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Synthesis and antimicrobial evaluation of cationic low molecular weight amphipathic 1,2,3-triazoles

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ABSTRACT

A library of 28 small cationic 1,4-substituted 1,2,3-triazoles was prepared for studies of antimicrobial activity. The structures addressed the pharmacophore model of small antimicrobial peptides and an amphipathic motif found in marine antimicrobials. Eight compounds showed promising antimicrobial activity, of which the most potent compound **10b** displayed minimum inhibitory concentrations of 4–8 μg/mL against *Streptococcus agalacticae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Enterococcus faecalis*. The simple syntheses and low degree of functionalization make these 1,4-substituted 1,2,3-triazoles interesting for further optimizations.

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Antimicrobial resistance to conventional antibiotic treatment is rapidly increasing, combined with lackluster efforts to develop novel classes of antibiotics by major pharmaceutical companies.[1–3] Infections caused by multi resistant bacteria is therefore one of the fastest growing medical threats to modern society.[4] Disturbingly, resistant bacteria have existed since the discovery of the first antibiotics. In recent years the race between growing resistance and progress of new antibiotics has intensified in favor of the bacteria. Unfortunately, no antibiotic has yet passed clinical trials for which there has not been reported cases of resistance.[5,6]

Most antibiotics applied today work through specific interactions with key intra- and extra-cellular targets in bacteria, and in a highly specific manner.[7] Due to the high target specificity, uncritical use of antibiotics easily selects for mutated bacteria to proliferate. A well known mechanism of resistance is expression of beta-lactamases that metabolizes beta-lactam based antibiotics.[8] An expanding field within antibiotic research in academia focuses on structures working through less specific mechanisms, like interactions with the bacterial cell membrane and non-specific interactions with intracellular targets.[9–12] The interest in these mechanisms of action comes from antimicrobial peptides (AMPs), that are important constituents of innate immunity in most living organisms. AMPs have a net positive charge (+2 to +9), consist of 12–50 residues, and fold into secondary structures with bactericidal properties.[13] These amphipathic structures, having a positively charged hydrophilic face and a lipophilic face, interact with anionic phospholipids on the surface of bacterial cell membranes. This is followed by membrane permeabilization by the lipophilic residues, leading to cell membrane disruption and ultimately cell lysis.[14,15]

Even though AMPs are considered to be highly active therapeutic compounds, there are some major issues in utilizing them on a large scale. Important drawbacks include low oral bio-availability, low metabolic stability, high manufacturer costs, and lack of patient-friendly administration methods aside from topical treatments.[16] Due to these obstacles, only a small number of antimicrobial agents utilized today are AMPs.[17] A way to circumvent the practical challenges associated with AMPs is to make smaller peptides and scaffold-based peptidomimetics that maintain the antimicrobial activity, but have improved pharmacokinetic properties. This has been demonstrated by Strøm et al., who have synthesized small beta-peptidomimetic structures (MW < 650) with high activity against a variety of resistant bacteria and with potential for *per oral* administration.[18,19] Recently, the group of Strøm[20] has reported a series of small cationic aminobenzamides (example shown as **E23** in Fig. 1) that mimic amphipathic structures found in marine antimicrobials such as synoxazolidinone A[21] and ianthelline[22] and display a membranolytic effect resembling many AMPs. The focus of this work was to further develop such amphipathic structures addressing both small AMPs and marine antimicrobials, and optimize these for antimicrobial activity. The di-functionalized 1,2,3-triazole was chosen as the core

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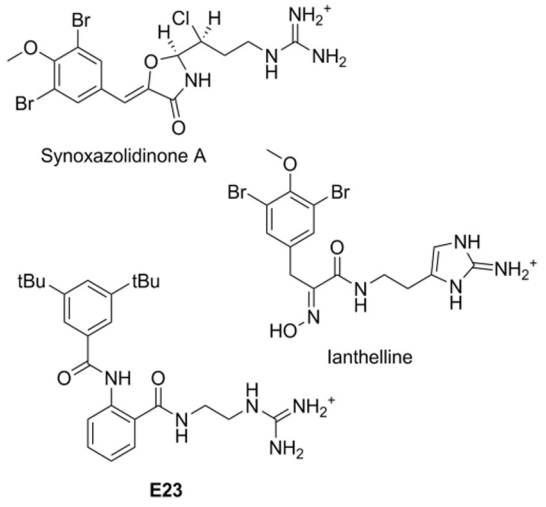
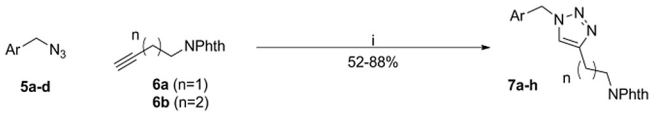


Fig. 1. Synoxazolidone A[21] (MRSA (MIC); 10 μg/mL), Ianthelline[22] (MRSA (MIC); 20 μg/mL), and **E23**; a natural product mimic by Strøm et al.[20] (MRSA (MIC); 4 μg/mL).

scaffold, due to the known biochemical properties of this type of structures.[23,24] Of importance was that triazoles are bioisosteres of amide bonds, which are more stable against proteolytic degradation than amides in AMPs.[25–27] The study included initial synthesis of 24 compounds to investigate the effects of varying between four lipophilic groups and three cationic groups, and including chain length variations. These results were followed up by synthesis of four optimized compounds based on the results from the initial series of di-functionalized 1,2,3-triazoles.

In order to synthesize a collection of disubstituted 1,2,3-triazole amphiphiles with the desired lipophilic- and cationic hydrophilic functionalities, the "click" chemistry protocol developed by Sharpless[28] and Meldal[29] was chosen. By using different catalysts for the "click" chemistry step, 1,2,3-triazoles with different substitution patterns can be prepared, i.e., 1,4-substitution when using copper (I) and 1,5-substitution when using ruthenium(II)-catalysis.[30] The



Scheme 1. (i) $CuSO_4{\cdot}5H_2O$ (5 mol %), Sodium ascorbate (10 mol%), Benzoic acid (10 mol%), tBuOH:H_2O (1:2), rt, 10 min – over night (Ar; **5a** = Ph, **5b** = naphthyl, **5c** = 3,5-di-*t*-Bu-Ph and **5d** = 3,5-CF_3-Ph).

1,4-substitution pattern was chosen here, due to the fact that the copper(I) catalysts used in these reactions are water insensitive (unlike their ruthenium counterparts), excluding the need for working under inert conditions. Thus, the first target compounds given in Fig. 2 (**1a**–**4f**) were prepared in order to screen the effects of different lipophilic aromatic groups and hydrophilic cationic nitrogen groups.

The "click" chemistry protocol requires two coupling partners carrying an azide and a terminal alkyne. It was found most convenient to insert the azide on the lipophilic moiety and the terminal alkyne on the nitrogen carrying functionality. The azides (**5a-d**, shown in Scheme 1) were synthesized from the respective commercially available bromides and alcohols, by well-established reactions (details are shown in the supporting information).[31–34] The alkynes carrying a handle for N-functionalization, were prepared from 3-butyn-1-ol and 1-chloropent-4-yne respectively, under Mitsunobu- or Finkelstein modified Gabriel-conditions (details shown in the supporting information).[35,36] This yielded **6a** and **6b** (shown in Scheme 1) with the same masked N-functionality and a difference of one methylene group in the carbon chain.

The alkynes (**6a** and **6b**) and azides (**5a-d**) were then combined to form [1,4]-1,2,3-triazoles (**7a-h**) using copper catalyzed "click"-chemistry conditions as shown in Scheme 1.[37] Thus, by using four different azides and two lipophiles, eight different "core" 1,2,3-triazoles ready for N-functionalization (**7a-h**) were prepared (see Scheme 1).

Three different cationic groups were evaluated; a primary amine (**a** and **d**), a tertiary amine (**b** and **e**) and a guanidine group (**c** and **f**) as shown in Scheme 2. The interest for the primary amine and the guanidine came from the functionalities found in AMPs,

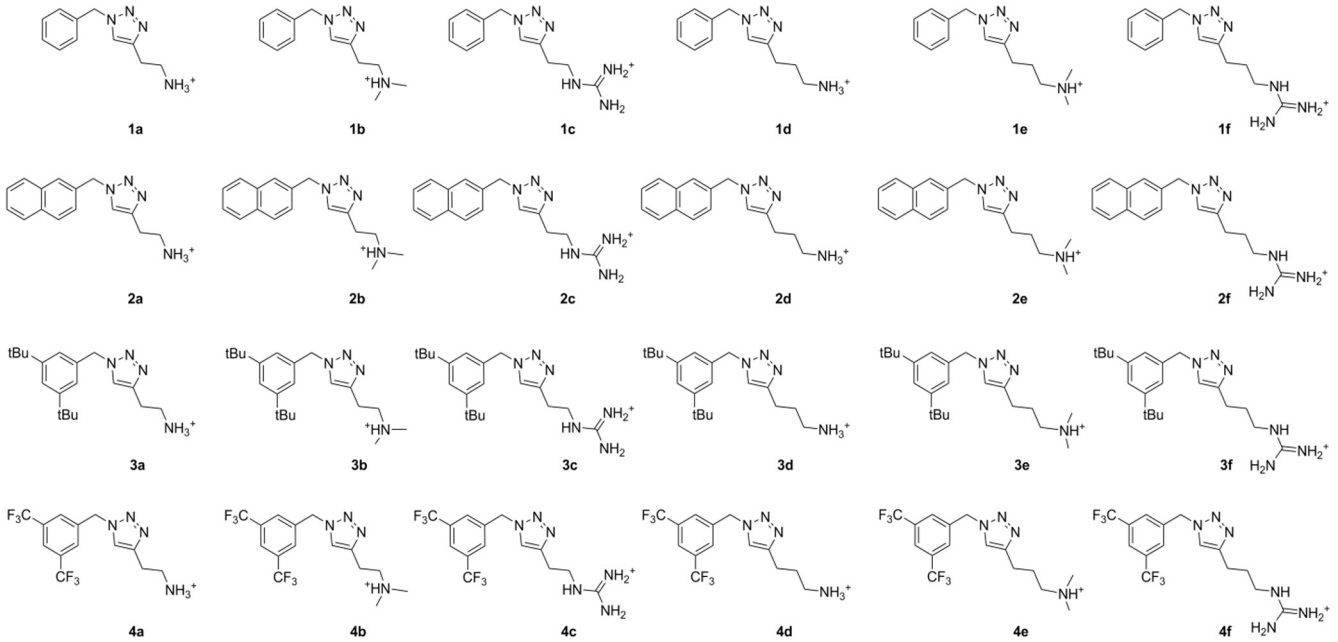
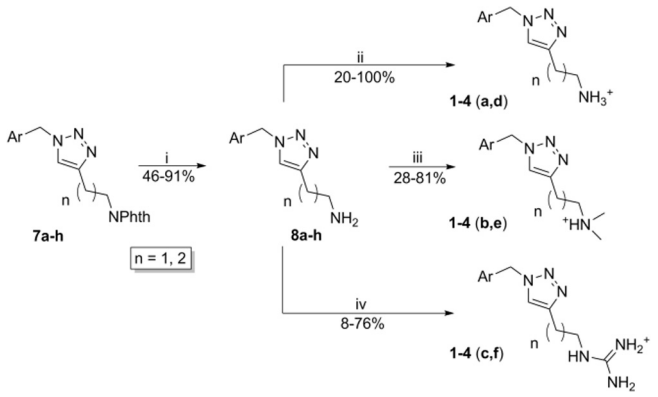


Fig. 2. Initial target [1,4]-1,2,3-triazoles **1a**–**4f** to be screened for antimicrobial effects. Counter-ion: Cl^-.



Scheme 2. (i) Hydrazine hydrate, toluene, reflux, (ii) HCl (conc. aq. or 2 M in Et_2O), iPrOH, MeCN or DCM, (iii) Formaldehyde, formic acid, MeCN, reflux, 1 h, acidic work up and (iv) 1H-pyrazole carboxamidine hydrochloride, MeCN, reflux 2–4 h.

were lysine and arginine residues contribute these groups to the amphiphile, thus taking a vital part in induction of antimicrobial activity.[13] A tertiary amine was expected to be a steric and electronic mid-point between the two naturally occurring cationic groups. In order to increase the steric bulk, without introducing additional nitrogens and resonance possibilities, the tertiary dimethylamino group was chosen as a mid-point between primary amines and guanidines. The eight protected 1,2,3-triazoles were deprotected using hydrazine hydrate according to a protocol developed by Gabriel.[38–40] The primary amines (**8a**-**h**) were subsequently functionalized in order to introduce the chosen functionality, and the primary amine HCl-salts **1**–**4** (**a**, **d**) (Scheme 2) were obtained by treatment with hydrochloric acid. The Eschweiler-Clarke reductive amination was utilized to create the tertiary amines **1**–**4** (**b**, **e**) (Scheme 2),[41,42] and an electrophilic guanidine reagent to create the guanidines **1**–**4** (**c**, **f**) (Scheme 2).[43] Performing the given transformations on all eight protected triazoles yielded the 24 different compounds depicted in Fig. 2 (**1a-4f**), in sufficient purity (>95% HPLC) for biological evaluation.

The 24 amphiphilic triazoles **1a-4f** were tested against three gram-positive and two gram-negative bacterial strains. In addition, all 24 compounds were subjected to toxicity studies against human fibroblasts (MRC-5). No activity was detected below 50 μg/mL, indicating low toxicity of the structures towards this type of human cells. Four of the 24 structures (**3a**, **3c**, **3e** and **3f**) showed promising activities against several cell lines. These were subjected to dilution assays in order to determine the minimum inhibitory concentrations (MICs) against the chosen bacteria. The MIC-values for the active compounds are shown in Table 1.

All the active compounds (**3a**, **3c**, **3e** and **3f**) contained the heavily hindered and non-polar 3,5-di-*tert*-butyl-phenyl functionality. This indicated that a bulky and non-polar lipophilic contribution was important for the activities in these structures. Furthermore, the guanidine hydrochloride functionality appeared to be related to the observed activities. As they (**3c** and **3f**, Fig. 2) were more potent than the tertiary dimethyl- and primary amines (**3a** and **3e**, Fig. 2), with the exception of **3a** against *E. coli*. The difference in activity was most pronounced against the gram-positive *S. aureus* and *Streptococcus* gr. B (*S. agalacticae*) bacteria, where a 2- to 4-fold increase in activity was observed for the guanidine compared to the other two cationic nitrogen groups.

So far, we have determined which lipophilic and cationic group that most likely promoted the highest activity against the five strains of bacteria tested. The third varying factor in the series of amphipathic 1,2,3-triazoles tested was the two or three carbon chain of the hydrophilic end of the triazole ring. A small increase in efficacy was observed for the longer **3f** compared to **3c** against *S. aureus* and *E. coli*. Furthermore, **3f** showed the overall highest activity against the gram-positive *S. aureus* and *S. agalacticae* (10 μg/mL), while there was a 4-fold decrease in the activity against gram-positive *E. faecalis* and the gram-negative *E. coli* and *P. aeruginosa* (40 μg/mL). Lowered activity against gram-negative compared to gram-positive bacteria is commonly observed, due to different outer membrane compositions.[44] However, it was surprising that the activity against *E. faecalis* was in the range of the gram-negative strains. In addition to the antimicrobial effects, some biofilm inhibition was observed in single concentration assays of these structures. The amphiphiles **2f** and **3** (except **3d**) showed biofilm inhibition at 50 μg/mL.

It was assumed from the pharmacological model[18–20] that a rather large lipophilic contribution would be important for achieving the desired antimicrobial effects. In order to rationalize our findings we attempted to use calculated pKa adjusted partition coefficients (ClogD) as an indicator for lipophilicity. The ClogD values were calculated (using the Marvinsketch software[45]) at physiological pH (pH = 7.40), showing the guanidines (**c** and **f**, Fig. 2) to be mostly protonated and the primary (**a** and **d**, Fig. 2) and tertiary amines (**b** and **e**, Fig. 2) to exist in more partitioned equilibria. However, when plotted against the values from the antimicrobial MIC-assays, no apparent connection was found between the ClogD and MIC-values. On the other hand, plotting all structures according to their retention times (Rt) from C18-HPLC as shown in Fig. 3, gave a more accurate picture of the effective lipophilic contributions. As the HPLC analyses were performed with an acid additive (0.1% TFA) in order to inhibit peak broadening, all of the compounds were assumed to exist mainly in their positively charged state. This indicated that the lipophilic nature of the charged structures is an important parameter for biological activity; e.g. **3f** is more active than **3e**, even though the calculated ClogD (displayed in Fig. 3) of **3e** is nearly the double of the one for **3f**. The fact that the Rts may be used as a rough indicator of antimicrobial activity may prove useful when targeting new potential candidates for optimization.

Compound **3f** from the initial screening and dose response assessments showed the highest antimicrobial activities, with MICs ranging from 10 to 40 μg/mL. In order to optimize the activities towards the target bacteria, a small and focused set of compounds was prepared based on the structure of **3f**. The first change was inspired by the planar benzamide peptide mimics

Table 1
Antimicrobial activity (MIC in μg/mL) for the 1,2,3-triazoles that showed any activity in the antibacterial assays. The "–"-sign in the table indicates no activity in the assay at the highest tested concentration (50 μg/mL).

Entry	3a	3c	3e	3f	Ref.[a]
E. faecalis[b]	–	40	–	40	10
S. aureus[b]	40	20	40	10	0.13
S. agalacticae[b]	40	10	50	10	4
E. coli[b]	40	50	–	40	0.5
P. aeruginosa[b]	50	40	–	40	0.5

[a] Ref.: Gentamicin.
[b] *E. faecalis* (ATCC 29212), *S. aureus* (ATCC 25923), *S. agalacticae* (ATCC 12386), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853).

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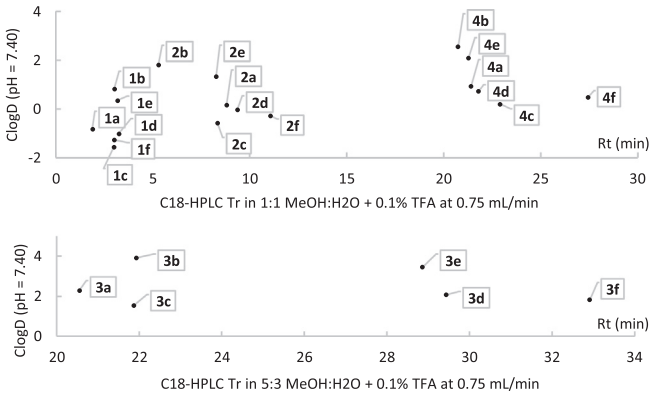
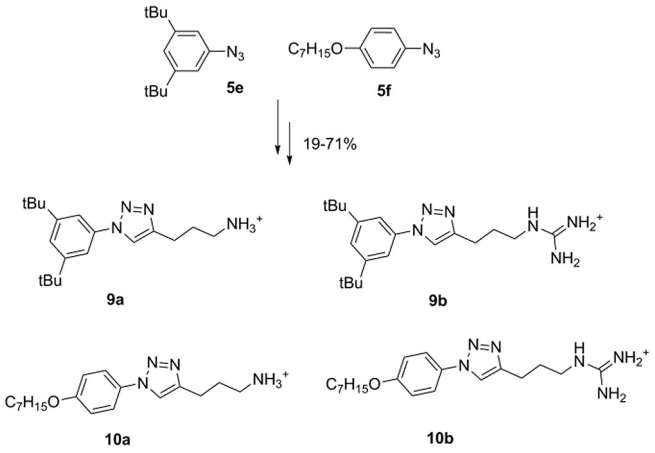


Fig. 3. Top plot: C18-Rt (MeOH/water, 1:1 + 0.1% TFA) for **1a-f**, **2a-f** and **4a-f** plotted against calculated ClogD at pH 7.40 (using the MarvinSketch suite). Bottom plot: C18-Rt (MeOH/water, 5:3 + 0.1% TFA) for **3a-f** plotted against calculated ClogD at pH 7.40 (using the MarvinSketch suite).



Scheme 3. Improved structures **9a-b** and **10a-b** based on **3f**, synthesized in three steps from **5e** and **5f** utilizing the chemistry displayed in Schemes 1 and 2. Counter ion: Cl^-.

presented by Strøm et al.,[20] where they achieved <10 μg/mL against the same bacteria. By using an aromatic azide instead of a benzylic azide in the "click"-coupling, the obtained amphiphiles would be more planar and rigid compared to **1a-4f** (Fig. 2). This was expected since the benzylic methylene group create an angle between the triazole ring and the lipophilic aromatic group and give more rotational freedom, which might be disfavourable for antibacterial activity. A second modification of **3f** in addition to removing the benzylic methylene group, was to change the structure of the lipophilic group. By going from the bulky *t*-Bu-functions to a linear alkyl chain with similar surface area and molecular weight, we hoped to mimic the membrane snorkeling effect of lysine and arginine rich proteins.[46] Thus, the four amphiphiles shown in Scheme 3 (**9a-b** and **10a-b**) were prepared for further studies.

The synthesis of **9a-b** and **10a-b** from the azides **5e** and **5f** were performed according to the methods presented in Schemes 1 and 2 with total yields ranging from 19 to 71% over three steps. The azides (**5e** and **5f**) were synthesized from commercially available iodophenol and 3,5-*t*-Bu-bromobenzene using a copper catalyzed synthesis presented by Zhu et al.[47] (experimental details are found in the supplementary information). The four triazole amphiphiles were subjected to the same bacterial strains as the initial 24 amphiphiles. The obtained MIC values are displayed in Table 2 together with the reference antibiotic gentamicin.

Removal of the benzylic methylene group and introduction of a more rigid and planar structure with possibility for conjugation lead to an approximate two-fold increase in activity against all the tested bacteria (**9b** compared to **3f**), and MIC-values as low as 4 μg/mL against *S. aureus* and *S. agalacticae*. Again, the guanidine hydrochloride (**9b** and **10b**) proved to be the most active hydrophile (compared to NH_3^+), as it led to a two-fold increase in activity against all bacteria (except for *E. coli*) compared to the ammonium hydrochlorides (**9a** and **10a**). Substituting the bulky 3,5-*t*-Bu group with a heptyl ether chain (**10a** and **10b**) led to a further two-fold increase in the activity against the gram-negative strains and the gram-positive *E. faecalis*. This may in turn be attributed to the heptyl chain's (**10b**) ability to penetrate deeper into the membrane compared to the *t*-Bu groups in **9b**. However, the exact mechanism of action for these compounds has not been investigated yet. It should also be noted that the activity of **10b** surpassed that of Gentamicin against *E. faecalis* and matched the activity against *S. agalacticae*.

As for the initial 24 amphiphiles, there was no evident correlation between the MIC-values and calculated CLogD. The most active compound (**10b**) had the lowest calculated CLogD of the four structures (Table 2). However, the retention times from C18-HPLC showed a better correlation, where the most active structure had the highest retention time on the C18-column (Table 2).

We have successfully synthesized 28 low molecular weight cationic triazole-based amphiphiles with different lipophilic and hydrophilic functionalities, and screened for antimicrobial effects against *S. agalacticae*, *S. aureus*, *P. aeruginosa*, *E. coli*, and *E. faecalis*. The most potent compound in our library (**10b**) displayed MIC-values between 4 and 8 μg/mL, which either matched or surpassed the activity of the marine natural product peptide mimics Synoxazolidone A and Ianthelline. The activity of **10b** also matched the activity against gram-negative bacteria for the benzamides presented by Strøm et al.[20] Thus, bioisosteres of amide bonds can be

Table 2
Antimicrobial activity (MIC in μg/mL) for the improved amphiphilic triazoles based on **3f**, calculated ClogD (pH = 7.4) and reverse phase HPLC retention times (min in 5:3 MeOH: H_2O + 0.1% TFA).

Entry	9a	9b	10a	10b	Ref.[a]
E. faecalis[b]	32	16	16	8	10
S. aureus[b]	16	4	16	4	0.13
S. agalacticae[b]	16	4	8	4	4
E. coli[b]	16	16	8	8	0.5
P. aeruginosa[b]	32	16	16	8	0.5
ClogD (calc.)[c]	2.00	1.75	1.41	1.16	–
RT (C18-HPLC)[d]	22.9	28.0	20.4	34.6	–

[a] Ref.: Gentamicin.
[b] *E. faecalis* (ATCC 29212), *S. aureus* (ATCC 25923), *S. agalacticae* (ATCC 12386), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853).
[c] Calculated at pH = 7.40 using the MarvinSketch suite.
[d] In 5:3 MeOH:H_2O + 0.1% TFA with 0.75 mL/min.

applied without compromising the activity of the substrates. This provides a higher degree of structural freedom when choosing substrates for this type of activity-driven library design of antimicrobial scaffolds. We believe our findings may serve as basis for further investigations into artificial peptide mimics.

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A. Supplementary material

Supporting information containing all experimental procedures (for both synthesis and biological assays) and full characterization of novel compounds is electronically available through the publisher's website. Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2017.01.092.

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