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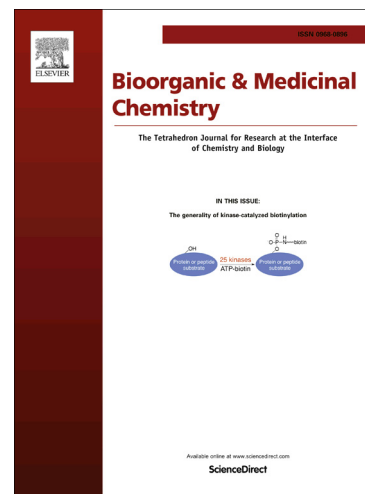
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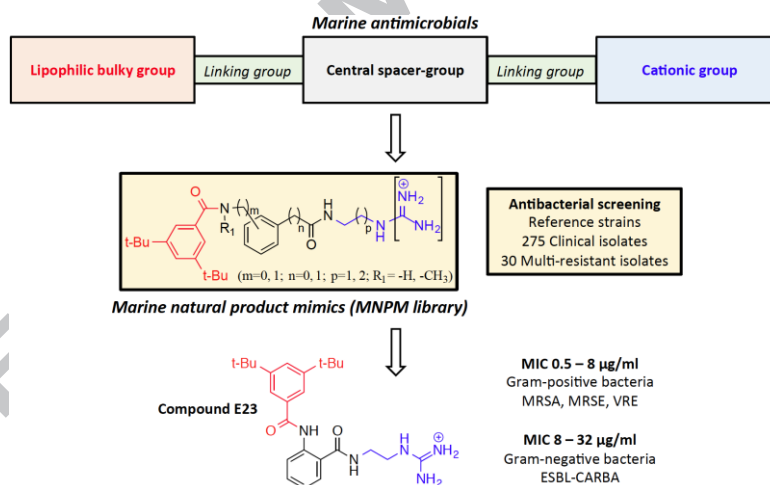
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Synthesis and antimicrobial activity of small cationic amphipathic aminobenzamide marine natural product mimics and evaluation of relevance against clinical isolates including ESBL-CARBA multi-resistant bacteria

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ABSTRACT

A library of small aminobenzamide derivatives was synthesised to explore a cationic amphipathic motif found in marine natural antimicrobials. The most potent compound **E23** displayed minimal inhibitory concentrations (MICs) of 0.5 – 2 µg/ml against several Gram-positive bacterial strains, including methicillin resistant *Staphylococcus epidermidis* (MRSE). **E23** was also potent against 275 clinical isolates including *Staphylococcus aureus*, *Enterococcus* spp., *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*, as well as methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant enterococci (VRE), and ESBL-CARBA multi-resistant Gram-negative bacteria. The study demonstrates how structural motifs found in marine natural antimicrobials can be a valuable source for making novel antimicrobial *lead-compounds*.

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1. Introduction

Modern society faces huge challenges with microbial resistance that in less than a generation might set us back to the pre-antibiotic era.¹ With reluctant enthusiasm from major pharmaceutical companies because of low profit prospects, responsibility resides on academia and small biotech companies to generate *lead-compounds* for development of novel antimicrobial agents to combat resistance. We have through investigations of antimicrobial peptides (AMPs),²⁻⁵ alkaloids,⁶ and peptidomimetics⁷⁻¹¹ shown how amphipathic molecules of various size and structures display antimicrobial activity as long as they fulfil a minimum pharmacophore of two cationic groups and two bulky lipophilic groups. However, we have also shown that the marine environment is a valuable source of other antimicrobial *hit-compounds* for rational drug-design, such as ianthelline,¹² synoxazolidinones,¹³ and 3-dehydroxytubastrine¹⁴ (Fig. 1). These and other marine antimicrobials such as aplysinamisines A and B,¹⁵ fasciospongine C,¹⁶ hyrtioseragamine A,¹⁷ ianthelliformisamines A-C,¹⁸ nakijiquinone H,¹⁹ and 5-bromoverongamine²⁰ also comprise cationic amphipathic structures. All these compounds have in common a structural motif that in its simplest form can be described as consisting of a

1) lipophilic group, 2) a spacer-group (linear or cyclic), and 3) a cationic group (amine or guanidine).

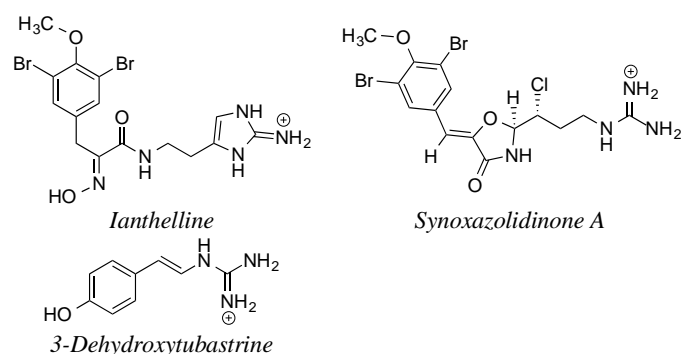


Figure 1. Examples of small cationic amphipathic marine antimicrobials.

In order to explore this as a general antimicrobial motif, we have in the present project synthesised a focused library of *marine natural product mimics* (MNPMs) with an

aminobenzamide spacer-group and derivatives thereof (Fig. 2). Our aim was to improve antimicrobial potency and investigate antimicrobial structure-activity relationships (SAR) also in conjunction to the pharmacophore model for small amphipathic AMPs and peptidomimetics.²⁻⁷ Initially, structural requirements for the lipophilic group were explored through a series of initial experiments with small substituted benzyl groups that gave low or no antimicrobial activity when included into our scaffolds (results not shown). Our investigations eventually resulted in the discovery of the *super bulky* 3,5-di-*tert*-butylphenyl group as most optimal for antimicrobial activity, and which was thereafter used in all compounds of the library. Thus, the library of MNPMs consisted of a 3,5-di-*tert*-butylphenyl group linked by an amide bond to the principal variable aminobenzamide spacer-group, which was further linked to different cationic groups (primary amine, tertiary amine or guanidine) of diverse chain length and orientation as shown in Fig. 2.

The library of MNPMs was initially screened for antimicrobial activity against a selection of Gram-positive and Gram-negative reference strains. To evaluate isolate variations, nine structurally diverse and potent MNPMs were screened against 25 randomly chosen clinical isolates of Gram-positive

Staphylococcus aureus and *Enterococcus* spp., and Gram-negative *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumonia*. These consecutive isolates were deposited at the Norwegian Organization for Surveillance of Resistant Microorganisms (NORM) in the period 2012-2014. Two promising MNPMs were further screened for antimicrobial activity against an expanded panel of 250 randomly chosen clinical isolates, and another panel of 30 multi-resistant clinical isolates including methicillin resistant *S. aureus* (MRSA), vancomycin resistant *Enterococci* (VRE), and Gram-negative isolates with extended spectrum β -lactamase – carbapenemase (ESBL-CARBA) resistance including *E. coli*, *P. aeruginosa*, *K. pneumonia*, and *Acinetobacter baumannii*. Initial investigations of the *mode-of-action* indicated a membranolytic effect resembling small cationic AMPs, although we cannot exclude additional intracellular targets.

The study demonstrates how a general structural motif found in small marine natural antimicrobials could be used as a valuable source for synthesizing less complex *lead-compounds* with activity against clinical and ESBL-CARBA multi-resistant isolates of pathogenic bacteria.

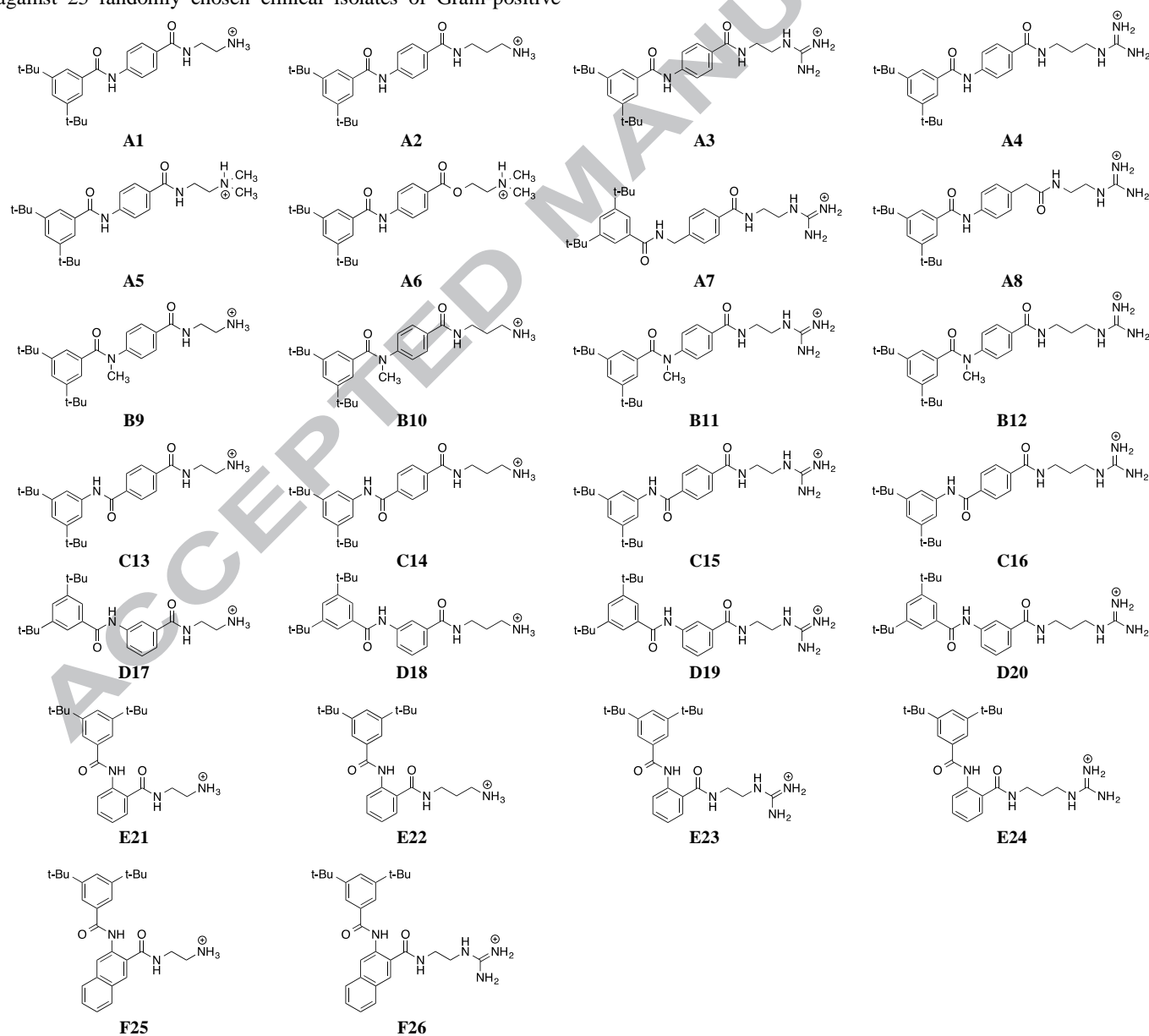


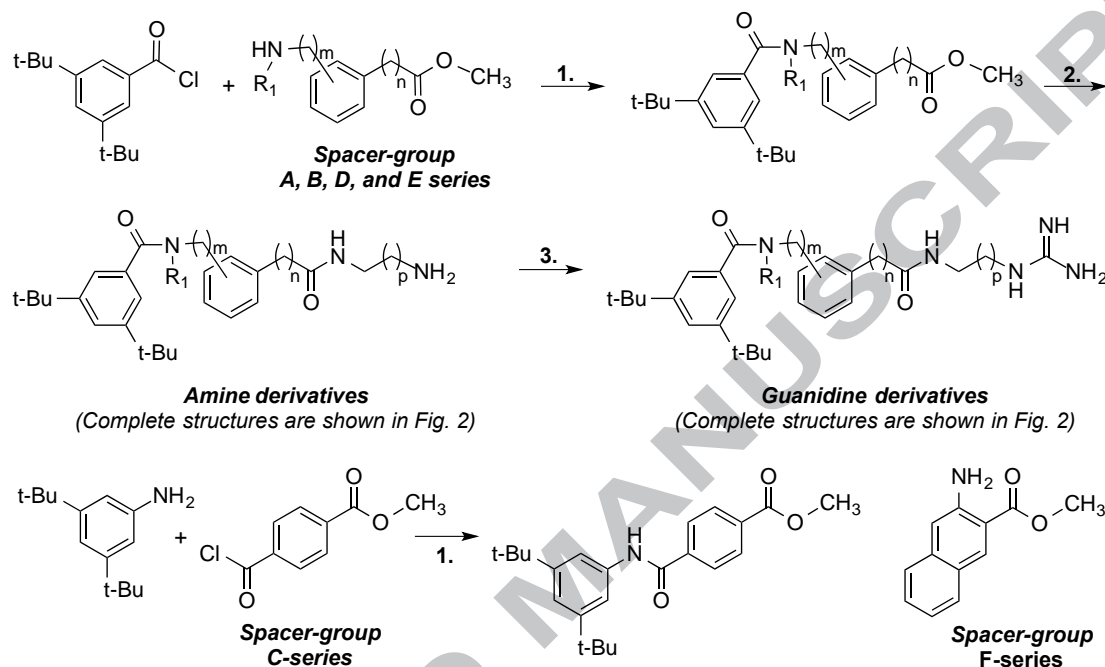
Figure 2. Library of MNPMs synthesised and investigated for antimicrobial activity. All MNPMs were isolated and tested as their HCl-salts.

2. Results and discussion

2.1 Synthesis

The library of MNPMs was synthesised according to Scheme 1. For all compounds, the first step was formation of an amide bond between the 3,5-di-*tert*-butylphenyl group and the aromatic spacer-group by reaction of the corresponding acyl-chlorides with the appropriate amines in the presence of base. The second amide or ester bridge between the aromatic spacer-group and the

cationic group was accomplished by reaction with an excess of the appropriate aliphatic amine or alcohol on the methyl ester group of the previous intermediate. Guanidine derivatives were prepared by guanylation with 1-amidino-1*H*-1,2,4-triazole hydrochloride. The MNPMs were precipitated as HCl salts by using 4 M HCl in dioxane. All MNPMs were purified by recrystallization in MeOH and Et₂O, and isolated and screened for antimicrobial activity as HCl-salts for optimized solubility in aqueous test-media.



Scheme 1. The scheme shows reactants and conditions for synthesis of the MNPMs investigated for antimicrobial activity. *Step 1.* 3,5-Di-*tert*-butylbenzoyl chloride, DIPEA, DMF, r.t., 18 h; *Step 2.* H₂N-CH₂(CH₂)_pNH₂ (p = 1, 2), 100 °C, 18 h; *Step 2.* for **A5**: *N,N*-dimethylethylenediamine, 100 °C, 18 h; *Step 2.* for **A6**: *N*-dimethylaminoethanol, NaOMe, 100 °C, 18 h; *Step 3.* 1-amidino-1*H*-1,2,4-triazole hydrochloride, DMF, r.t., 18 h. (m = 0 or 1, n = 0 or 1, p = 1 or 2, R₁ = H or CH₃).

2.2 Antimicrobial activity against bacterial reference strains

Antimicrobial activity of the synthesised MNPMs was first evaluated by determination of minimal inhibitory concentrations (MICs) against a panel of reference strains including Gram-positive *S. aureus*, methicillin resistant *Staphylococcus epidermidis* (MRSE), *Bacillus subtilis*, and *Corynebacterium glutamicum*, and Gram-negative *E. coli* and *P. aeruginosa* (Table 1). A broth microdilution method was used to determine MIC values whereas toxicity was determined as haemolytic activity (EC₅₀) against human red blood cells (RBC). A selectivity index (SI) was also calculated and defined as the ratio between haemolytic activity (EC₅₀) and MIC against *S. aureus*, which is commonly used as a reference strain.²¹ Determination of SI values was used as an aid to identify promising MNPMs for further antimicrobial screening studies.

The majority of the MNPMs prepared showed MIC values ≤ 8 µg/ml against the Gram-positive bacteria and ≤ 16 µg/ml against the Gram-negative bacteria (Table 1). The SI of the MNPMs varied from 0.3 to 37 when comparing haemolytic activity with antimicrobial activity against *S. aureus*. As described below, overall highest antimicrobial potency and selectivity was achieved for **E23**, which had MIC values of 0.5 – 2 µg/ml against the Gram-positive bacteria and SI of 37. Commercially available reference antibiotics gave comparable MIC values to our most potent MNPMs when tested in our assay, and especially against the Gram-positive bacteria. Thus, vancomycin displayed MIC of

2 µg/ml against *S. epidermidis*, erythromycin gave MIC of 1 – 2.5 µg/ml against the remaining Gram-positive bacteria (*S. aureus*, *B. subtilis*, and *C. glutamicum*), and polymyxin B showed MIC of 2.5 µg/ml against *E. coli* and *P. aeruginosa*. Overall, the MIC values for the reference antibiotics were generally similar to values obtained against common quality control bacteria.²²

The initial A-series having a 4-aminobenzamide spacer-group (Fig. 2) resulted in MNPMs displaying MIC values of 2 – 16 µg/ml against the Gram-positive bacteria and 8 – >128 µg/ml against the Gram-negative bacteria for **A1** - **A8** (Table 1). The most potent MNPMs of the A-series were the guanidine derivatives **A3** and **A4**, together with **A8** (as described below). An SI of 15.5 was achieved for **A4** whereas **A3** had an SI of 9.5.

The amine MNPMs **A1** and **A2** were less potent against *S. aureus*, *S. epidermidis* (MRSE) and *P. aeruginosa*, than the preceding guanidine MNPMs and more toxic against human RBC giving lower SI's. The results also showed that altering the length of the aliphatic chain connecting the 4-aminobenzamide spacer-group and cationic group had little influence on antimicrobial activity, as observed by similar antimicrobial potencies for **A1** and **A2**, and **A3** and **A4**.

MNPMs **A5** and **A6**, contained a tertiary cationic dimethyl amino group, in addition to the ester group of **A6**, and both showed comparable potencies as the amine analogues **A1** and **A2** against the Gram-positive reference strains. However,

antimicrobial activity against the Gram-negative reference strains was strongly reduced. Only **A5** displayed activity against *E. coli*, whereas **A6** was altogether inactive against both the Gram-negative reference strains (tested up to 128 µg/ml). Thus, introduction of the ester group in **A6** clearly had a negative impact on antimicrobial activity, which might be related to reduced stability and ester-hydrolysis. The results for both **A5** and **A6** also showed that the tertiary dimethyl amino group was not optimal as cationic group, especially with respect to Gram-negative bacteria. Thus, only primary amine and guanidine groups were chosen as cationic groups in the following series of MNPMs.

MNPMs **A7** and **A8** were guanylated and had spacer-groups with an additional methylene group included in order to evaluate the influence of introducing increased flexibility, easier rotation and wider conformational diversity. Derivative **A8** showed the same high antimicrobial potency as **A3** and **A4**, and was a little more potent against three of the Gram-positive reference strains than **A7**. However, we experienced very low aqueous solubility for **A7** and **A8**, and especially when determining haemolytic activity. Thus, accurate SI determination for **A8** was not possible since highest achievable test-concentration for **A8** was 64 µg/ml in the RBC assay, which resulted in 35% haemolysis at this concentration. MNPMs **A7** and **A8** were therefore discontinued in further screening studies.

The B-series contained a 2-(methylamino)benzamide spacer-group, i.e. having an *N*-methylated amide connected to the lipophilic group, which resulted in highly variable MIC values of 2 to >128 µg/ml. Low or no antimicrobial activity was observed against the Gram-negative reference strains for all four derivatives of the B-series. Highest antimicrobial activity against Gram-positive reference strains was observed for the guanidine derivatives **B11** and **B12**, and it was noteworthy that these were also practically non-toxic to RBC with EC₅₀ values >240 µg/ml and SI's of 15. The amine MNPMs **B9** and **B10** could be described as close to inactive, although some activity was observed for **B9**, which only differed from **B10** by having a shorter cationic chain.

There was an important difference in antimicrobial potency between MNPMs of the A-series and the B-series. MNPMs of the A-series showed rather small differences in antimicrobial potency for amine analogues and guanidine derivatives, while the difference between amine analogues and guanidine analogues in the *N*-methylated B-series was more pronounced. This was especially obvious against Gram-positive bacteria. Furthermore, despite that the activity of the guanidine derivatives of the B-series was somewhat lower, the toxicity was much lower than that of the guanidine derivatives of the A-series. Thus, *N*-methylation of the amide group had a substantial influence on bioactivity that may not only be a result of increased lipophilicity. It could also be a conformational effect caused by steric repulsions between the *N*-methylated amide group and the *ortho*-protons of the spacer-group twisting the adjacent aromatic groups out of plane.

The C-series contained a terephthalamide spacer-group, i.e. having a reversed amide bond connected to the lipophilic group, and were otherwise close analogues of **A1** – **A4**. However, reversing the orientation of this amide bond had little effect as observed by comparable MIC and haemolytic activity of the A- and C-series, and as shown by pairwise comparing derivatives **C13/A1**, **C14/A2**, **C15/A3** and **C16/A4**.

The D-series contained a 3-aminobenzamide spacer-group and displayed MIC values from 2 – 16 µg/ml against both Gram-

positive and Gram-negative bacteria. As observed for the previous A-series, the guanidine derivatives **D19** and **D20** were overall more potent than the amine analogues **D17** and **D18**. Compound **D20** displayed the highest SI of 14.8 within the D-series.

The E-series was based on a 2-aminobenzamide spacer-group, i.e. with *ortho*-substitution or 1,2-positioning of the lipophilic and cationic groups on the spacer-group, and resulted in some of the most potent MNPMs obtained against Gram-positive bacteria. Thus, **E23** and **E24** with a cationic guanidine group displayed MIC values of 0.5 – 4 µg/ml against the Gram-positive bacteria, and were more potent than the corresponding amine derivatives **E21** and **E22**. Thus, confirming the general *guanidine rule* of the present MNPMs series. MIC values against the Gram-negative bacteria were in the range 8 – 64 µg/ml and comparable to MNPMs described above. A reason to the high potency of the E-series could be related to a more rigid conformation stabilized by intra-molecular hydrogen bonding between the two closely 1,2-positioned amide groups. Little effect on toxicity against RBC was observed and **E23** displayed an SI of 37, which was the highest SI value achieved among all 26 MNPMs investigated.

The last F-series included a 3-amino-2-naphthamide spacer-group, which increased the lipophilicity and size of the spacer-group, but maintained the *ortho*-substitution (or 1,2-positioning) of the lipophilic and cationic groups as in the E-series. Both derivatives **F25** and **F26** were highly potent and in the same potency range as the E-series against Gram-positive bacteria with MIC values of 1 – 8 µg/ml. However, surprisingly low antimicrobial activity was observed against the Gram-negative *P. aeruginosa* reference strain and especially for **F25**. Additionally, introduction of the 3-amino-2-naphthamide spacer-group had a negative impact on the aqueous solubility of **F26**, and determination of haemolytic activity for **F26** was challenging to accomplish.

In summary, highest antimicrobial activity was achieved against Gram-positive bacteria; in which the potencies varied much less between the different series A-F compared to antimicrobial activity against Gram-negative bacteria. Structurally, guanidine derivatives were both more potent against bacteria and displayed lower haemolytic activity compared to amine derivatives. Similar enhanced antimicrobial activity has also been reported for guanidine-containing α -peptide- β -peptoid chimeras compared to amino analogues.²³ *N*-Methylation of one of the amide bonds resulted in substantial reduced antimicrobial potency of the amine-analogues in the B-series, and also reduced Gram-negative susceptibility for the guanidine derivatives in the same series. Reversing the orientation of the amide bond in the C-series had little effect on antimicrobial potency, and altering the substitution pattern to *meta*-substitution (or 1,3-positioning) of the aminobenzamide spacer-group had little effect in the D-series. However, *ortho*-substitution (or 1,2-positioning) of the *super bulky* 3,5-di-*tert*-butylphenyl group and cationic side-chains in the E- and F-series was highly favourable with respect to antimicrobial activity. This may involve increased rigidification by intra-molecular H-bonding between the two closely 1,2-positioned amide groups and co-planarity of the aromatic groups resulting in optimized bacterial target interactions. Thus, the guanidine and *ortho*-substituted derivative **E23** was a promising compound for further screening studies, as described below.

Table 1. Antimicrobial activity (MIC in $\mu\text{g/ml}$) against reference strains and haemolytic activity against human RBC (EC_{50} in $\mu\text{g/ml}$) of synthesized MNPMs.

Series	Entry	Mw ^b	Antimicrobial activity ^a - $\mu\text{g/ml}$						RBC	SI ^c
			<i>S. aureus</i>	<i>S. epidermidis</i>	<i>B. subtilis</i>	<i>C. glutamicum</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	EC ₅₀	RBC/ <i>S. aureus</i>
A	A1	432.0	8	8	4	4	8	16	28	3.5
	A2	446.0	8	8	4	4	8	32	31	3.9
	A3	474.1	4	4	4	2	8	16	38	9.5
	A4	488.1	4	4	4	2	8	16	62	15.5
	A5	460.1	16	8	8	4	32	>128	>16 ^d	>1.0
	A6	461.0	8	8	4	4	>128	>128	28	3.5
	A7	488.1	8	8	8	2	16	16	52	6.5
	A8	488.1	4	4	4	2	16	16	>64 ^d	>16.0
B	B9	446.0	128	64	64	32	64	128	43	0.3
	B10	460.0	>128	>128	128	64	>128	>128	172	<1.3
	B11	488.1	16	16	16	4	128	128	243	15.2
	B12	502.1	16	16	16	2	128	128	244	15.3
C	C13	432.0	8	8	4	8	8	16	21	2.6
	C14	446.0	8	8	8	4	8	16	28	3.5
	C15	474.1	4	4	4	2	8	16	44	11.0
	C16	488.1	4	8	4	2	8	8	42	10.5
	D17	432.0	16	8	8	8	16	16	39	2.4
D	D18	446.0	8	8	8	4	16	16	32	4.0
	D19	474.1	4	4	4	2	8	8	37	9.3
	D20	488.1	4	8	4	4	8	16	59	14.8
	E21	432.0	8	8	4	4	16	64	29	3.6
E	E22	446.0	8	8	4	4	16	32	23	2.9
	E23	474.1	1	2	1	0.5	8	16	37	37.0
	E24	488.1	4	2	2	1	8	32	39	9.8
F	F25	482.1	2	8	4	2	8	>128	23	11.5
	F26	524.1	2	2	1	1	16	64	>16 ^d	>8.0

^a *Staphylococcus aureus* ATCC 9144; Methicillin resistant *Staphylococcus epidermidis* (MRSE) RP62A, CCUG 31568 (ATCC 35984); *Bacillus subtilis* 168,²⁴ 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 *S. aureus*.

^d Precipitation in test buffer observed at higher concentrations.

2.3 Antimicrobial profile against clinical isolates

As part of investigating clinical relevance, we selected nine structurally diverse and potent MNPMs and determined their antimicrobial profile against 25 clinical isolates of Gram-positive *S. aureus* and *Enterococcus* spp., and Gram-negative *E. coli*, *P. aeruginosa*, and *K. pneumonia* (Table 2). Thus, representative derivatives from all the different series except the non-potent B-series were included.

The results correlated with the initial screening against the reference strains with few exceptions (Table 2 – top section). Thus, MIC values obtained against the clinical isolates were in the range 2 – 16 $\mu\text{g/ml}$ against all isolates, although higher MIC values were observed against *P. aeruginosa* and *K. pneumonia* for some of the MNPMs. Although two new groups of bacteria were included, antimicrobial activity against *Enterococcus* spp. was comparable to the reference strain *S. aureus*, and antimicrobial activity against *K. pneumonia* and *E. coli* were comparable, too. The perhaps most important difference in potency between the reference strains and clinical isolates was observed for **A3** in case of *P. aeruginosa*, in which **A3** surprisingly was inactive against all clinical isolates (MIC > 64 $\mu\text{g/ml}$), but displayed a MIC of 16 $\mu\text{g/ml}$ against the *P. aeruginosa* reference strain (Table 1).

As a further confirmation of antimicrobial efficacy, the two MNPMs **D19** and **E23** were tested against an extended panel of 250 randomly chosen clinical isolates (Table 2 – lower section).

Structurally **D19** and **E23** were geometric isomers, in which **D19** contained a *meta*-substituted (or 1,3-positioned) spacer-group and **E23** an *ortho*-substituted (or 1,2-positioned) spacer-group, but were otherwise similar by both having the same cationic guanidine group. The results showed that when 250 additional isolates were included, the antimicrobial potencies of both **D19** and **E23** were still in accordance with the first 25 isolates investigated. Shown in Table 2 – lower section, is the concentration that inhibited $\geq 90\%$ of these isolates (MIC₉₀; i.e. MIC value for ≥ 45 isolates). Thus, **D19** displayed MIC₉₀ of 4 – 8 $\mu\text{g/ml}$ against the Gram-positive isolates, and MIC₉₀ of 16 $\mu\text{g/ml}$ against the Gram-negative isolates. However, **E23** was even more potent and displayed MIC₉₀ of 2 – 4 $\mu\text{g/ml}$ against the Gram-positive isolates, and MIC₉₀ of 8 – 32 $\mu\text{g/ml}$ against the Gram-negative isolates. Thus, **E23** confirmed the efficacy of *ortho*-substitution (or 1,2-positioning) of the *super bulky* 3,5-di-*tert*-butylphenyl group and cationic side-chain on the 2-aminobenzamide spacer-group, and confirmed its potential as a promising broad-spectrum antimicrobial *lead-compound*.

Table 2. Antimicrobial activity (MIC in $\mu\text{g/ml}$) against 25 randomly chosen clinical isolates. MIC₉₀ is the concentration of MNPMs that inhibited $\geq 90\%$ of all clinical isolates when screened against 50 additional isolates within each group of bacteria.

Entry	MIC - $\mu\text{g/ml}$				
	<i>S. aureus</i>	<i>Enterococcus</i> spp.	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>K. pneumoniae</i>
No. of isolates:	5	5	5	5	5
A1	8	16	8	16	16
A3	4	8	8	>64	16
C15	4	8	8	8	16
C16	4	8	8	8	16
D19	4	16	16	8	16
E21	8	8	16	>64	32
E23	2	8	8	16	16
F25	2	2	8	>64	64
F26	4	8	16	>64	32

No. of isolates:	MIC ₉₀ - $\mu\text{g/ml}$				
	<i>S. aureus</i>	<i>Enterococcus</i> spp.	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>K. pneumoniae</i>
No. of isolates:	50	50	50	50	50
D19	4	8	16	16	16
E23	2	4	8	32	16

2.4 Antimicrobial activity against 30 multi-resistant isolates

As a final investigation of *in vitro* antimicrobial efficacy, **D19** and **E23** were also tested against 30 multi-resistant clinical isolates (Table 3). Included in the screening were Gram-positive methicillin resistant *S. aureus* (MRSA), vancomycin resistant *Enterococci* (VRE) and Gram-negative isolates with extended spectrum β -lactamase – carbapenemase (ESBL-CARBA) resistance, including New Delhi metallo- β -lactamase 1 (NDM-1) resistance. The results for **D19** and **E23** revealed similar antimicrobial potency as for the reference strains and clinical isolates in the screenings described above. Highest antimicrobial activity was observed against Gram-positive MRSA and VRE, whereas the Gram-negative ESBL-CARBA isolates showed more variations among different isolates (tested up to 32 $\mu\text{g/ml}$). In this last screening against multi-resistant isolates both **D19** and **E23** emerged as promising broad-spectrum antimicrobial *lead-compounds*. Surprisingly **D19** was more potent than **E23** against some of the Gram-negative isolates.

2.5 Membrane integrity investigations

To investigate membrane disruptive properties of the MNPMs, three structurally diverse but equipotent derivatives **A3**, **D20**, and **E22** were investigated by a modified whole-cell real-time membrane integrity assay with *B. subtilis* 168 carrying the plasmid pCSS962.²⁵ This particular *B. subtilis* strain expresses luciferase and emits luminescence if externally added D-luciferin enters bacterial cells upon membrane disruption.

A dose-response effect was observed for all three compounds tested and increase in luminescence was detected at concentrations close to the MIC (4 $\mu\text{g/ml}$) against *B. subtilis* 168, i.e. showing a clear membranolytic effect (Fig. 3). At the highest concentration (25 $\mu\text{g/ml}$), the luminescence induction was faster for the *para*-substituted (or 1,4-positioned) guanidine derivative **A3** and *ortho*-substituted (or 1,2-positioned) amine derivative **E22** than the *meta*-substituted (or 1,3-positioned) guanidine derivative **D20** (Fig. 4). Chlorhexidine, a bactericidal agent known for its cell wall and membrane-disruptive activities was used as a reference compound.²⁶⁻²⁸

Table 3. Antimicrobial activity (MIC in $\mu\text{g/ml}$) of **D19** and **E23** against multi-resistant isolates.

Multi-resistant isolate	D19	E23	ESBL-CARBA ¹
<i>S. aureus</i> N315	8	4	
<i>S. aureus</i> NCTC 10442	8	4	
<i>S. aureus</i> isolate 85/2082	8	4	
<i>S. aureus</i> isolate WIS	8	4	
<i>S. aureus</i> IHT 99040	>8	>4	
<i>E. faecium</i> 50673722	8	8	
<i>E. faecium</i> 50901530	8	4	
<i>E. faecium</i> K36-18	8	8	
<i>E. faecium</i> 50758899	8	8	
<i>E. faecium</i> TUH50-22	8	4	
<i>E. coli</i> 50579417	32	32	OXA-48
<i>E. coli</i> 50639799	16	16	VIM-29
<i>E. coli</i> 50676002	16	>32	NDM-1
<i>E. coli</i> 50739822	32	32	NDM-1
<i>E. coli</i> 50857972	16	16	IMP-26
<i>P. aeruginosa</i> K34-7	16	>32	VIM-2
<i>P. aeruginosa</i> K34-73	>32	>32	VIM-4
<i>P. aeruginosa</i> K44-24	16	>32	IMP-14
<i>P. aeruginosa</i> 50692172	16	32	NDM-1
<i>P. aeruginosa</i> 50692520	16	32	VIM
<i>K. pneumoniae</i> K47-25	>32	32	KPC-2
<i>K. pneumoniae</i> K66-45	16	32	NDM-1
<i>K. pneumoniae</i> 50531633	16	16	NDM-1+OXA-181
<i>K. pneumoniae</i> 50625602	16	16	OXA-245
<i>K. pneumoniae</i> 50667959	16	32	VIM-1
<i>A. baumannii</i> K12-21	32	32	OXA-58
<i>A. baumannii</i> K44-35	32	16	OXA-23
<i>A. baumannii</i> K47-42	32	16	OXA-23
<i>A. baumannii</i> K55-13	>32	32	OXA-24
<i>A. baumannii</i> K63-58	32	32	OXA-23

¹ESBL-CARBA: Extended spectrum β -lactamase – carbapenemase producing isolates. OXA, oxacillinase; VIM, Verona integron-encoded metallo- β -lactamase; NDM, New Delhi metallo- β -lactamase; IMP, imipenem-type carbapenemase; KPC, *K. pneumoniae* carbapenemase.

Although more extensive studies are needed to conclusively prove the difference in the kinetics of membrane-interaction between the tested compounds, Fig. 4 shows that the kinetics of the luminescence emission was in general similar for the MNPMs and chlorhexidine at 25 $\mu\text{g/ml}$. However, although the MIC of chlorhexidine against *B. subtilis* was as low as 0.3 $\mu\text{g/ml}$ (data not shown), a pronounced membrane-disruptive effect on *B. subtilis* 168 was only shown at concentrations above 12.5 $\mu\text{g/ml}$; corresponding to approximately 40 times its MIC. Chlorhexidine has previously been reported to have a complex mode of action depending on the concentration, i.e. up to certain concentrations it can cause leakage of intracellular constituents, while at higher concentrations it leads to coagulation of cytoplasmic contents.^{27, 28} The tested MNPMs were more efficient than chlorhexidine in terms of membrane disruption and seemed thereby to have the bacterial cell membrane as their primary target. Overall, both the MNPMs and chlorhexidine affected membrane integrity, although the molecular mechanisms leading to membrane leakage were presumably different. Finally, according to the light emission kinetics, MNPMs from different series were likely to differ from each other in the way they interact with bacterial cell membranes.

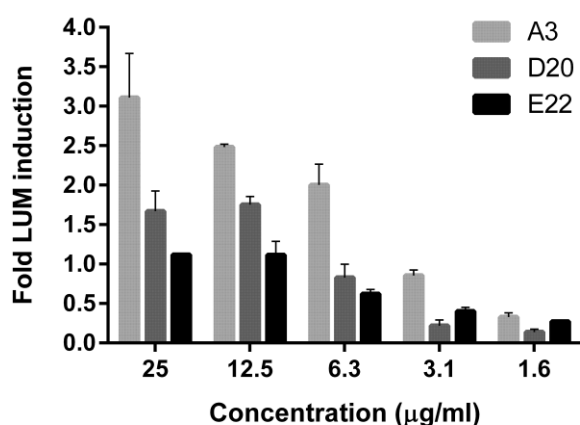


Figure 3. Dose-dependent relative luminescence emission by *B. subtilis* 168 (pCSS962) treated with MNPMs **A3**, **D20** and **E22**. Luminescence (LUM) induction was monitored during the first 30 seconds after addition of the bacterial suspension (already containing D-luciferin) to the samples. The fold LUM induction is the ratio between the maximum luminescence in the samples and the water control treated with 20 µg/ml chlorhexidine (or 31 µg/ml for **A3**). The mean of two experiments ± SD is presented.

3. Conclusions

The present report demonstrates design and synthesis of small cationic and amphipathic MNPMs with higher antimicrobial potencies against both Gram-positive and Gram-negative bacteria than the marine natural antimicrobials that inspired the project. The potencies of the synthesised MNPMs also challenged the pharmacophore model of small cationic AMPs and peptidomimetics that target bacterial cell membranes. Thus, we have shown how aminobenzamide MNPMs with a single cationic charge (amine or guanidine) in combination with a *super bulky* 3,5-di-*tert*-butylphenyl group can ensure high antimicrobial potency against bacterial reference strains, clinical isolates, and multi-resistant isolates. As part of our strategy we have had emphasis on using inexpensive starting materials, efficient and few steps, and environmental friendly chemistry to increase the commercial prospective of any *lead-compound* generated from the project. The perhaps most promising MNPM prepared was **E23**, which displayed MIC values between 0.5 – 8 µg/ml against all Gram-positive isolates investigated, including multi-resistant isolates of MRSA and VRE. **E23** displayed also MIC values between 8 – 32 µg/ml against 250 clinical Gram-negative isolates, and 16 – 32 µg/ml against ESBL-CARBA multi-resistant isolates where very few, if any treatment alternatives exist. In summary, based on these *in vitro* screening results we have developed a series of highly potent antimicrobial MNPMs, demonstrated important structural properties for antimicrobial activity, and identified a promising *lead-compound* for further drug-development.

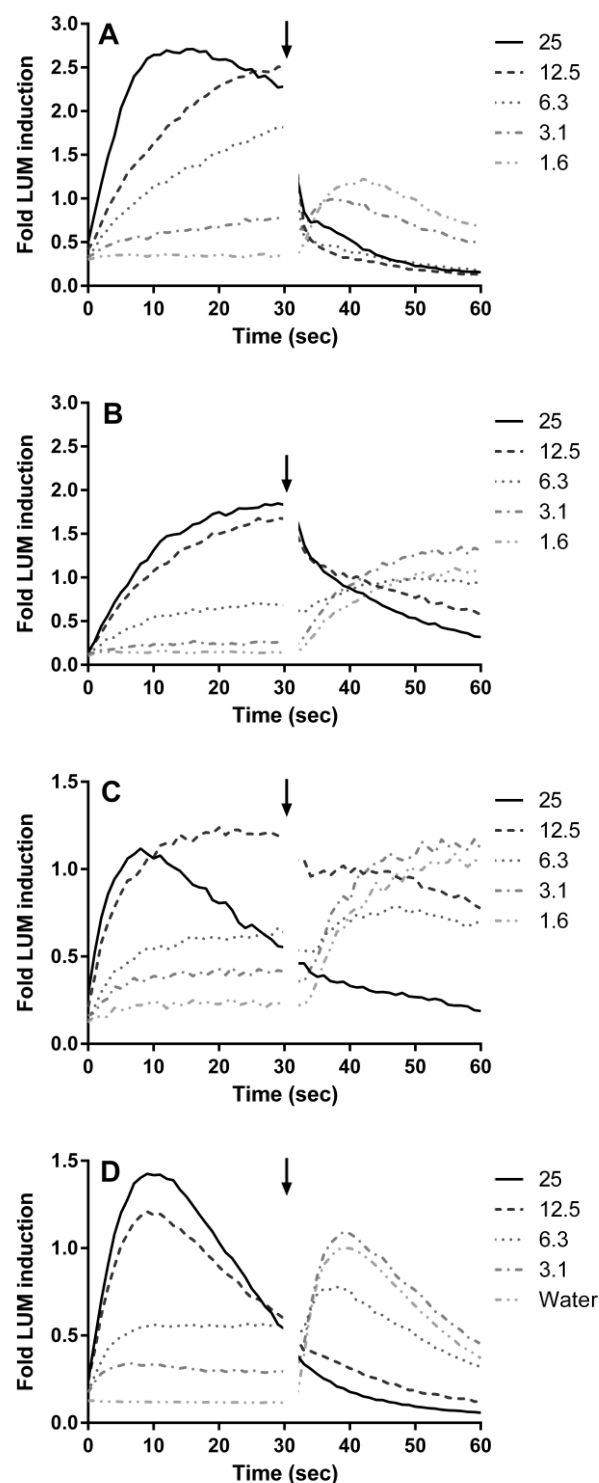


Figure 4. Kinetics of the relative luminescence emission by *B. subtilis* 168 (pCSS962) treated with different concentrations (µg/ml) of A) **A3**, B) **D20**, C) **E22**, and D) the reference antimicrobial agent chlorhexidine. Bacteria were incubated in presence of the compounds and D-luciferin. Water was used as an untreated control whereas chlorhexidine at 20 µg/ml (or 31 µg/ml for **A3**) was used as a membranolytic control and was added to all samples after 30 seconds of incubation (indicated by an arrow). In total, the luminescence (LUM) was monitored for up to 120 sec, but only the first 60 seconds are presented. The fold induction of LUM in the samples, compared to the maximum LUM of the water control after the addition of chlorhexidine is presented. Data from single experiments are shown as representatives for two independent experiments.

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6. Experimental section

6.1 Chemicals and equipment

Reagents and solvents used for synthesis were purchased from Sigma-Aldrich Inc., USA. Waters Alliance 2695 Separations Module (Waters Inc., USA) accompanied by Micromass Qattro LC (Micromass, UK) was used as HPLC-MS system for reaction monitoring. ¹H and ¹³C NMR spectra were recorded on a 400 MHz NMR spectrometer (Varian, USA). Residual solvent peak for methanol-d₄ was used as reference for chemical shifts, i.e. δH: 3.310 ppm and δC: 49.000 ppm. High-resolution MS data were acquired on a Waters LC-MS system (Milford, MA, USA) composed of an Acquity UPLC coupled to an LCT-Premier time-of-flight MS with electrospray ionization. Melting points were determined in open capillary tubes with a Buchi B-540 melting point apparatus.

6.2 Synthesis of starting materials

3,5-Di-tert-butylbenzoyl chloride: 3,5-Di-tert-butylbenzoic acid (5 g, 50 mM) was heated under reflux in an excess of SOCl₂ (50 ml) for 4 h with a few drops of DMF added. After removal of excess of SOCl₂ *in vacuo*, the residue 3,5-di-tert-butylbenzoyl chloride was obtained in approx. quantitative yield and used without further purification.

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*-toluenesulfonic 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*-toluenesulfonic acid (7.6 g, 40 mmol) for 18 h. Methanol was evaporated *in vacuo* and a saturated solution of NaHCO₃ was added to the residue. The product was isolated by extraction with ethyl acetate. Organic solvent was evaporated under reduced pressure and methyl 4-methylaminobenzoate was obtained in approx. quantitative yield.

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 NaHCO₃. 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-naphthoic acid was 70%

(3.5 g). Methyl 3-amino-2-naphthoate was synthesized according to a somewhat modified method described elsewhere.²⁹ Briefly, concentrated H₂SO₄ (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, where after the mixture was cooled and methanol evaporated *in vacuo*. The residue was poured onto ice and neutralized with saturated NaCO₃. The resulting yellowish precipitate was filtered, washed with water, and dried. Mass of obtained methyl 3-amino-2-naphthoate was 2.7 g (70% yield).

6.3 Synthesis of MNPM test compounds

N-(4-((2-Aminoethyl)carbamoyl)phenyl)-3,5-di-*tert*-butylbenzamide hydrochloride (**A1**): Methyl 4-aminobenzoate (302 mg, 2 mmol) and an excess of DIPEA (1 ml, 6 mmol) were mixed in DMF (4 ml). 3,5-Di-*tert*-butylbenzoyl chloride (290 mg, 1.9 mmol) was added to the reaction mixture with stirring. After 18 h at room temperature, the reaction mixture was poured into 1 M HCl (aq.), the organic phase was isolated, and methyl 4-(3,5-di-*tert*-butylbenzamido)benzoate was purified by recrystallization from MeOH/H₂O (85% yield). Methyl 4-(3,5-di-*tert*-butylbenzamido)benzoate (294 mg, 0.8 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 dissolved in ethyl acetate and washed with water. Ethyl acetate was removed *in vacuo* and *N*-(4-((2-aminoethyl)carbamoyl)phenyl)-3,5-di-*tert*-butylbenzamide was obtained in 90% yield.

N-(4-((2-Aminoethyl)carbamoyl)phenyl)-3,5-di-*tert*-butylbenzamide (198 mg, 0.5 mmol) was 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. Solvents were removed *in vacuo*. Obtained *N*-(4-((2-aminoethyl)carbamoyl)phenyl)-3,5-di-*tert*-butylbenzamide hydrochloride was purified in quantitative yield by recrystallization from MeOH/Et₂O. Briefly, *N*-(4-((2-aminoethyl)carbamoyl)phenyl)-3,5-di-*tert*-butylbenzamide hydrochloride (100 mg, 0.23 mmol) was dissolved in 1 ml of MeOH under reflux. Et₂O was added drop wise until precipitation was complete. ¹H NMR (CD₃OD): 7.91 (d, 2H, J = 8.8 Hz), 7.86 (d, 2H, J = 8.8 Hz), 7.79 (d, 2H, J = 2 Hz), 7.69 (t, 1H, J = 2 Hz), 3.66 (t, 2H, J = 6 Hz), 3.16 (t, 2H, J = 6 Hz), 1.38 (s, 18H). ¹³C NMR (CD₃OD): 169.1, 168.6, 151.1, 142.3, 134.1, 128.8, 127.9, 126.0, 121.6, 120.1, 39.8, 37.6, 34.5, 30.4. HRMS-ESI: C₁₅H₁₆ClN₄O₃ [M+H]⁺ calcd: 396.2651, found: 396.2638; mp 287-289 °C (decomp.).

MNPMs **A2**, **A5**, **A6**, **B9**, **B10**, **D17**, **D18**, **E21**, **E22**, and **F25** were synthesised in a similar manner. MNPMs **C13** and **C14** were synthesised likewise, but starting from 3,5-di-*tert*-butylaniline and methyl 4-(chlorocarbonyl)benzoate (Scheme 1).

N-(4-((3-Aminopropyl)carbamoyl)phenyl)-3,5-di-*tert*-butylbenzamide hydrochloride (**A2**). ¹H NMR (CD₃OD): 7.88 (d, 2H, J = 9.2 Hz), 7.86 (d, 2H, J = 9.2 Hz), 7.79 (d, 2H, J = 1.6 Hz), 7.69 (t, 1H, J = 1.6 Hz), 3.51 (t, 2H, J = 6.8 Hz), 3.01 (t, 2H, J = 6.8 Hz), 1.98 (p, 2H, J = 6.8 Hz), 1.38 (s, 18H). ¹³C NMR (CD₃OD): 168.9, 168.6, 151.1, 142.2, 134.1, 128.9, 127.7, 125.9, 121.6, 120.2, 36.9, 36.0, 34.5, 30.4, 27.56. HRMS-ESI: C₂₅H₃₆N₃O₂ [M+H]⁺ calcd: 410.2808, found: 410.2791; mp 184-186 °C (decomp.).

3,5-Di-*tert*-butyl-*N*-(4-((2-(dimethylamino)ethyl)carbamoyl)phenyl)benzamide hydrochloride (**A5**). ¹H NMR (CD₃OD): 7.93 (d, 2H, J = 8.8 Hz),

7.87 (d, 2H, J = 8.8 Hz), 7.79 (d, 2H, J = 1.6 Hz), 7.69 (t, 1H, J = 1.6 Hz), 3.73-3.81 (m, 2H), 3.37-3.44 (m, 2H), 3.00 (s, 6H), 1.38 (s, 18H). ¹³C NMR (CD₃OD): 169.1, 168.6, 151.1, 142.4, 134.1, 129.1, 127.9, 126.0, 121.6, 120.1, 57.5, 42.5, 35.0, 34.5, 30.3. HRMS-ESI: C₂₆H₃₈N₃O₂ [M+H]⁺ calcd: 424.2964, found: 424.2959; mp 96-99 °C (decomp.).

2-(Dimethylamino)ethyl 4-(3,5-di-*tert*-butylbenzamido)benzoate hydrochloride (**A6**). ¹H NMR (CD₃OD): 8.10 (d, 2H, J = 9 Hz), 7.90 (d, 2H, J = 9 Hz), 7.79 (d, 2H, J = 1.6 Hz), 7.70 (t, 1H, J = 1.6 Hz), 4.61-4.67 (m, 2H), 3.45-3.54 (m, 2H), 2.92 (s, 6H), 1.39 (s, 18H). ¹³C NMR (CD₃OD): 168.7, 165.6, 151.2, 143.8, 134.0, 130.4, 126.0, 124.4, 121.6, 119.9, 59.0, 56.4, 42.9, 34.5, 30.3. HRMS-ESI: C₂₆H₃₇N₂O₃ [M+H]⁺ calcd: 425.2804, found: 425.2792; mp 187-189 °C (decomp.).

N-(4-((2-Aminoethyl)carbamoyl)phenyl)-3,5-di-*tert*-butyl-*N*-methylbenzamide hydrochloride (**B9**). ¹H NMR (CD₃OD): 7.79 (d, 2H, J = 8.8 Hz), 7.36 (t, 1H, J = 1.6 Hz), 7.23 (d, 2H, J = 8.8 Hz), 7.16 (d, 2H, J = 1.6 Hz), 3.62 (t, 2H, J = 6 Hz), 3.50 (s, 3H), 3.13 (t, 2H, J = 6 Hz), 1.16 (s, 18H). ¹³C NMR (CD₃OD): 172.2, 168.4, 150.4, 148.2, 134.4, 131.3, 128.1, 126.7, 123.6, 123.0, 39.5, 37.3, 37.1, 34.2, 30.2. HRMS-ESI: C₂₅H₃₆N₃O₂ [M+H]⁺ calcd: 410.2808, found: 410.2792; mp >101-103 °C (decomp.).

N-(4-((3-Aminopropyl)carbamoyl)phenyl)-3,5-di-*tert*-butyl-*N*-methylbenzamide hydrochloride (**B10**). ¹H NMR (CD₃OD): 7.76 (d, 2H, J = 8.4 Hz), 7.36 (t, 1H, J = 1.6 Hz), 7.20 (d, 2H, J = 8.4 Hz), 7.15 (d, 2H, J = 1.6 Hz), 3.50 (s, 3H), 3.45 (t, 2H, J = 6.8 Hz), 2.96 (t, 2H, J = 6.8 Hz), 1.94 (p, 2H, J = 6.8 Hz), 1.16 (s, 18H). ¹³C NMR (CD₃OD): 172.1, 168.0, 150.4, 148.1, 134.4, 131.5, 127.9, 126.7, 123.5, 123.1, 37.0, 36.9, 36.0, 34.2, 30.2, 27.4. HRMS-ESI: C₂₆H₃₈N₃O₂ [M+H]⁺ calcd: 424.2964, found: 424.2955; mp >80-83 °C (decomp.).

*N*¹-(2-Aminoethyl)-*N*⁴-(3,5-di-*tert*-butylphenyl)terephthalamide hydrochloride (**C13**). ¹H NMR (CD₃OD): 8.35 (d, 2H, J = 8.4 Hz), 8.00 (d, 2H, J = 8.4 Hz), 7.59 (d, 2H, J = 1.6 Hz), 7.28 (t, 1H, J = 1.6 Hz), 3.70 (t, 2H, J = 5.6 Hz), 3.21 (t, 2H, J = 5.6 Hz), 1.34 (s, 18H). ¹³C NMR (CD₃OD): 168.8, 166.4, 151.2, 138.1, 137.6, 136.2, 127.4, 127.3, 118.5, 115.5, 36.6, 37.4, 34.4, 30.4. HRMS-ESI: C₂₄H₃₄N₃O₂ [M+H]⁺ calcd: 396.2651, found: 396.2635; mp 271-273 °C (decomp.).

*N*¹-(3-Aminopropyl)-*N*⁴-(3,5-di-*tert*-butylphenyl)terephthalamide hydrochloride (**C14**). ¹H NMR (CD₃OD): 8.03 (d, 2H, J = 8.4 Hz), 7.97 (d, 2H, J = 8.4 Hz), 7.60 (d, 2H, J = 1.6 Hz), 7.27 (t, 1H, J = 1.6 Hz), 3.53 (t, 2H, J = 6.8 Hz), 3.02 (t, 2H, J = 6.8 Hz), 1.99 (p, 2H, J = 6.8 Hz), 1.34 (s, 18H). ¹³C NMR (CD₃OD): 168.5, 166.4, 151.2, 138.0, 137.6, 136.4, 127.5, 127.1, 118.5, 115.5, 37.0, 36.1, 34.4, 30.4, 27.5. HRMS-ESI: C₂₅H₃₆N₃O₂ [M+H]⁺ calcd: 410.2808, found: 410.2795; mp 209-211 °C (decomp.).

N-(3-((2-Aminoethyl)carbamoyl)phenyl)-3,5-di-*tert*-butylbenzamide hydrochloride (**D17**). ¹H NMR (CD₃OD): 8.30-8.25 (m, 1H), 7.85-7.80 (m, 1H), 7.81 (d, 2H, J = 1.6 Hz), 7.69 (t, 1H, J = 1.6 Hz), 7.68-7.63 (m, 1H), 7.49 (t, 1H, J = 8 Hz), 3.69 (t, 2H, J = 6 Hz), 3.19 (t, 2H, J = 6 Hz), 1.39 (s, 18H). ¹³C NMR (CD₃OD): 169.5, 168.6, 151.1, 138.9, 134.2, 134.0, 128.7, 125.9, 124.5, 122.9, 121.5, 120.4, 39.6, 37.4, 34.5, 30.4. HRMS-ESI: C₂₄H₃₄N₃O₂ [M+H]⁺ calcd: 396.2651, found: 396.2650; mp 185-187 °C (decomp.).

N-(3-((3-Aminopropyl)carbamoyl)phenyl)-3,5-di-*tert*-butylbenzamide hydrochloride (**D18**). ¹H NMR (CD₃OD): 8.26-

8.22 (m, 1H), 7.86-7.81 (m, 1H), 7.80 (d, 2H, J = 1.6 Hz), 7.69 (t, 1H, J = 1.6 Hz), 7.65-7.60 (m, 1H), 7.47 (t, 1H, J = 8 Hz), 3.52 (t, 2H, J = 6.8 Hz), 3.02 (t, 2H, J = 6.8 Hz), 1.98 (p, 2H, J = 6.8 Hz), 1.38 (s, 18H). ¹³C NMR (CD₃OD): 169.2, 168.6, 151.1, 138.9, 134.5, 134.0, 128.7, 125.9, 124.4, 122.7, 121.5, 120.2, 40.0, 36.1, 34.5, 30.4, 27.5. HRMS-ESI: C₂₅H₃₆N₃O₂ [M+H]⁺ calcd: 410.2808, found: 410.2797; mp 273-275 °C (decomp.).

N-(2-((2-Aminoethyl)carbamoyl)phenyl)-3,5-di-*tert*-butylbenzamide hydrochloride (**E21**). ¹H NMR (CD₃OD): 8.50 (dd, 1H, J = 8.4 Hz, J = 1.2 Hz), 7.86 (d, 2H, J = 1.6 Hz), 7.82 (dd, 1H, J = 8.4 Hz, J = 1.2 Hz), 7.71 (t, 1H, J = 1.6 Hz), 7.57 (ddd, 1H, J = 8.8 Hz, J = 8.4 Hz, J = 1.2 Hz), 7.23 (ddd, 1H, J = 8.8 Hz, J = 8.4 Hz, J = 1.2 Hz), 3.68 (t, 2H, J = 6 Hz), 3.17 (t, 2H, J = 6 Hz), 1.40 (s, 18H). ¹³C NMR (CD₃OD): 170.8, 167.3, 151.4, 138.9, 134.0, 132.1, 127.78, 126.1, 123.3, 121.6, 121.3, 121.3, 39.5, 37.5, 34.6, 30.4. HRMS-ESI: C₂₄H₃₄N₃O₂ [M+H]⁺ calcd: 396.2651, found: 396.2637; mp 259-261 °C (decomp.).

3,5-Di-*tert*-butyl-*N*-(2-((3-aminopropyl)carbamoyl)phenyl)benzamide hydrochloride (**E22**). ¹H NMR (CD₃OD): 8.59 (dd, 1H, J₁ = 8.4 Hz, J₂ = 1.2 Hz), 7.87 (d, 2H, J = 1.6 Hz), 7.78 (dd, 1H, J₁ = 8.4 Hz, J₂ = 1.2 Hz), 7.71 (t, 1H, J = 1.6 Hz), 7.56 (ddd, 1H, J₁ = 8.4 Hz, J₂ = 7.2 Hz, J₃ = 1.2 Hz), 7.22 (ddd, 1H, J₁ = 8.4 Hz, J₂ = 7.2 Hz, J₃ = 1.2 Hz), 3.53 (t, 2H, J = 6.8 Hz), 3.02 (t, 2H, J = 6.8 Hz), 1.99 (p, 2H, J = 6.8 Hz), 1.40 (s, 18H). ¹³C NMR (CD₃OD): 170.2, 166.9, 151.5, 139.1, 134.0, 132.1, 127.6, 126.0, 123.2, 121.3, 121.1, 121.0, 37.2, 36.1, 34.6, 30.4, 27.4. HRMS-ESI: C₂₅H₃₆N₃O₂ [M+H]⁺ calcd: 410.2808, found: 410.2805; mp 268-270 °C (decomp.).

N-(2-Aminoethyl)-3-(3,5-di-*tert*-butylbenzamido)-2-naphthamide hydrochloride (**F25**). ¹H NMR (CD₃OD): 8.86 (s, 1H), 8.39 (s, 1H), 7.95 (dd, 1H, J₁ = 8 Hz, J₂ = 0.4 Hz), 8.00 (d, 2H, J = 1.6 Hz), 7.88 (dd, 1H, J₁ = 8 Hz, J₂ = 0.4 Hz), 7.73 (t, 1H, J = 1.6 Hz), 7.61 (ddd, 1H, J₁ = 8.4 Hz, J₂ = 6.8 Hz, J₃ = 0.8 Hz), 7.51 (ddd, 1H, J₁ = 8.4 Hz, J₂ = 6.8 Hz, J₃ = 0.8 Hz), 3.75 (t, 2H, J = 6 Hz), 3.24 (t, 2H, J = 6 Hz), 1.42 (s, 18H). ¹³C NMR (CD₃OD): 171.0, 167.5, 151.5, 135.1, 134.5, 134.0, 129.3, 128.9, 128.3, 128.2, 127.1, 126.1, 125.7, 122.6, 121.3, 118.6, 39.3, 37.1, 34.6, 30.4. HRMS-ESI: C₂₈H₃₆N₃O₂ [M+H]⁺ calcd: 446.2808, found: 446.2787; mp 285-287 °C (decomp.).

3,5-Di-*tert*-butyl-*N*-(4-((2-guanidinoethyl)carbamoyl)phenyl)benzamide hydrochloride (**A3**) was synthesized according to a modified method described elsewhere.³⁰ Briefly, *N*-(4-((2-aminoethyl)carbamoyl)phenyl)-3,5-di-*tert*-butylbenzamide (198 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, Et₂O (10 ml) was added to the liquid filtrate, and the mixture was stirred for 1 h at room temperature. The ether layer was decanted off, a new portion of Et₂O (10 ml) was added, and the mixture stirred for 10 h at room temperature. Et₂O was removed from the solid precipitate. Obtained 3,5-di-*tert*-butyl-*N*-(4-((2-guanidinoethyl)carbamoyl)phenyl)benzamide hydrochloride was purified by recrystallization from MeOH/Et₂O in 91% yield (216 mg). ¹H NMR (CD₃OD): 7.89 (d, 2H, J = 9.2 Hz), 7.85 (d, 2H, J = 9.2 Hz), 7.79 (d, 2H, J = 1.6 Hz), 7.69 (t, 1H, J = 1.6 Hz), 3.57 (t, 2H, J = 6.4 Hz), 3.43 (t, 2H, J = 6.4 Hz), 1.38 (s, 18H). ¹³C NMR (CD₃OD): 168.9, 168.6, 157.5, 151.1, 142.2, 134.1, 128.9, 127.8, 126.0, 121.6, 120.2, 40.7, 38.6, 34.5, 30.3. HRMS-ESI: C₂₅H₃₆N₅O₂ [M+H]⁺ calcd: 438.2869, found: 438.2855; mp 307-309 °C (decomp.).

Compounds **A4**, **A7**, **A8**, **B11**, **B12**, **C15**, **C16**, **D19**, **D20**, **E23**, **E24**, and **F26** were synthesized in similar manner (Scheme 1).

3,5-Di-*tert*-butyl-*N*-(4-((3-guanidinopropyl)carbamoyl)phenyl)benzamide hydrochloride (**A4**). ¹H NMR (CD₃OD): 7.89 (d, 2H, J = 9.2 Hz), 7.85 (d, 2H, J = 9.2 Hz), 7.79 (d, 2H, J = 1.6 Hz), 7.68 (t, 1H, J = 1.6 Hz), 3.47 (t, 2H, J = 6.8 Hz), 3.27 (t, 2H, J = 6.8 Hz), 1.90 (p, 2H, J = 6.8 Hz), 1.38 (s, 18H). ¹³C NMR (CD₃OD): 168.6, 168.5, 157.3, 151.1, 142.0, 134.1, 129.3, 127.7, 125.9, 121.6, 120.2, 38.7, 36.6, 34.5, 30.4, 28.7. HRMS-ESI: C₂₆H₃₈N₅O₂ [M+H]⁺ calcd: 452.3026, found: 452.3020; mp 205-207 °C (decomp.).

3,5-Di-*tert*-butyl-*N*-(4-((2-guanidinoethyl)carbamoyl)benzyl)benzamide hydrochloride (**A7**). ¹H NMR (CD₃OD): 7.83 (d, 2H, J = 8 Hz), 7.74 (d, 2H, J = 1.6 Hz), 7.64 (t, 1H, J = 1.6 Hz), 7.46 (d, 2H, J = 8 Hz), 4.63 (s, 2H), 3.55 (t, 2H, J = 6 Hz), 3.41 (t, 2H, J = 6 Hz), 1.35 (s, 18H). ¹³C NMR (CD₃OD): 169.6, 169.3, 157.5, 151.1, 143.4, 133.3, 132.4, 127.2, 127.1, 125.6, 121.2, 42.8, 40.6, 38.6, 34.5, 30.4. HRMS-ESI: C₂₆H₃₈N₅O₂ [M+H]⁺ calcd: 452.3026, found: 452.3015; mp 155-157 °C (decomp.).

3,5-Di-*tert*-butyl-*N*-(4-((2-guanidinoethyl)amino)-2-oxoethyl)phenyl)benzamide hydrochloride (**A8**). ¹H NMR (CD₃OD): 7.78 (d, 2H, J = 1.6 Hz), 7.67 (t, 1H, J = 1.6 Hz), 7.64 (d, 2H, J = 8.4 Hz), 7.31 (d, 2H, J = 8.4 Hz), 3.54 (s, 2H), 3.36 (t, 2H, J = 6 Hz), 3.29 (t, 2H, J = 6 Hz), 1.38 (s, 18H). ¹³C NMR (CD₃OD): 173.6, 168.6, 157.4, 151.1, 137.4, 134.2, 131.5, 129.1, 125.7, 121.7, 121.5, 41.9, 40.6, 38.2, 34.5, 30.4. HRMS-ESI: C₂₆H₃₈N₅O₂ [M+H]⁺ calcd: 452.3026, found: 452.3011; mp 258-260 °C (decomp.).

3,5-Di-*tert*-butyl-*N*-(4-((2-guanidinoethyl)carbamoyl)phenyl)-*N*-methylbenzamide hydrochloride (**B11**). ¹H NMR (CD₃OD): 7.76 (d, 2H, J = 8.8 Hz), 7.36 (t, 1H, J = 1.6 Hz), 7.22 (d, 2H, J = 8.8 Hz), 7.15 (d, 2H, J = 1.6 Hz), 3.51 (t, 2H, J = 6 Hz), 3.51 (s, 3H), 3.37 (t, 2H, J = 6 Hz), 1.16 (s, 18H). ¹³C NMR (CD₃OD): 172.2, 168.2, 157.5, 150.4, 148.2, 134.4, 131.5, 128.0, 126.7, 123.5, 123.1, 40.6, 38.5, 37.0, 34.2, 30.2. HRMS-ESI: C₂₆H₃₈N₅O₂ [M+H]⁺ calcd: 452.3026, found: 452.3016; mp >149-151 °C (decomp.).

3,5-Di-*tert*-butyl-*N*-(4-((3-guanidinopropyl)carbamoyl)phenyl)-*N*-methylbenzamide hydrochloride (**B12**). ¹H NMR (CD₃OD): 7.75 (d, 2H, J = 8.4 Hz), 7.36 (t, 1H, J = 1.6 Hz), 7.21 (d, 2H, J = 8.4 Hz), 7.15 (d, 2H, J = 1.6 Hz), 3.51 (s, 3H), 3.41 (t, 2H, J = 6.8 Hz), 3.21 (t, 2H, J = 6.8 Hz), 1.83 (p, 2H, J = 6.8 Hz), 1.16 (s, 18H). ¹³C NMR (CD₃OD): 172.1, 167.7, 157.3, 150.4, 148.0, 134.4, 131.9, 127.9, 126.7, 123.5, 123.1, 38.6, 37.0, 36.5, 34.2, 30.2, 28.6. HRMS-ESI: C₂₇H₄₀N₅O₂ [M+H]⁺ calcd: 466.3182, found: 466.3162; mp >139-141 °C (decomp.).

*N*¹-(3,5-di-*tert*-butylphenyl)-*N*¹-(2-guanidinoethyl)terephthalamide hydrochloride (**C15**). ¹H NMR (CD₃OD): 8.03 (d, 2H, J = 8.8 Hz), 7.97 (d, 2H, J = 8.8 Hz), 7.59 (s, 2H), 7.28 (s, 1H), 3.60 (t, 2H, J = 6 Hz), 3.45 (t, 2H, J = 6 Hz), 1.34 (18s). ¹³C NMR (CD₃OD): 168.6, 166.4, 157.5, 151.2, 138.1, 137.6, 136.4, 127.5, 127.2, 118.5, 115.5, 40.6, 38.7, 34.4, 30.4. HRMS-ESI: C₂₅H₃₆N₅O₂ [M+H]⁺ calcd: 438.2869, found: 438.2869; mp 305-307 °C (decomp.).

*N*¹-(3,5-Di-*tert*-butylphenyl)-*N*¹-(3-guanidinopropyl)terephthalamide hydrochloride (**C16**). ¹H NMR (CD₃OD): 8.02 (d, 2H, J = 8 Hz), 7.95 (d, 2H, J = 8 Hz), 7.59 (s,

2H), 7.28 (s, 1H), 3.49 (t, 2H, J = 6.8 Hz), 3.29 (t, 2H, J = 6.8 Hz), 1.92 (p, 2H, J = 6.8 Hz), 1.34 (s, 18H). ¹³C NMR (CD₃OD): 168.1, 166.5, 157.3, 151.2, 137.9, 137.6, 136.8, 127.4, 127.1, 118.5, 115.5, 38.7, 36.7, 34.4, 30.5, 28.6. HRMS-ESI: C₂₆H₃₈N₅O₂ [M+H]⁺ calcd: 452.3026, found: 452.3022; mp 151-153 °C (decomp.).

3,5-Di-tert-butyl-N-(3-((2-guanidinoethyl)carbamoyl)phenyl)benzamide hydrochloride (**D19**). ¹H NMR (CD₃OD): 8.29-8.23 (m, 1H), 7.814-7.78 (m, 1H), 7.80 (d, 2H, J = 1.6 Hz), 7.69 (t, 1H, J = 1.6 Hz), 7.65-7.59 (m, 1H), 7.48 (t, 1H, J = 8 Hz), 3.58 (t, 2H, J = 6 Hz), 3.44 (3t, 2H, J = 6 Hz), 1.39 (s, 18H). ¹³C NMR (CD₃OD): 168.3, 168.6, 157.5, 151.1, 138.9, 134.5, 134.0, 128.8, 125.9, 124.4, 122.8, 121.5, 120.2, 40.6, 38.6, 34.5, 30.4. HRMS-ESI: C₂₅H₃₆N₅O₂ [M+H]⁺ calcd: 438.2869, found: 438.2862; mp 282-284 °C (decomp.).

3,5-Di-tert-butyl-N-(3-((3-guanidinopropyl)carbamoyl)phenyl)benzamide hydrochloride (**D20**). ¹H NMR (CD₃OD): 8.25-8.21 (m, 1H), 7.84-7.78 (m, 1H), 7.80 (d, 2H, J = 1.6 Hz), 7.69 (t, 1H, J = 1.6 Hz), 7.64-7.58 (m, 1H), 7.47 (t, 1H, J = 8 Hz), 3.48 (t, 2H, J = 6.8 Hz), 3.28 (t, 2H, 6.8 Hz), 1.90 (p, 2H, J = 6.8 Hz), 1.39 (s, 18H). ¹³C NMR (CD₃OD): 169.0, 168.6, 157.3, 151.1, 138.9, 134.8, 134.0, 128.6, 125.9, 124.2, 122.7, 121.5, 120.2, 38.7, 36.6, 34.5, 30.4, 28.6. HRMS-ESI: C₂₆H₃₈N₅O₂ [M+H]⁺ calcd: 452.3026, found: 452.3024; mp 285-287 °C (decomp.).

3,5-Di-tert-butyl-N-(2-((2-guanidinoethyl)carbamoyl)phenyl)benzamide hydrochloride (**E23**). ¹H NMR (CD₃OD): 8.56 (dd, 1H, J₁ = 8.4 Hz, J₂ = 0.8 Hz), 7.87 (d, 2H, J = 1.6 Hz), 7.77 (dd, 1H, J₁ = 8 Hz, J₂ = 1.6 Hz), 7.71 (t, 1H, J = 1.6 Hz), 7.57 (ddd, 1H, J₁ = 8.4 Hz, J₂ = 8.4 Hz, J₃ = 1.6 Hz), 7.22 (ddd, 1H, J₁ = 8.4 Hz, J₂ = 8 Hz, J₃ = 0.8 Hz), 3.59 (t, 2H, J = 6 Hz), 3.46 (t, 2H, J = 6 Hz), 1.40 (s, 18H). ¹³C NMR (CD₃OD): 170.4, 167.0, 157.5, 151.5, 138.9, 133.9, 132.1, 127.6, 126.0, 123.2, 121.5, 121.3, 121.2, 40.3, 38.4, 34.6, 30.4. HRMS-ESI: C₂₅H₃₆N₅O₂ [M+H]⁺ calcd: 438.2869, found: 438.2857; mp 173-175 °C (decomp.).

3,5-Di-tert-butyl-N-(2-((3-guanidinopropyl)carbamoyl)phenyl)benzamide hydrochloride (**E24**). ¹H NMR (CD₃OD): 8.55 (dd, 1H, J₁ = 8 Hz, J₂ = 0.8 Hz), 7.86 (d, 2H, J = 1.6 Hz), 7.76 (dd, 1H, J₁ = 8 Hz, J₂ = 0.8 Hz), 7.70 (t, 1H, J = 1.6 Hz), 7.55 (ddd, 1H, J₁ = 8.8 Hz, J₂ = 8 Hz, J₃ = 0.8 Hz), 7.22 (ddd, 1H, J₁ = 8.8 Hz, J₂ = 8 Hz, J₃ = 0.8 Hz), 3.49 (t, 2H, J = 6.8 Hz), 3.29 (t, 2H, J = 6.8 Hz), 1.92 (p, 2H, J = 6.8 Hz), 1.40 (s, 18H). ¹³C NMR (CD₃OD): 170.2, 166.9, 157.2, 151.5, 139.1, 134.0, 132.1, 127.6, 126.0, 123.2, 121.3, 121.1, 121.0, 37.2, 36.1, 34.6, 30.4, 27.4. HRMS-ESI: C₂₆H₃₈N₅O₂ [M+H]⁺ calcd: 452.3026, found: 452.3025; mp 86-89 °C (decomp.).

3-(3,5-Di-tert-butylbenzamido)-N-(2-guanidinoethyl)-2-naphthamide hydrochloride (**F26**). ¹H NMR (CD₃OD): 8.96 (s, 1H), 8.36 (s, 1H), 7.93 (dd, 1H, J₁ = 8.4 Hz, J₂ = 0.4 Hz), 7.91 (d, 2H, J = 1.6 Hz), 7.87 (dd, 1H, J₁ = 8.4 Hz, J₂ = 0.4 Hz), 7.72 (t, 1H, J = 1.6 Hz), 7.59 (ddd, 1H, J₁ = 8.4 Hz, J₂ = 6.8 Hz, J₃ = 1.2 Hz), 7.49 (ddd, 1H, J₁ = 8.4 Hz, J₂ = 6.8 Hz, J₃ = 1.2 Hz), 3.65 (t, 2H, J = 6 Hz), 3.51 (t, 2H, J = 6 Hz), 1.41 (s, 18H). ¹³C NMR (CD₃OD): 170.5, 167.1, 157.5, 151.5, 135.1, 134.6, 134.0, 129.2, 128.8, 128.3, 128.2, 127.1, 126.0, 125.6, 122.3, 121.3, 118.2, 40.3, 38.6, 34.6, 30.4. HRMS-ESI: C₂₉H₃₈N₅O₂ [M+H]⁺ calcd: 488.3026, found: 488.3022; mp 250-252 °C (decomp.).

6.4 Biological test methods

Bacterial reference strains used in the initial antimicrobial screening are displayed under Table 1. The expanded screenings were performed against a collection of 275 randomly chosen clinical isolates of the species denoted in Table 2. The collection of *Enterococcus* spp. contained isolates of both *E. faecium* (20 isolates) and *E. faecalis* (35 isolates), due to the limited amount of clinical isolates available for this group of bacteria. The collection of 30 multi-drug resistant isolates used in the last screening is described in Table 3 and was obtained from the Norwegian National Advisory Unit on Detection of Antimicrobial Resistance (K-res), University Hospital Northern-Norway (UNN).

Minimum inhibitory concentration (MIC) assay: Working solutions of compounds were prepared with up to 100% DMSO and stored at -20 °C. To facilitate dissolution, if necessary, they were heated (40 - 80 °C) before use. All dilutions were prepared in double-distilled water. The final concentration of DMSO in the test series was ≤ 1% and did not affect the assay results. MIC was determined using a microdilution susceptibility test according to CLSI M07-A9³¹ with some modifications. The bacterial inoculum in Mueller-Hinton broth (MHB, Difco Laboratories, USA) was adjusted to approximately 2.5-3 x 10⁴ cells/ml, and incubated in a ratio 1:1 with test compounds in polystyrene 96-well flat-bottom microplates (Nunc, Roskilde, Denmark). Positive control (without antimicrobials) and negative control (without bacteria) were included. The reference antibiotics were vancomycin hydrochloride (powder for infusion, Hospira Enterprises B.V., Almere, Netherlands) for *S. epidermidis*, erythromycin (Apotekproduksjon AS, Oslo, Norway) for the other Gram-positive bacterial strains, and polymyxin B sulfate (Sigma-Aldrich, St. Louis, MO, USA) for the Gram-negative bacteria. The microplates were incubated in an EnVision microplate reader (Perkin-Elmer, Turku, Finland) placed in an incubator set to 35 °C for 48 h. The MIC value was defined as the lowest concentration of compound resulting in no bacterial growth as determined by OD₆₀₀ measurement. The compounds were tested in two parallels.

Antimicrobial screening against clinical isolates: MIC was determined as described above with few exceptions; working solutions of compounds were prepared from concentrated DMSO stocks stored at room temperature; the bacterial inoculum density was increased 40x, enterococci were incubated in Brain Heart Infusion broth (BHIB, Difco Laboratories, USA), the microplates were incubated for 24 h and compounds were tested in four parallels. MIC₉₀ was defined as the MIC value at which ≥ 90 % of the isolates were inhibited. The value was calculated using the formula $n \times 0.9$ where n is the number of isolates tested within each group of bacteria.³²

Determination of haemolytic activity: Haemolytic activity against RBC was determined according to Paulsen et al.,⁴ with minor modification. Briefly, blood was collected from a healthy, consenting adult in a vacutainer tube with heparin (BD Vacutainer LH, No. 36849, Plymouth, UK). RBCs were washed 3 x with phosphate-buffered saline (PBS, pH 7.4), and resuspended to give a 10% RBC suspension. The test was performed in 96 well U-shaped microtiter plates (Nunclon™ Surface, Nunc, Roskilde, Denmark). Serial dilutions of the test compounds were made in water or 1-2% DMSO. Final concentration of RBCs per sample was approximately 10⁸ RBC/ml, as determined by a Sysmex XN-2000 Hematology system (Sysmex America, Inc). After 1 h of incubation in a

shaker at 37 °C, the plates were centrifuged at 450 × g for 10 min. The supernatants were carefully transferred to flat-bottomed polycarbonate microtiter plates (Nunc, Roskilde, Denmark) and absorbance corresponding to haemoglobin release was measured at 550 nm. Haemolysis was confirmed by visual inspection. Baseline haemolysis was determined by incubating cells with 0.5% DMSO in 50% PBS, and 100% haemolysis was determined at 0,05% (w/v) Triton X-100 in 50% PBS. The percentage of haemolysis was determined using the formula: $((A_{\text{sample}} - A_{\text{baseline}})/(A_{\text{Complete haemolysis}} - A_{\text{baseline}})) \times 100$. The compound concentration giving 50% lysis (EC_{50}) was determined. The test was performed in two to four parallels. The results shown are from one representative experiment.

Membrane integrity assay: The test strain was *B. subtilis* 168 carrying the plasmid pCSS962, constructed according to.²⁵ The real-time membrane integrity assay was performed as described before by Virta et al.²⁵ with modifications. Overnight cultures were grown in MHB with chloramphenicol (5 µg/ml, Merck KGaA, Darmstadt, Germany). The bacteria were pelleted by centrifugation for 10 min at 4500 rpm, before they were resuspended in MHB to give an OD_{600} of 0.1. D-luciferin potassium-salt (pH 7.4, SynChem Inc, IL, USA) was added to a final concentration of 1 mM and the background luminescence was measured. Black round-bottomed 96-well plates (Nunc,

Roskilde, Denmark), containing dilutions of the test compounds (20 µl per well), were loaded into a Synergy H1 Hybrid Reader (BioTek, Winooski, VT, USA). During the first step, aliquots (180 µl) of the inoculum with D-luciferin were successively (well by well) injected to the test wells by an automatic injector with tracking of the luminescence emission every second for 30 sec. During the second step, chlorhexidine acetate (1 µg/ml buffered solution, Fresenius Kabi, Halden Norway) was added to all samples at membrane-disruptive concentrations (either 20 or 31 µg/ml). The monitoring of the luminescence emission continued for up to additional 90 sec. The residual light emission peak would indicate lysis of the cells that have survived the first treatment step. The experiments were performed two times, the mean ± SD is presented.

Supplementary Material

¹H-NMR spectra of synthesized compounds are included as “Supporting Material”.

