamino)benzoate was in accordance to Gao et al.[40] and methyl 4-amino-3-methylbenzoate was in accordance to Yang et al.[41]

Methyl isonipecotate was of technical grade (Sigma-Aldrich; purity approx. 80%) and was purified by vacuum distillation before use.

Methyl 3-amino-2-naphthoate: 3-Amino-2-naphthoic acid was of technical grade (Sigma-Aldrich; purity approx. 80%) and was purified before use. Crude 3-amino-2-naphthoic acid (5 g, 27 mM) was dissolved in 500 ml of saturated solution of $NaHCO_3$. The solution was filtered and concentrated HCl was added drop wise to the solution until pH 7, in which 3-amino-2-naphthoic acid started to precipitate. The yellowish precipitate was isolated by filtration. Yield of purified 3-amino-2-naphtoic acid was 70% (3.5 g). Methyl 3-amino-2-naphthoate was synthesized according to a modified method described elsewhere.[42] Briefly, concentrated H_2SO_4 (12 ml) was added to methanol (70 ml), followed by 3-amino-2-naphthoic acid (3.5 g, 19 mmol). The solution was refluxed for 18 h, the mixture was cooled and methanol evaporated *in vacuo*. The residue was poured onto ice and neutralized with saturated $NaHCO_3$. The resulting yellowish precipitate was filtered, washed with water, and dried. An amount of 2.7 g of methyl 3-amino-2-naphthoate was obtained (70% yield). Spectroscopic data were in accordance to Theeraladanon et al.[43]

4.3 Synthesis of MNPM test compounds

N-(2-Aminoethyl)-4-((3,5-di-tert-butylphenyl)sulfonamido)benzamide hydrochloride (**G1**): Methyl 4-aminobenzoate (604 mg, 4 mmol) and 3,5-di-*tert*-butylbenzene-1-sulfonyl chloride (549 mg, 1.9 mmol) were heated together at 150 °C with stirring until the melt solidified. The reaction mixture was allowed to cool down to room temperature, and 2 M solution of HCl was added. The solid residue was filtered out and crystallised from MeOH/H_2O. Methyl 4-((3,5-di-*tert*-butylphenyl)sulfonamido)benzoate was obtained in 85% yield. Methyl 4-((3,5-di-*tert*-butylphenyl)sulfonamido)benzoate (300 mg, 0.7 mmol) was dissolved in an excess of ethylenediamine (3 ml, 45 mmol) and the reaction mixture was stirred at 100 °C for 18 h. The excess of ethylenediamine was removed *in vacuo* and the residue was dissolved in acetonitrile and washed with hexane. The solvent was removed *in vacuo* and *N*-(2-aminoethyl)-4-((3,5-di-*tert*-butylphenyl)sulfonamido)benzamide was obtained in 92% yield and used directly in the next step without further purification for synthesis of its guanylated analogue **G2** (see below for **G2**). Prior to biological screenings, *N*-(2-aminoethyl)-4-((3,5-di-*tert*-butylphenyl)sulfonamido)benzamide (200 mg, 0.5 mmol) was further dissolved in MeOH (1 ml), an excess of 4 M solution of HCl in dioxane (0.5 ml) was added, and the mixture stirred for 30 min at room temperature to achieve the HCl salt. Solvents were removed *in vacuo*. Obtained *N*-(2-aminoethyl)-4-((3,5-di-*tert*-butylphenyl)sulfonamido)benzamide hydrochloride was purified in quantitative yield by crystallisation from MeOH/Et_2O. Briefly, *N*-(2-aminoethyl)-4-((3,5-di-*tert*-butylphenyl)sulfonamido)benzamide hydrochloride (100 mg, 0.2 mmol) was dissolved in 1 ml of MeOH under reflux. Et_2O was added drop wise until precipitation was complete. ^{1}H NMR (CD_3OD): 7.75 (d, 2H, J = 8.8 Hz), 7.63 (s, 3H), 7.20 (d, 2H, J = 8.8), 3.61 (t, 2H, J = 6 Hz), 3.12 (t, 2H, J = 6 Hz), 1.27 (s, 18H). ^{13}C NMR (CD_3OD): 168.9, 152.0, 141.6, 138.9, 128.8, 128.3, 126.7, 120.9, 119.1, 39.7, 37.3, 34.6, 30.1. HRMS-ESI: $C_{23}H_{34}N_3O_3S$ $[M+H]^+$ calcd: 432.2321, found: 432.2324; mp 264-267 °C (decomp.).

Compounds **H7**, **H8**, **I11**, **J15**, and **J16** were synthesized in a similar manner (Scheme 1).

N-(2-Aminoethyl)-4-(((3,5-di-tert-butylphenyl)sulfonamido)methyl)benzamide hydrochloride (**H7**) ^{1}H NMR (CD_3OD): 7.79 (d, 2H, J = 8 Hz), 7.71 (s, 2H), 7.70 (s, 1H), 7.33 (d, 2H, J = 8 Hz), 4.15 (s, 2H), 3.67 (t, 2H, J = 6 Hz), 3.17 (t, 2H, J = 6 Hz), 1.36 (s, 18H). ^{13}C NMR (CD_3OD): 169.2, 152.1, 141.9, 140.1, 132.3, 127.4, 127.2, 126.4, 120.7, 45.9, 39.7, 37.4, 34.7, 30.2. HRMS-ESI: $C_{24}H_{36}N_3O_3S$ $[M+H]^+$ calcd: 446.2477, found: 446.2460; mp 133-135 °C (decomp.).

N-(2-Aminoethyl)-2-(4-((3,5-di-tert-butylphenyl)sulfonamido)phenyl)acetamide hydrochloride (**H8**) ^{1}H NMR (CD_3OD): 7.63 (t, 1H, J_1 = 1.6 Hz), 7.56 (s, 2H, J_1 = 1.6 Hz), 7.20 (d, 2H, J = 8 Hz), 7.06 (d, 2H, J = 8 Hz), 3.48 (s, 2H), 3.43 (t, 2H, J_1 = 6 Hz), 3.05 (t, 2H, J_1 = 6 Hz), 1.28 (s, 18H). ^{13}C NMR (CD_3OD): 173.8, 151.8, 138.8, 136.7, 131.7, 129.5, 126.4, 121.6, 120.9, 41.5, 39.5, 37.0, 34.6, 30.1. HRMS-ESI: $C_{24}H_{36}N_3O_3S$ $[M+H]^+$ calcd: 446.2477, found: 446.2462; mp > 80-83 °C (decomp.).

N-(2-Aminoethyl)-3-((3,5-di-tert-butylphenyl)sulfonamido)benzamide hydrochloride (**I11**) ^{1}H NMR (CD_3OD): 7.71 (t, 1H, J = 2 Hz), 7.62 (t, 1H, J = 1.6 Hz), 7.56 (d, 2H, J = 1.6 Hz), 7.56 (dd, 1H, J_1 = 8 Hz, J_2 = 2 Hz), 7.32 (t, 1H, J = 8 Hz), 7.19 (d, 1H, J_1 = 8 Hz, J_2 = 2 Hz), 3.63 (t, 2H, J = 6 Hz), 3.14 (t, 2H, J = 6 Hz), 1.26 (s, 18H). ^{13}C NMR (CD_3OD): 168.9, 151.9, 138.7, 138.5, 134.6, 128.9, 126.6, 124.4, 122.9, 120.9, 120.5, 39.7, 37.7, 34.6, 30.1. HRMS-ESI: $C_{23}H_{34}N_3O_3S$ $[M+H]^+$ calcd: 432.2321, found: 432.2300; mp > 79-82 °C (decomp.).

N-(2-Aminoethyl)-2-((3,5-di-tert-butylphenyl)sulfonamido)benzamide hydrochloride (**J15**) ^{1}H NMR (CD_3OD): 7.73 (d, 1H, J_1 = 8 Hz), 7.64 (s, 1H), 7.56 (s, 2H), 7.49 (d, 1H, J_1 = 8 Hz), 7.44 (t, 1H, J_1 = 8 Hz), 7.18 (t, 1H, J_1 = 8 Hz), 3.64 (t, 2H, J_1 = 6 Hz), 3.18 (t, 2H, J_1 = 6 Hz), 1.27 (s, 18H). ^{13}C NMR (CD_3OD): 170.1, 152.0, 138.4, 138.1, 132.2, 128.2, 126.8, 124.1, 122.5, 121.7, 121.0, 39.6, 37.1, 34.6, 30.1. HRMS-ESI: $C_{23}H_{34}N_3O_3S$ $[M+H]^+$ calcd: 432.2321, found: 432.2310; mp > 112-114 °C (decomp.).

N-(2-Aminoethyl)-3-((3,5-di-tert-butylphenyl)sulfonamido)-2-naphthamide hydrochloride (**J16**) ^{1}H NMR (CD_3OD): 8.27 (s, 1H), 7.86 (d, 1H, J = 8 Hz), 7.71 (d, 1H, J = 8 Hz), 7.70 (s, 1H), 7.56 (s, 1H), 7.55 (t, 1H, J = 8 Hz), 7.53 (s, 2H), 7.48 (t, 1H, J = 8 Hz), 3.68 (t, 2H, J = 6 Hz), 3.23 (t, 2H, J = 6 Hz), 1.15 (s, 18H). ^{13}C NMR (CD_3OD): 170.2, 151.9, 137.5, 134.6, 133.7, 129.8, 129.6, 128.5, 128.2, 126.7, 126.7, 126.1, 123.6, 121.2, 120.0, 39.8, 37.2, 34.5, 30.0. HRMS-ESI: $C_{27}H_{36}N_3O_3S$ $[M+H]^+$ calcd: 482.2477, found: 482.2466; mp 165-167 °C (decomp.).

N-(2-Aminoethyl)-4-((3,5-di-tert-butyl-N-methylphenyl)sulfonamido)benzamide hydrochloride (**G3**): 4-Methylaminobenzoate (330 mg, 2 mmol), 3,5-di-*tert*-butylbenzene-1-sulfonyl chloride (549 mg, 1.9 mmol) and $NaHCO_3$ (170 mg, 2 mmol) were heated together at 150 °C with stirring until the melt solidified. The reaction mixture was allowed to cool down to room

temperature, and 2 M solution of HCl was added. The solid residue was filtered out and recrystallised from MeOH/H_2O. Methyl 4-(3,5-di-*tert*-butyl-*N*-methylphenylsulfonamido)benzoate was obtained in 87% yield. The rest of the synthesis was performed according to the method described for **G1**.

N-(2-Aminoethyl)-4-((3,5-di-tert-butyl-N-methylphenyl)sulfonamido)benzamide hydrochloride (**G3**) ^{1}H NMR (CD_3OD): 7.84 (d, 2H, J = 8.8 Hz), 7.72 (t, 1H, J = 1.6 Hz), 7.29 (d, 2H, J = 1.6 Hz), 7.24 (2, 2H, J = 8.8 Hz), 3.65 (t, 2H, J = 6 Hz), 3.16 (t, 2H, J = 6 Hz), 3.14 (s, 3H), 1.26 (s, 18H). ^{13}C NMR (CD_3OD): 168.6, 152.0, 145.1, 135.1, 131.7, 127.7, 127.1, 125.5, 121.5, 39.6, 37.4, 36.7, 34.6, 30.1. HRMS-ESI: $C_{24}H_{36}N_3O_3S$ $[M+H]^+$ calcd: 446.2477, found: 446.2480; mp 137-139 °C (decomp.).

Compounds **G4** and **I12** were synthesized in a similar manner (Scheme 1).

N-(3-Aminopropyl)-4-((3,5-di-tert-butyl-N-methylphenyl)sulfonamido)benzamide hydrochloride (**G4**) ^{1}H NMR (CD_3OD): 7.85 (d, 2H, J = 8.4 Hz), 7.74 (s, 1H), 7.29 (s, 2H), 7.25 (d, 2H, J = 8.4 Hz), 3.52 (t, 2H, J = 6.4 Hz), 3.16 (s, 2H), 3.01 (t, 2H, J = 6.4 Hz), 1.99 (p, 2H, J = 6.4 Hz), 1.28 (s, 18H). ^{13}C NMR (CD_3OD): 168.2, 152.0, 144.9, 135.0, 131.9, 127.6, 127.0, 125.5, 121.5, 36.9, 36.6, 36.1, 34.6, 30.1, 27.6. HRMS-ESI: $C_{25}H_{38}N_3O_3S$ $[M+H]^+$ calcd: 460.2634, found: 460.2612; mp 121-124 °C (decomp.).

N-(2-Aminoethyl)-3-((3,5-di-tert-butyl-N-methylphenyl)sulfonamido)benzamide hydrochloride (**I12**) ^{1}H NMR (CD_3OD): 7.85 (d, 1H, J = 8 Hz), 7.75 (s, 1H), 7.69 (s, 1H), 7.47 (t, 1H, J = 8 Hz), 7.29 (s, 2H), 7.26 (d, 1H, J = 8 Hz) 3.67 (t, 2H, J = 6 Hz), 3.18 (t, 2H, J = 6 Hz), 3.16(s, 3H), 1.29 (s, 18H). ^{13}C NMR (CD_3OD): 168.5, 152.0, 142.2, 134.9, 134.2, 129.5, 128.8, 127.1, 125.8, 125.7, 121.7, 39.6, 37.4, 36.9, 34.6, 30.1. HRMS-ESI: $C_{24}H_{36}N_3O_3S$ $[M+H]^+$ calcd: 446.2477, found: 446.2462; mp > 95-97 °C (decomp.).

4-((3,5-Di-tert-butylphenyl)sulfonamido)-N-(2-guanidinoethyl)benzamide hydrochloride (**G2**) was synthesized as previously described in Igumnova et al.[3] Briefly, *N*-(2-aminoethyl)-4-((3,5-di-*tert*-butylphenyl)sulfonamido)benzamide (200 mg, 0.5 mmol) was dissolved in DMF (0.5 ml) and 1-amidino-1*H*-1,2,4-triazole hydrochloride (74 mg, 0.5 mmol) was added to the reaction mixture. The reaction vessel was sealed with a septum, and the reaction mixture stirred for 18 h at room temperature. The reaction mixture was filtered, Et_2O (10 ml) was added to the filtrate, and the mixture was stirred for 1 h at room temperature. The ether layer was decanted off, a new portion of Et_2O (10 ml) was added, and the mixture stirred for 10 h at room temperature. Et_2O was removed from the solid precipitate. Obtained 4-((3,5-di-*tert*-butylphenyl)sulfonamido)-*N*-(2-guanidinoethyl)benzamide hydrochloride was purified by recrystallization from MeOH/Et_2O in 91% yield (216 mg). ^{1}H NMR (CD_3OD): 7.74 (d, 2H, J = 8.8 Hz), 7.62 (s, 3H), 7.19 (d, 2H, J = 8.8 Hz), 3.51 (t, 2H, J = 6 Hz), 3.38 (t, 2H, J = 6 Hz), 1.27 (s, 18H). ^{13}C NMR (CD_3OD): 168.6, 157.5, 152.0, 141.5, 138.9, 129.0, 128.1, 126.6, 120.8, 119.3, 40.6, 38.5, 34.6, 30.1. HRMS-ESI: $C_{24}H_{36}N_5O_3S$ $[M+H]^+$ calcd: 474.2539, found: 474.2538; mp 247-249 °C (decomp.).

Compounds **G5**, **G6**, **H9**, **H10**, **I13**, **I14**, **J17**, **J18**, **K19**, **K20**, **K21**, **K22**, **L23**, **L24**, **L25**, and **L26** were synthesized in similar manner (Scheme 1).

4-((3,5-Di-tert-butyl-N-methylphenyl)sulfonamido)-N-(2-guanidinoethyl)benzamide hydrochloride (**G5**) ^{1}H NMR (CD_3OD): 7.82 (d, 2H, J = 8.8 Hz), 7.72 (t, 2H, J = 1.6 Hz), 7.28 (d, 2H, J = 1.6 Hz), 7.23 (d, 2H, J = 8.8 Hz), 3.55 (t, 2H, J = 6 Hz), 3,41 (t, 2H, J = 6 Hz), 3.14 (s, 3H), 1.26 (s, 18H). ^{13}C NMR (CD_3OD): 168.3, 157.5, 152.0, 145.0, 135.0, 131.8, 127.6, 127.0, 125.5, 121.5, 40.7, 38.6, 36.6, 34.6, 30.0. HRMS-ESI: $C_{25}H_{38}N_5O_3S$ $[M+H]^+$ calcd: 488.2695, found: 488.2685; mp > 98-102 °C (decomp.).

4-((3,5-Di-tert-butyl-N-methylphenyl)sulfonamido)-N-(3-guanidinopropyl)benzamide hydrochloride (**G6**) ^{1}H NMR (CD_3OD): 7.83 (d, 2H, J = 8.8 Hz), 7.74 (s, 1H), 7.29 (s, 2H), 7.24 (2, 2H, J = 8.8 Hz), 3.48 (t, 2H, J = 6.4 Hz), 3.28 (t, 2H, J = 6.4 Hz), 3.16 (s, 3H), 1.90 (p, 2H, J = 6.4 Hz), 1.28 (s, 18H). ^{13}C NMR (CD_3OD): 167.9, 157.3, 152.0, 144.8, 135.0, 132.2, 127.5, 127.0, 125.5, 121.5, 38.6, 36.6, 36.6, 34.6, 30.1, 28.7. HRMS-ESI: $C_{26}H_{40}N_5O_3S$ $[M+H]^+$ calcd: 502.2852, found: 502.2858; mp 119-121 °C (decomp.).

4-(((3,5-Di-tert-butylphenyl)sulfonamido)methyl)-N-(2-guanidinoethyl)benzamide hydrochloride (**H9**) ^{1}H NMR (CD_3OD): 7.75 (d, 2H, J = 8 Hz), 7.70 (s, 3H), 7.32 (d, 2H, J = 8 Hz), 4.15 (s, 2H), 3.56 (t, 2H, J = 6 Hz), 3.42 (t, 2H, J = 6 Hz), 1.36 (s, 18H). ^{13}C NMR (CD_3OD): 168.9, 157.5, 152.1, 141.8, 140.2, 132.5, 127.5, 127.1, 126.4, 120.7, 45.9, 40.7, 38.5, 34.7, 30.2. HRMS-ESI: $C_{25}H_{38}N_5O_3S$ $[M+H]^+$ calcd: 488.2695, found: 488.2676; mp 193-195 °C (decomp.).

2-(4-((3,5-Di-tert-butylphenyl)sulfonamido)phenyl)-N-(2-guanidinoethyl)acetamide hydrochloride (**H10**) ^{1}H NMR (CD_3OD): 7.63 (s, 1H), 7.56 (s, 2H), 7.18 (d, 2H, J = 8.4 Hz), 7.05 (d, 2H, J = 8.4 Hz), 3.46 (s, 2H), 3.37-3.25 (m, 4H), 1.28 (s, 18H). ^{13}C NMR (CD_3OD): 173.4, 157.5, 151.8, 138.8, 136.7, 131.9, 129.4, 126.4, 121.6, 120.9, 41.5, 40.6, 38.2, 34.6, 30.1. HRMS-ESI: $C_{25}H_{38}N_5O_3S$ $[M+H]^+$ calcd: 488.2695, found: 488.2690; mp > 135-137 °C (decomp.).

3-((3,5-Di-tert-butylphenyl)sulfonamido)-N-(2-guanidinoethyl)benzamide hydrochloride (**I13**) ^{1}H NMR (CD_3OD): 7.70 (t, 1H, J = 1.6 Hz), 7.64 (t, 1H, J = 1.6 Hz), 7.58 (d, 2H, J = 1.6 Hz), 7.58-7.53 (m, 1H), 7.34 (t, 1H, J = 8 Hz), 7.25-7.19 (m, 1H), 3.55 (t, 2H, J = 6 Hz), 3.41 (t, 2H, J = 6 Hz), 1.28 (s, 18H). ^{13}C NMR (CD_3OD): 168.7, 157.5, 151.9, 138.6, 138.5, 134.8, 128.9, 126.6, 124.4, 122.8, 120.9, 120.4, 40.6, 38.6, 34.6, 30.1. HRMS-ESI: $C_{24}H_{36}N_5O_3S$ $[M+H]^+$ calcd: 474.2539, found: 474.2531; mp 140-143 °C (decomp.).

3-((3,5-Di-tert-butyl-N-methylphenyl)sulfonamido)-N-(2-guanidinoethyl)benzamide hydrochloride (**I14**) ^{1}H NMR (CD_3OD): 7.78 (dd, 1H, J_1 = 8 Hz, J_2 = 1.6 Hz), 7.73 (t, 1H, J = 1.6 Hz), 7.64 (t, 1H, J = 1.6 Hz), 7.436 (t, 1H, J = 8 Hz), 7.27 (d, 2H, J = 1.6 Hz), 7.23 (dd, 1H, J_1 = 8 Hz, J_2 = 1.6 Hz) 3.53 (t, 2H, J = 6 Hz), 3.40 (t, 2H, J = 6 Hz), 3.13(s, 3H), 1.26 (s, 18H). ^{13}C NMR (CD_3OD): 168.2, 157.5, 152.0, 142.2, 134.8, 134.4, 129.6, 128.8, 127.1, 125.7, 125.4, 121.7, 40.7, 38.6, 36.9, 34.6, 30.1. HRMS-ESI: $C_{25}H_{38}N_5O_3S$ $[M+H]^+$ calcd: 488.2695, found: 488.2684; mp 77-79 °C (decomp.).

2-((3,5-Di-tert-butylphenyl)sulfonamido)-N-(2-guanidinoethyl)benzamide hydrochloride (**J17**) ^{1}H NMR (CD_3OD): 7.66 (d, 1H, J = 8 Hz), 7.61 (t, 1H, J = 1.6 Hz), 7.55 (d, 1H, J = 8 Hz), 7.53 (d, 2H, J = 1.6 Hz), 7.43 (d, 1H, J = 8 Hz), 7.14 (t, 1H, J = 8 Hz), 3.47 (t, 2H, J = 6 Hz), 3.36 (t, 2H, J = 6 Hz), 1.24 (s, 18H). ^{13}C NMR (CD_3OD): 169.6, 157.5, 152.0, 138.7, 138.3, 132.2, 128.0, 126.7, 123.9, 122.0, 121.7, 120.9, 40.4, 38.4, 34.6, 30.1. HRMS-ESI: $C_{24}H_{36}N_5O_3S$ $[M+H]^+$ calcd: 474.2539, found: 474.2535; mp > 103-105 °C (decomp.).

3-((3,5-Di-tert-butylphenyl)sulfonamido)-N-(2-guanidinoethyl)-2-naphthamide hydrochloride (**J18**) ^{1}H NMR (CD_3OD): 8.26 (s, 1H), 7.87 (d, 1H, J = 8 Hz), 7.86 (s, 1H), 7.77 (d, 1H, J = 8 Hz), 7.57 (t, 1H, J = 8 Hz), 7.55 (s, 1H), 7.54 (s, 2H), 7.48 (t, 1H, J = 8 Hz), 3.54 (t, 2H, J = 6 Hz), 3.44 (t, 2H, J = 6 Hz), 1.15 (s, 18H). ^{13}C NMR (CD_3OD): 171.1, 158.9, 153.3, 139.2, 136.1, 135.5, 131.1, 130.8, 129.9, 129.7, 129.7, 128.1, 128.1, 124.1, 122.6, 120.9, 41.9, 39.9, 35.9, 31.4. HRMS-ESI: $C_{28}H_{38}N_5O_3S$ $[M+H]^+$ calcd: 524.2695, found: 524.2696; mp > 76-79 °C (decomp.).

4-((3,5-Di-tert-butylphenyl)sulfonamido)-N-(2-guanidinoethyl)-3-methylbenzamide hydrochloride (**K19**) ^{1}H NMR (CD_3OD): 7.66 (t, 1H, J = 1.6 Hz), 7.62 (dd, 1H, J_1 = 8 Hz, J_2 = 1.6 Hz), 7.61 (d, 2H, J = 1.6 Hz), 7.48 (d, 2H, J = 1.6 Hz), 7.33 (d, 2H, J = 8 Hz), 3.52 (t, 2H, J = 6 Hz), 3.39 (t, 2H, J = 6 Hz), 1.91 (s, 3H), 1.25 (s, 18H). ^{13}C NMR (CD_3OD): 168.6, 157.5, 152.0, 139.4, 138.7, 133.3, 131.1, 129.6, 126.5, 125.6, 125.1, 120.7, 40.7, 38.5, 34.6, 30.1, 16.4. HRMS-ESI: $C_{25}H_{38}N_5O_3S$ $[M+H]^+$ calcd: 488.2695, found: 488.2678; mp > 169-172 °C (decomp.).

1-((3,5-Di-tert-butylphenyl)sulfonyl)-N-(2-guanidinoethyl)piperidine-4-carboxamide hydrochloride (**K20**) ^{1}H NMR (CD_3OD):7.78 (s, 1H), 7.60 (s, 2H), 4.83-4.76 (m, 2H), 3.39-

3.24 (m, 4H), 3.38-3.25 (m, 2H), 3.25-3.14 (m, 1H), 1.94-1.82 (m, 2H), 1.82-1.65 (m, 2H), 1.39 (s, 18H). ^{13}C NMR (CD_3OD): 176.5, 157.5, 152.3, 135.5, 126.9, 121.4, 45.5, 41.5, 40.6, 38.0, 34.7, 30.2, 27.9. HRMS-ESI: $C_{23}H_{40}N_5O_3S$ $[M+H]^+$ calcd: 466.2852, found: 466.2847; mp > 184-186 °C (decomp.).

(S)-1-((3,5-Di-tert-butylphenyl)sulfonyl)-N-(2-guanidinoethyl)pyrrolidine-2-carboxamide hydrochloride (**K21**) ^{1}H NMR (CD_3OD):7.83 (s, 1H), 7.71 (s, 2H), 4.08-4.00 (m, 1H), 3.66-3.56 (m, 1H), 3.56-3.35 (m, 4H), 3.26-3.16 (m, 1H), 2.02-1.73 (m, 3H), 1.64-1.52 (m, 1H), 1.40 (s, 18H). ^{13}C NMR (CD_3OD): 174.4, 157.5, 152.7, 135.6, 127.4, 121.5, 62.5, 49.4, 40.5, 38.1, 34.7, 30.7, 30.2, 24.1. HRMS-ESI: $C_{22}H_{38}N_5O_3S$ $[M+H]^+$ calcd: 452.2695, found: 452.2690; mp > 82-84 °C (decomp.).

3,5-Di-tert-butyl-N-(4-guanidinobutyl)benzenesulfonamide hydrochloride (**K22**) ^{1}H NMR (CD_3OD): 7.70 (s, 3H), 3.17 (t, 2H, *J* = 6.8 Hz), 2.86 (t, 2H, *J* = 6.8 Hz), 1.69-1.50 (m, 4H), 1.37 (s, 18H). ^{13}C NMR (CD_3OD): 158.6, 153.6, 141.2, 127.8, 122.0, 43.4, 42.0, 36.1, 31.6, 27.9, 27.0. HRMS-ESI: $C_{19}H_{35}N_4O_2S$ $[M+H]^+$ calcd: 383.2481, found: 383.2475; mp > 168-170 °C (decomp.).

4-((3,5-Dimethylphenyl)sulfonamido)-N-(2-guanidinoethyl)benzamide hydrochloride (**L23**) ^{1}H NMR (CD_3OD): 7.71 (d, 2H, *J* = 8.8 Hz), 7.43 (s, 2H), 7.21 (s, 1H), 7.19 (d, 2H, *J* = 8.8 Hz), 3.50 (t, 2H, *J* = 6 Hz), 3.37 (t, 2H, *J* = 6 Hz), 2.31 (s, 6H). ^{13}C NMR (CD_3OD): 168.7, 157.5, 141.4, 139.4, 139.1, 134.1, 128.7, 128.1, 124.2, 118.6, 40.6, 38.5, 19.7. HRMS-ESI: $C_{18}H_{24}N_5O_3S$ $[M+H]^+$ calcd: 390.1600, found: 390.1602; mp 257-259 °C (decomp.).

4-((3,5-Bis(trifluoromethyl)phenyl)sulfonamido)-N-(2-guanidinoethyl)benzamide hydrochloride (**L24**) ^{1}H NMR (CD_3OD): 8.30 (s, 2H), 8.25 (s, 1H), 7.78 (d, 2H, *J* = 8.8 Hz), 7.23 (d, 2H, *J* = 8.8 Hz), 3.51 (t, 2H, *J* = 6 Hz), 3.38 (t, 2H, *J* = 6 Hz). ^{13}C NMR (CD_3OD): 168.4, 157.5, 142.5, 140.3, 132.4 (q, *J* = 34 Hz), 130.1, 128.5, 127.2 (q, *J* = 4 Hz), 126.3 (q, *J* = 4 Hz), 122.5 (q, *J* = 273 Hz), 119.8, 40.6, 38.5. HRMS-ESI: $C_{18}H_{18}N_5O_3SF_6$ $[M+H]^+$ calcd: 498.1035, found: 498.1032; mp 215-217 °C (decomp.).

N-(2-Guanidinoethyl)-4-(naphthalene-2-sulfonamido)benzamide hydrochloride (**L25**) ^{1}H NMR (CD_3OD): 8.41 (d, 1H, *J* = 1.6 Hz), 7.97 (d, 2H, *J* = 8.4 Hz), 7.92 (d, 1H, *J* = 8 Hz), 7.79 (dd, 1H, J_1 = 8 Hz, J_2 = 1.6 Hz), 7.68 (d, 2H, *J* = 8.8 Hz), 7.66-7.58 (m, 2H), 7.23 (d, 2H, J = 8.8 Hz), 3.47 (t, 2H, *J* = 6 Hz), 3.34 (t, 2H, *J* = 6 Hz). ^{13}C NMR (CD_3OD): 168.7, 157.4, 141.3, 136,4, 134.9, 131.9, 129.1, 129.0, 128.8, 128.7, 128.3, 128.1, 127.5, 127.3, 121.8, 118.9, 40.6, 38.5. HRMS-ESI: $C_{20}H_{22}N_5O_3S$ $[M+H]^+$ calcd: 412.1443, found: 412.1423; mp > 157-159 °C (decomp.).

4-((3,5-Di-tert-butyl-4-methoxyphenyl)sulfonamido)-N-(2-guanidinoethyl)benzamide hydrochloride (**L26**) ^{1}H NMR (CD_3OD): 7.74 (d, 2H, *J* = 8.8 Hz), 7.67 (s, 2H), 7.19 (d, 2H, *J* = 8.8 Hz), 3.66 (s, 3H), 3.51 (t, 2H, *J* = 6 Hz), 3.36 (t, 2H, *J* = 6 Hz), 1.35 (s, 18H). ^{13}C NMR (CD_3OD): 168.6, 163.26, 157.5, 144.8, 141.6, 133.3, 128.9, 128.1, 125.4, 119.2, 64.0, 40.7, 38.5, 35.6, 30.7. HRMS-ESI: $C_{25}H_{38}N_5O_4S$ $[M+H]^+$ calcd: 504.2645, found: 504.2642; mp 247-249 °C (decomp.).

4.4 Biological test methods

The initial antimicrobial screening included bacterial reference strains listed under Table 1. The expanded screenings were performed against the collection of 275 clinical isolates deposited at the *Norwegian Organization for Surveillance of Resistant Microorganisms* (NORM) (2012-2014), and the collection of 30 multidrug-resistant clinical isolates from the *The Norwegian National Advisory Unit on Detection of Antimicrobial Resistance.*[8-10] Bacteria included are denoted in the respective tables (Table 2, Table 3). The collection of *Enterococcus* spp. contained isolates of both *E. faecium* and *E. faecalis.*[3] Multidrug resistance was defined as resistance to at least one class of antimicrobial agents.[44]

Minimum inhibitory concentration (MIC) assay: To determine the MIC, a microdilution susceptibility test was performed according to CLSI M07-A9[45] with modifications described previously.[3] Briefly, the bacterial inoculum in Mueller-Hinton broth (MHB, Difco Laboratories, USA) adjusted to approximately 2.5-3 x 10^4 cells/ml, was added to flat-bottom microplates (Nunc, Roskilde, Denmark) in a ratio 1:1 with serial dilutions of the test compounds. The solutions of the compounds were prepared with up to 100% DMSO, but the final DMSO concentration in the test wells did not exceed 1%. After the plates were incubated at 35 °C for 48 h, the MIC value defined as the lowest concentration of compound resulting in no bacterial growth, was determined by OD_{600} measurement.

Antimicrobial screening against clinical isolates was performed as previously described,[3] with approximately 1-1.2 x 10^6 cells/ml and 24 h of incubation time. MIC_{90} was defined as the MIC value that inhibited ≥90% of the isolates.[46]

Hemolytic assay: The lytic activity against human RBCs was determined as described before.[3] Briefly, blood from a healthy adult was collected in heparinized tubes. Washed RBC suspension in phosphate-buffered saline (PBS, pH 7.4), was adjusted to 10% and incubated in a ratio 9:1 with serial dilutions of the compounds at 37 °C for 1 h. After centrifugation at 450×g for 10 min, the supernatants were transferred to flat-bottom microplates (Nunc, Roskilde, Denmark) and OD_{550} was measured. Incubation of RBCs in 50% PBS and 0.5% DMSO gave baseline hemolysis, while incubation in 50% PBS and 0.05% Triton X-100 gave complete (100%) hemolysis. EC_{50} was defined as the concentration of compound giving 50% lysis.

Determination of cytotoxicity against MRC-5 cells: The test compounds were screened for cytotoxicity at two-fold concentrations up to 100 µg/ml against normal human lung fibroblast (MRC-5, ATCC CCL-171™) cells.[47] MRC-5 cells were cultured and assayed in Dulbecco's modified Eagle's medium (D-MEM, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS, Merck) and 5 ml of L-alanyl-Lglutamine (200 mM, Merck) and incubated in 5% CO_2 at 37 °C. Briefly, the cells were seeded in 96-well microtiter plates (Thermo Fisher Scientific) at 4000 cells/well and incubated for 24 h to allow the cells to adhere before the cell media was replaced and the compounds (50 µl) were added. The plates were then incubated for 72 h. Subsequently, 10 µl of MTS solution (Cell Titer 96 Aqueous One Solution Reagent, Promega) was added to each well, and the cells were incubated for 1 h at 37 °C. The absorbance was measured at 485 nm using a DTX multimode detector (Beckman Coulter). The negative control was defined as cells assayed with their respective cell media with 1% DMSO (1% was the highest DMSO concentration used in wells with compound), and positive control as cells assayed with 0.5% Triton X-100 (Sigma-Aldrich). Cell survival was calculated as follows: Cell survival (%) = (absorbance treated wells − absorbance positive control)/(absorbance negative control − absorbance positive control) × 100. EC_{50} values for cytotoxicity against MRC-5 cells refer to the concentration (µg/ml) causing 50% inhibition of cell survival.

Membrane integrity assay: The membrane integrity of *B. subtilis* 168 transformed with the plasmid pCSS962 according to Virta et al., [29] was assayed as previously described.[3] Briefly, aliquots of bacterial suspension with an OD_{600}=0.1 containing 1 mM D-luciferin potassium-salt (pH 7.4, SynChem Inc, IL, USA) were automatically injected to black microplates (Nunc, Roskilde, Denmark) with dilutions of the compounds. The luminescence emission was monitored before and after the addition of the membrane-disruptive control chlorhexidine acetate (Fresenius Kabi, Halden Norway).

Biofilm eradication assay: Eradication of mature biofilms of *S. epidermidis* RP62A (MRSE) (ATCC 35984 / CCUG 31568) was determined based on Klingenberg et al.[48] and Christensen et al.[49] Briefly, overnight cultures were prepared in Tryptic soy broth (TSB; BD, USA) at 37 °C with agitation. The cultures were centrifuged for 5 min at 13 000 rpm and the pellets resuspended in 0.9% NaCl. The suspension was filtered through a syringe filter with pore size of 5 µm (Acrodisc®, Pall Corporation, Neuquay Cornwall, UK) to remove aggregates and adjusted to 2 McFarland in 0.9% NaCl, before being diluted in TSB with 1% glucose (TSB_{glu}) to a final inoculum of approximately 6 x 10^6 CFU/ml. A volume of 50 µl of

the bacterial suspension was added into flat-bottom microplates (Nunclon™ Surface, Nunc, Roskilde, Denmark). The plates were sealed, incubated for 24 ±2 h at 37 °C without shaking and then washed 2x with PBS. Dilutions of test compounds in 50% TSB_{glu} were added to the biofilms. Untreated samples containing 50% TSB_{glu} were run on the same plates. Following 24 ±2 h of incubation at 37 °C without shaking, the plates were washed 3x and dried overnight at room temperature or for 1 hour at 55 °C before staining with 0.1% crystal violet for 5 min. The plates were washed 3x with tap water, and crystal violet was solubilized with 70% ethanol. OD_{600} was measured after a brief agitation. All compounds and concentrations were tested in 4 to 8 parallels on 2 to 3 different plates. For each parallel series on a plate, the highest and the lowest values (outliers) were excluded. Percent biofilm biomass in comparison to the untreated control was calculated.

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A. Supplementary data

Supplementary data (^{1}H-NMR spectra of synthesized compounds) associated with this article can be found, in the online version, at…