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Article

Structure-Activity Study of an All-d Antimicrobial Octapeptide D2D

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Abstract:

The increasing emergence of multi-drug resistant bacteria is a serious threat to public health worldwide. Antimicrobial peptides have attracted attention as potential antibiotics since they are present in all multicellular organisms and act as a first line of defence against invading pathogens. We have previously identified a small all-d antimicrobial octapeptide amide kk(1-nal)fk(1-nal)k(nle)-NH2 (D2D) with promising antimicrobial activity. In this work, we have performed a structure-activity relationship study of D2D based on 36 analogues aimed at discovering which elements are important for antimicrobial activity and toxicity. These modifications include an alanine scan, probing variation of hydrophobicity at lys5 and lys7, manipulation of amphipathicity, N-and C-termini deletions and lys-arg substitutions. We found that the hydrophobic residues in position 3 (1-nal), 4 (phe), 6 (1-nal) and 8 (nle) are important for antimicrobial activity and to a lesser extent cationic lysine residues in position 1, 2, 5 and 7. Our best analogue 5, showed MICs of 4 µg/mL against A. baumannii, E. coli, P. aeruginosa and S. aureus with a hemolytic activity of 47% against red blood cells. Furthermore, compound 5 kills bacteria in a concentration-dependent manner as shown by time-kill kinetics. Circular dichroism (CD) spectra of D2D and compounds 1–8 showed that they likely fold into α-helical secondary structure. Small angle x-ray scattering (SAXS) experiments showed that a random unstructured polymer-like chains model could explain D2D and compounds 1, 3, 4, 6 and 8. Solution structure of compound 5 can be described with a nanotube structure model, compound 7 can be described with a filament-like structure model, while compound 2 can be described with both models. Lipid interaction probed by small angle X-ray scattering (SAXS) showed that a higher amount of compound 5 (~50–60%) inserts into the bilayer compared to D2D (~30–50%). D2D still remains the lead compound, however compound 5 is an interesting antimicrobial peptide for further investigations due to its nanotube structure and minor improvement to antimicrobial activity compared to D2D.

Keywords: antimicrobial peptides; d-peptides; minimum inhibitory concentration; hemolytic activity; time-kill kinetics; circular dichroism; small angle X-ray scattering
1. Introduction

Multidrug-resistant (MDR) bacteria is a major global health problem [1]. Recently, WHO published a list of MDR bacteria which are of critical concern [2]. Especially worrying are the Gram-negative pathogens *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and several species of Enterobacteriaceae [3]. The development of carbapenem resistance in these species has led to the re-introduction of the last resort antibiotic Colistin, which was initially abandoned due to side-effects. Since 2000, approximately 30 new antibiotics have been marketed worldwide of which five were first-in-class antibiotics [4]. However, these five new antibiotic classes linezolid, daptomycin, retapamulin, fidaxomicin and bedaquiline only target Gram-positive bacteria so there is an unmet need for new antibiotics targeting Gram-negative bacteria [4].

In recent years, antimicrobial peptides (AMPs) have attracted considerable interest as potential antibiotics [5]. Antimicrobial peptides are a part of the innate immune system in all higher organisms. They display broad-spectrum antimicrobial activity at low concentrations, fast killing, and often a membrane-specific mechanism of action [6]. Because the bacterial membrane is a fundamental part of the bacterial cell envelope, it is believed this makes it more difficult for microbes to develop effective mechanisms of resistance against AMPs as compared to traditional antibiotics [7]. Furthermore, AMPs may also have immunomodulatory properties [8]. The drawbacks of AMPs as peptide-based antibiotics include susceptibility to proteases and potential toxicity [9]. The stability of promising synthetic AMPs are typically improved by cyclization [10] or insertion of non-proteinogenic building blocks [11]. These include α-amino acids [12], peptoids (N-substituted glycines) [13], peptidomimetics such as, α-peptide/β-peptides [14], lysine-based α-peptides/α-peptoids [15], lysine-based α-peptide/β-peptoids [16], and α/γ N-Acylated-N-Aminoethylpeptides (AApeptides) [17]. Previously, we have identified an all-α-peptide, D2D, which shows promising activity against clinical isolates of Methicillin Resistant *Staphylococcus pseudintermedius* and *Pseudomonas aeruginosa*. Furthermore, the compound showed moderate toxicity against red blood cells and resistance to proteolytic degradation [18]. D2D consists of 4 cationic residues lys$^1$, lys$^2$, lys$^5$, lys$^7$ and 4 hydrophobic residues 1-naphthylalanine (1-nal)$^3$, phe$^4$, 1-nal$^6$, norleucine (nle)$^8$. D2D is a full α-amino acid analog derived from a structure-activity relationship study of the peptoid D2. Furthermore D2, was selected in a previous study after screening from a combinatorial library of de novo designed compounds for their activity against *S. pseudintermedius* and toxicity in vitro against red blood cells [19].

In this work, we present a structure-activity study of D2D (see Figure 1) based on 36 analogs including ala-scan, N-and C-termini deletions and lys-arg replacements. The analogs were tested against *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 29213), *Pseudomonas aeruginosa* (ATCC 27853) and *Acinetobacter baumannii* (ATCC 19606).
2. Results and Discussion

A total of 37 peptides including the lead compound D2D (Table 1) were synthesized. See supplementary materials for structures, HPLC chromatograms and MALDI-TOF-MS spectra.

Table 1. Sequence, MIC and hemolytic activity of 36 analogues of D2D.

<table>
<thead>
<tr>
<th>No.</th>
<th>Sequence</th>
<th>E.C.</th>
<th>S.A.</th>
<th>P.A.</th>
<th>A.B.</th>
<th>%H</th>
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<td>74</td>
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In order to evaluate the role of each individual amino acid for the antimicrobial activity and toxicity of D2D, we performed alanine scan by systematically replacing all residues with α-alanine (Table 1). This identified four residues to be important for activity (compounds 3 (1-nal), 4 (phe), 6 (1-nal) and 8 (nle) (Table 1)). When these residues were replaced with α-alanine the MIC changed from 4–8 μg/mL to between 64 and > 128 μg/mL for E. coli and S. aureus while P. aeruginosa and A. baumannii ranged from 16 to > 128 μg/mL. The 1-naphthylalanine (1-nal) at position 6 appeared to be the most important amino acid in the sequence for maintaining activity. These substitutions (3, 4, 6 and 8) also decreased the hemolytic activity compared to D2D. The alanine scan identified four of eight residues (Compounds 1, 2, 5 and 7, all lys) with improved or similar MIC values compared to D2D. The MIC values ranged from 4–16 μg/mL for E. coli, P. aeruginosa, A. baumannii and 2–8 μg/mL for S. aureus. Compound 1 improved the activity against S. aureus by 4-fold, but decreased activity against P. aeruginosa by 2-fold compared to D2D. Compound 2 had decreased activity against E. coli, P. aeruginosa and A. baumannii by 2-fold, but retained the activity against S. aureus compared to D2D. Compound 5 showed improved activity against E. coli and S. aureus and comparable activity to D2D against P. aeruginosa and A. baumannii.

Compound 7 showed a 2-fold reduction in MIC against P. aeruginosa, but retained activity against other tested bacteria compared to D2D. Compound 1 and 5 showed higher hemolytic activity compared to D2D. Compound 2 and 7 greatly increased the hemolytic activity compared to D2D.

As mentioned previously, an ala-scan is often used to identify the importance of the side-chain in antimicrobial peptides. Representative examples include indolicidin [20], Temporin 1AI [21] and anoplin [22]. What is typically found is that cationic residues like Lys or Arg are replaceable, while substitution of hydrophobic residues has a great negative effect on MIC but a positive effect on hemolytic activity.

In an ala-scan of the α-helical decapeptide amide anoplin, GLLKRIKTL, replacing residue Arg<sub>5</sub>, Lys<sub>7</sub> or Thr<sub>8</sub> with alanine were found to be more active than anoplin by extending the hydrophobic phase of the helix [22], while substituting the five hydrophobic residues resulted in increased MICs. In the present study, we investigated all-α-peptides. Previously, we have shown that D2D is approximately four-fold more active than its l-enantiomer [18]. This is in alignment with several studies which report that the d-enantiomer is more active than the corresponding l-peptide. For example, Manabe et al.

Table 1. Cont.

<table>
<thead>
<tr>
<th>No.</th>
<th>Sequence&lt;sup&gt;[b]&lt;/sup&gt;</th>
<th>E.C. &lt;sup&gt;[a]&lt;/sup&gt;</th>
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<th>P.A. &lt;sup&gt;[e]&lt;/sup&gt;</th>
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<td>2</td>
<td>16</td>
<td>8</td>
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</table>

[a]: Minimum Inhibitory Concentration (MICs) were determined in triplicate by the broth microdilution method in accordance with CLSI standards. MICs are reported in μg/mL. To convert to μM use μM = ((μg/mL)/Mw) · 1000; [b]: Compounds tested in this study. All D-peptides were synthesized as C-terminal amides. Changes relative to D2D are highlighted. Unusual amino acids in the sequences: 1-naphthylalanine (1-nal) and norleucine (nle) [c]: E. coli (ATCC 25922); [d]: S. aureus (ATCC 29213); [e]: P. aeruginosa (ATCC 27853); [f]: A. baumannii (ATCC 19606); [g] % Hemolysis at 150 μM.

2.1. Alanine Scan of D2D

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compared the antimicrobial activity of a sapesin B analog, KLKLKLKLK-NH₂, to its corresponding all-d analogue [23]. A significantly higher antimicrobial activity against S. aureus was found for the d-peptide (1 and 16 µg/mL, respectively). Similarly Oddo et al. reported that an all d-peptide amide, kklkklrlkyl was significantly more active against A. baumannii than the corresponding l-enantiomer [24].

2.2. Investigation of Hydrophobicity at Position 5 and 7 Analogues

A series of substituted analogues were designed based on the above results. These included substitutions at residue lys⁵ and lys⁷ based on the improved or retained antimicrobial activity of compound 5 and 7 compared to D2D. Furthermore, placing D2D on a Schiffer-Edmundson wheel projection [25] (Figure 2) revealed a partly amphipathic helix, with lys¹, lys³, and lys² on the hydrophilic surface of the helical wheel and 1nal⁶, 1nal⁹, phe⁴, and nle⁸ on the hydrophobic side. The amino acid substitutions were selected to study the effects of substituting more or less hydrophobic amino acids than alanine. The chosen amino acids were d-1-Naphthylalanine, d-Dap, d-Nle, d-Phe, d-Ser, d-Thr, d-Tyr, and d-Val.

![Figure 2. Helical wheel of D2D. The red colour indicates cationic amino acids and the blue colour indicates hydrophobic amino acids.](image)

Position 5 (Compounds 9–16 Table 1): The MIC values for position 5 substitutions ranged from 4–64 µg/mL for E. coli, 2–16 µg/mL for S. aureus, 16–128 µg/mL for P. aeruginosa and 8–64 µg/mL for A. baumannii. The substitutions at position 5 did not improve the MIC values overall compared to 5 and D2D, but some peptides had improved activity against S. aureus. Substitutions at position 5 generally increased the hemolytic activity compared to D2D (23% hemolysis) compounds 11, 12, 15, and 16 being more hemolytic than D2D and compound 9, 10, 13, and 14 having moderate hemolytic activity compared to D2D at 150 µM.

Position 7 (Compounds 17–24 Table 1): The MIC values for position 7 substitutions ranged from 8–16 µg/mL for E. coli, 2–16 µg/mL for S. aureus, 16–64 µg/mL for P. aeruginosa and 4–16 µg/mL for A. baumannii. The substitutions at position 7 (compounds 17–24) did not improve the MIC values overall compared to compound 5 and D2D, but the antimicrobial activity of the position 7 peptides is overall better compared to position 5 substitutions (compounds 9–16). The substitutions at position 7 have significantly larger hemolytic activity compared to position 5 substitutions, with 18 being the only peptide with a hemolytic activity below 50% at 150 µM.

2.3. Manipulating Amphipaticity and Truncations (Compounds 25–32)

Compound 25 and 26:

Since many small cationic antimicrobial peptide adopt α-helical structures, we designed compounds 25 and 26 assuming possible gain from creating more balance between the hydrophobic
and cationic residues. To obtain a better balance between these domains we exchanged 1-nal⁶ with lys⁷ compound 25 and lys⁸ and nle⁸ analogue 26 (Figure 2). In both cases, this creates a perfect amphipathic helix. The MIC values for both 25 and 26 were 128 µg/mL for E. coli, 64 µg/mL for S. aureus and 32 µg/mL for P. aeruginosa and A. baumannii. These rearrangements in the sequence lead to a significant loss in activity. The hemolytic activity was increased for compound 25 (50%), while compound 26 (18%) is almost the same as D2D (23%). The difference in hemolytic activity could be explained by the cationic lys⁸ at the C-terminal end for compound 26.

N- and C-terminus truncations abolish activity of D2D (Compounds 27–32 Table 1):

The influence of the N- and C-terminal regions for the antimicrobial activity and toxicity were investigated. Six truncated analogues of D2D were synthesized; compounds 27–29 being the N-terminal truncations and compounds 30–32 being the C-terminal truncations. Only [des-lys¹]D2D, compound 27, showed some activity. Our finding that truncated analogues are not active is agreement with other structural activity studies of AMPs, e.g., fallaxin [26] and anoplin [22].

2.4. Manipulating Cationicity by Lys-Arg Substitutions (Compound 33–36)

The influence of an increased cationicity for the antimicrobial activity and toxicity was investigated by replacing all lysines one at a time with arginine. Arginine forms three hydrogen bonds (pKa 12.5) compared to lysine which forms two (pKa 10.5). The MIC values for the arginine substitutions was 8 µg/mL for E. coli, 2–8 µg/mL for S. aureus, 16 µg/mL for P. aeruginosa and 8–16 µg/mL for A. baumannii. The antimicrobial activity did not improve, but was more or less retained for the arginine substitutions compared to D2D. The hemolytic activity for analogues 33, 34, 35, and 36 increased compared to D2D. A study of the antimicrobial peptide amide BP100, KKLFKKILKYL, showed that a single substitution with arginine at lys⁸ improved antimicrobial activity without any detectable increase in toxicity [24]. Another study of antimicrobial peptides consisting of exclusively arginine and tryptophan of varying length showed that arginine compared to lysine could yield some antimicrobial peptides with enhanced antimicrobial activity [27]. However, improvements in the antimicrobial activity was not seen for 33–36.

2.5. Time-kill Kinetics of D2D and Analogue 5

Compound 5 (CS) and D2D were evaluated for in vitro efficacy in time kill experiments (Figure 3). They were tested against ATCC strains: A. baumannii (Figure 3A), E. coli (Figure 3B), P. aeruginosa (Figure 3C) and S. aureus (Figure 3D). Compound 5 was tested at concentrations of 1 × MIC, 3 × MIC and 5 × MIC in all experiments. For comparison D2D was only included at 5 × MIC which seems to be slightly more active than compound 5. There is a clear concentration-dependent bacterial killing observed for compound 5, as is often seen for cationic AMPs [11]. D2D and compound 5, both reduce the number of viable cells by 4–5 logs against E. coli and A. baumannii at 5 × MIC (Figure 3A,B). Whereas, only D2D is capable of killing 1 log of P. aeruginosa at 5 × MIC (Figure 3C), clearly demonstrating some differentiation in species specificity between the compounds. For the three Gram-negative species tested, regrowth of cultures was evident after 24 h. This is probably related to the concentration dependent killing, as the peptides get sequestered by killed cells leaving unaffected cells to regrow. Both compounds were shown to be capable of killing S. aureus with a 5 log reduction in viable cells at 5 × MIC. From the data, it seems that there could be a slight re-growth of S. aureus after 24 h, however it is much less pronounced than it was for the Gram-negative species, indicating that bacterial killing probably was more pronounced. Because the MIC of compound 5 was the same for all species (4 µg/mL), this indicates that the concentration dependent killing is differentiated from Gram-negative to Gram-positive bacteria, i.e., less compound is needed for antimicrobial killing of Gram-positive S. aureus or is sequestered than for Gram-negative species. Furthermore, it is clear that P. aeruginosa is much less sensitive to compound 5 and D2D in our time kill experiments, indicating that while the MIC is relative similar against all Gram-negative species tested (at 5 × 10⁵ CFU), growth conditions at higher cell densities in exponentially growing cultures may influence activity/sequestration. Our
data also indicate that small modifications to AMPs (Table 1) can have relatively large effect on species-specific activity as seen for *P. aeruginosa*. However, this is only indicative and it needs a more detailed analysis.

Figure 3. Time-Kill Kinetics. Compound 5 (C5) and D2D were evaluated for in vitro efficacy in time kill experiments. Exponentially growing cells (1 × 10⁸ cells) of ATCC strains *A. baumannii* 19606 (A), *E. coli* 25922 (B), *P. aeruginosa* 27853 (C) and *S. aureus* 29213 (D) were treated with C5 at 1 × MIC (– – – – ), 3 × MIC ( – – – ), 5 × MIC ( – – – – ) (MIC = 4 µg/mL), D2D at 5 × MIC ( – – – – ) and non-treated cells ( – – – ). Sampling of viable cells was performed at time points 0, 1, 3, 5 and 24 h (X-axis). Viable cell counts (CFU ml⁻¹, Y-axis), was done by spot plating of washed cells.

2.6. CD-Experiments

The solution structure of compounds 1–8 were compared with the D2D sequence by circular dichroism spectroscopy at 37 °C in a simple membrane-mimicking solvent mixture of water and trifluoroethanol (Figure 4). D2D and all eight analogs display far UV CD that can point toward folding with α-helical or possibly β-sheet secondary structure, but a detailed analysis is hindered due to the strong spectral contributions from the aromatic 1-naphthylalanine sidechains. The positive CD in the range from 200–240 nm and negative below 200 nm for 1–8 all is consistent with the expected mirror image spectra of an L-amino acid sequence with α-helical secondary structure [28]. The alanine scan has consequences for the UV absorption due to the single (in 3 and 6) or double presence of the chromophoric sidechain of naphthylalanine (in the rest) which displays an absorbance band centered at 223 nm (Figure 5). The far-UV CD spectra are also affected by these aromatic side-chains. This is mainly observed in the highest wavelength region, between 220–240 nm, where a negatively signed component is seen for peptides with two D-1-naphthylalanines present (Figure 4A), which is likely due an exciton coupling effect between the aromatic groups. The exception to this is observed for compound 5, which does not display a first negative band component, despite the sequence similarity to D2D (Figure 4B). This might be interpreted as a case where the positive peak of the peptide n-π
* transition eliminates a contribution from the π-1-naphthylalanines— or that these have a changed signature due to a difference in folding of 5 compared to e.g., D2D.

Figure 4. Far-UV circular dichroism spectra of D2D and 1–8 in a 50% trifluoroethanol water mixture at 37 °C. The left panel compares 1–8 (A) while the right panel compares 5 to D2D (B).

Figure 5. Far-UV absorption spectra for D2D and 1–8 in a 50% trifluoroethanol water mixture at 37 °C. as obtained from conversion of the high tension signal of the detector during measurement of CD. The absorbance peak with maximum at 223 nm originates in the naphthylalanine side chain, which is present at positions three and six (except in compound 3 and 6 where one such instance of 1-nal is replaced by ala).

2.7. Peptide Self-Assembly Nanostructure Investigated by Small Angle X-ray Scattering

The nanostructure of peptide D2D and compound 1–8 (alanine-scane) in solution was studied in detail using SAXS at ambient temperature. In Figure 6A the scattering curve from the lead compound D2D and the most active compound 5 is plotted together for comparison. As seen from the figure, there are significant differences in the structure of these two peptides. While the scattering curve for D2D can be recognized as the classical scattering pattern expected from random unstructured polymer-like chains, the results for compound 5 indicates formation of much larger self-assembled structures. Fit analysis of the data from compound 5 reveals that the scattering can be well explained using a fit model of defined hollow nano-tubes with an outer radius of 33 Å and a shell thickness of 4 Å. The structure is retained over the whole concentration range from 2.5–10 mg/mL (lower concentrations...
were not measured as the scattering signal to noise ratio is too low for the lab-SAXS instrument and the peptide too susceptible to radiation damage to be measured using synchrotron SAXS). Increasing the temperature to 37 °C and 45 °C did not dissolve the structure (see supplementary information Figure S1).

Further studies showed that compounds 1, 3, 4, 6 and 8 all resemble D2D as random polymer-like chains (see supplementary information, Figure S3), while compounds 2 and 7 assemble into larger filament like structures as seen in Figure 6B. The scattering curve for compound 2 can be explained by a nanotube structure where the cross section of the tubes is much smaller (14 Å) than for compound 5. The scattering could also be explained by a filament-like structure with dimensions 65x32x > 400 Å consisting of 85% water. Compound 7 on the other hand does not fit with a nano-tube model but has a higher slope at low q (power of 2) indicating sheet-formation. Due to the limited q range on the lab-source SAXS instrument a defined length cannot be directly accessed from the scattering curve.

The detector image (see supplementary information Figure S2) reveal that compound 2 filaments aligns parallel in the capillary resulting in anisotropic scattering (seen as asymmetric beam on the detector). The sample was measured for 14 h to see whether the injection into the capillary was the cause of the filament-ordering. However, the results revealed that the asymmetry of the scattering on the detector increased over time. This indicate that during the peptide injection the filaments are partly broken up, and reforms in the capillary over time seen as an increased asymmetric scattering pattern. However as seen in Figure S2 the scattering curve does not change over time indicating that the increase in length cannot be seen in the probed q-range (the “broken up” filaments at time = 0 are already above 400 Å).

Even though both compound 5 and 7 both have the same elongated structure these peptides do not align in the capillary during the measurements in the same way. This effect might be explained by increased length of the tubes or mechanical stiffness for compound 2 compared with compound 5 and 7. However, as the exact length of these peptides is not visible in the available q-range and the mechanical properties have not been studied, this remains inconclusive.

2.8. Lipid Interaction Probed by Small Angle X-ray Scattering

As bacterial membrane destruction is reported as one of the most important modes of actions of antimicrobial peptides we mixed the most active compound 5 and the lead compound D2D with lipid
vesicles mimicking membranes of bacterial and measured the samples with synchrotron-SAXS (see Figure 7A,B).

As bacterial membrane destruction is reported as one of the most important modes of actions of antimicrobial peptides we mixed the most active compound and the lead compound with lipid vesicles mimicking membranes of bacterial and measured the samples with synchrotron-SAXS (see Figure 7A,B).

**Figure 7.** Lipid-peptide interaction of D2D and 5 studied using synchrotron SAXS, and the resulting lipid bilayer structure (of DMPC-DMPG-DMPE-PEG bilayers) as obtained from detailed mathematical modeling of the scattering curves. Scattering of compound D2D (A) and compound 5 (B) mixed with lipids in ration 1:5, 1:10 and 1:20 together with the model fit curves in red. The curves have been offset with a factor of 100 for better visualization. Based on the fit analysis volume probability plots have been calculated for D2D (C) and compound 5 (D) showing that a higher amount of compound 5 inserts into the membrane than D2D. Both peptides seem to position in the interface between the headgroups and tail region of the outer leaflet of the lipid bilayer.

The results were analysed using a detailed scattering model that allow extraction of peptide position in the bilayer as well as the peptide effect on the structure and thickness of the lipid bilayer [29]. From the fit parameters (see supplementary information Table S2) a volume probability plot showing the structure of the bilayer after peptide insertion can be calculated, and has been plotted in Figure 7C for peptide D2D and Figure 7D for compound 5. As seen from the plot a higher amount of compound 5
(−50–60%) inserts into the bilayer compared to D2D (−30–50%). This is seen directly from the scattering curve in Figure 7A,B by a smaller shift in the first minima at intermediate q for D2D. When comparing to previous results found for natural unstructured peptides like indolicidin, where ~75–100% of the peptide has been found to insert into bilayers of the same lipid composition [29], the synthetic n-peptides in this study have a slightly lower affinity for the membrane.

Further D2D does not seem to affect the membrane structure in any significant way at this concentration, even at higher ratios where only minor reductions of the bilayer thickness of about 1 Å was found which is within statistical errors. These results indicate that there is no evidence for membrane deformation or any particular indication of pore formation. However peptide membrane insertion has been suggested to cause lipid disordering resulting increased leakage of cell fluids [30].

3. Materials and Methods

TentaGel R RAM (0.19 mmol/g) were purchased from Rapp Polymere GmbH. TFA (trifluoroacetic acid) and piperidine were purchased from Iris-Biotech GmbH. Fmoc (9-fluorenylmethyloxycarbonyl) protected amino acids were purchased from Sigma-Aldrich, Iris-Biotech GmbH, Novabiochem and Alfa Aesar. Disposable reactors (5 mL polypropylene) fitted with a PTFE filter were acquired from Fa. Gerhardt, Kassel Germany. N,N-diisopropylethylamine (DIEA), triisopropylsilane (TIS), Mueller-Hinton Broth II (MHBII), Phosphate buffered saline (PBS tablets) and α-cyano-4-Hydroxycinnamic acid (ACCA) were from Sigma-Aldrich. 1-Hydroxy-7-azabenzotriazole (HOAt) and (1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) were from GL Biochem Shanghai. Melittin were from Serva. DMF (dimethylformamide), DCM (dichloromethane), ACN (acetonitrile) and Et₂O (diethyl ether) were from VWR, Pennsylvania, USA. All reagents and solvents were used without further purification. Biorad Microseal film, CAPP Origami reagent reservoir, clear V and U-shaped 96-well polypropylene plate, clear flat bottom 96-well polystyrene ELISA plate, Eppendorf Protein LoBind tubes (2 mL).

3.1. Synthesis of Peptides

The manual Fmoc solid-phase synthesis of peptides was carried out in disposable syringes, equipped with a fritted filter [31]. Crude products were purified by preparative RP-HPLC until ≥95% purity was obtained (analytical RP-HPLC). HPLC system consisting of WatersTM 600 Pump, In-line Degasser, 600 Controller and 2996 Photodiode Array Detector, the column used was a WatersTM XBridgeTM BEH130 C18, 5 µm, 10 × 250 mm with H₂O:ACN gradient. The appropriate fractions were concentrated and lyophilized. Purity was determined by analytical reverse-phase HPLC system consisting of WatersTM 717 plus Autosampler, In-line Degasser AF, 600 Controller and 2996 Photodiode Array Detector, the column used was a WatersTM SymmetryTM C18, 5 µm, 4.6 × 250 mm on an acetonitrile-water gradient. Finally, the products were characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Bruker MicroflexTM), using α-cyano-4-hydroxycinnamic acid as matrix.

3.2. Bacterial Strains

E. coli (ATCC 25922), S. aureus (ATCC 29213), P. aeruginosa (ATCC 27853) and A. baumannii (ATCC 19606).

3.3. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed by broth dilution in MHB-II media using a protocol adapted from [32], with minor modifications. Briefly, the compounds were prepared in standard stocks of 1 mg/mL in milli-Q H₂O. Antimicrobial dilutions were prepared in two times the final concentration (128 μg/mL) of the compounds. Bacterial suspensions were prepared from overnight cultures (MHB-II) by diluting to OD₆₀₀ = 0.001 (Approximately 1 × 10⁶ CFU). An amount of 100 µL MHB-II was transferred to all wells in column 1–9, excluding the antibiotics well (A1–A9).
An amount of 200 µL of MHB-II was transferred to all wells in column 10–12. An amount of 200 µL peptide stock was transferred to each well from A1–9. A 2-fold serial dilution from row A–G was obtained by transferring 100 µL from row A to B, and continued until row G. Resulting in final concentration range of peptides: 128, 64, 32, 16, 8, 4, and 2 (µg/mL). Finally, 100 µL bacterial suspension (1 × 10^6 CFU) were added to all wells giving a final bacterial inoculum of approximately 5 × 10^5 colony forming units (CFU). The plates were incubated for 18–20 h at 37 °C.

3.4. Time Kill Curves

The time kill experiments were performed by treating exponentially growing cultures with D2D and compound 5. Overnight cultures grown in MHB-II media, were diluted 1:10,000 to ensure exponentially growing cultures (8–10 generations), before cultures reached 1 × 10^8 CFU approximately OD600 = 0.1. When cultures reached 1 × 10^8 they were split into blood glass tubes containing 3 mL each and then treated with the respective drugs at the concentrations as shown in Figure 3. Sampling was performed at 0, 1, 3, 5 and 24 h, by removing 250 µL culture. Samples were spun down at 4000 G for 5 min and supernatant removed, cells were washed in 1 mL 0.9% NaCl, spun down again, before being suspended in 250 µL 0.9% NaCl. Finally, 10 fold dilution series were prepared in 0.9% NaCl. From these 10 µL were spotted (in triplicate) onto LB agar. CFU were counted after 24 h incubation at 37 °C. Experiments were performed in triplicate from individual colonies. Data analysis was performed using GraphPad prism 5 and plotted as the mean of triplicated with standard deviation.

3.5. Hemolytic Activity

The percentage of hemolysis at 150 µM was determined for all compounds as previously described [33]. Briefly, two-fold serial dilutions (2.35 to 150 µM) of compounds in PBS were mixed with a 0.5% v/v suspension of fresh human red blood cells (RBC) in the same buffer. After 1 h incubation at 37 °C, plates were centrifuged and aliquots of the supernatants were transferred to clear ELISA plates. Absorbance at 414 nm was determined and normalised with respect to a negative (PBS, 0%) and positive (melittin, 100%) control.

3.6. Circular Dichroism

Circular dichroism (CD) spectra were measured on a Jasco J-815 spectropolarimeter in the far-UV region from 260–190 nm with a 1 nm interval, a 2 nm bandwidth using a digital integration time of 4 s and a scan speed of 20 nm per min. During measurement, the high-tension signal applied to the detector was also recorded and was subsequently converted to absorbance. Data represents the average of five individual scans subtracted a corresponding reference measurement on pure solvent. The peptide samples D2D and 1–8 were dissolved in milli-Q water with 50% (v/v) 2,2,2-trifluoroethanol (TFE). Each sample was placed in a 2 mm quartz cuvette from Hellma. The temperature was kept at 37 °C during measurements using a Jasco CDF-426S thermostated sample holder accessory. Data was treated using Jasco Spectra Analysis and plotted using MicroCal OriginPro 2018. No smoothing has been applied. The quantitative interpretation of secondary structure for the studied peptides is hampered by the spectral contributions of the aromatic sidechains and the analysis has thus centered on a comparative study of the band shape and sign and the Y-axis unit is reported as the measured elipticity signal in mdeg.

3.7. Small Angle X-ray Scattering on Pure Peptides in Solution

SAXS experiments of D2D and the alanine-scan (compounds 1–8) were performed using a Bruker NANOSTAR equipped with a microfocus X-ray source (IμS Cu, InCoatec, Germany) and a VÅNTEC-2000 detector. Raw scattering data was calibrated to absolute intensity scale using water as a primary standard and radially averaged in order to obtain the 1D scattered intensity profile as a function of the scattering vector \( q = 4 \pi \sin (\theta/2)/\lambda \), where \( \theta \) is the scattering angle and \( \lambda \) is the X-ray wavelength of 1.54 Å. The samples were dissolved in 50 mM Tris buffer, pH 7.4 (10 mg/mL, 5 mg/mL
and 2.5 mg/mL) and injected manually into a temperature-controlled quartz cell located in a vacuum chamber and measured for 180 min.

Random polymer-like chains:
The scattering of the peptide D2D was analysed using a random polymer-like chain model:

$$I_{\text{chain}}(q) = \phi \cdot V_p \cdot \Delta \rho^2 \cdot P_{\text{chain}}(q)$$  \hspace{1cm} (1)

where $\phi$ is the volume fraction of the peptide, $V_p$ is the volume of the polymer, $\Delta \rho$ is the excess scattering length density and $P_{\text{chain}}(q)$ is the form factor of the free peptide chains given by the Debye expression for Gaussian chains:

$$g_{P_{\text{chain}}}(q) = \frac{2 \exp[-(qR_g)^2]}{(qR_g)^4}$$  \hspace{1cm} (2)

where $R_g$ is the radius gyration of the peptide chains.

Filament-like sheet model:
The scattering for compounds 2 and 7 was analysed using a rectangular sheet-like model:

$$I_{\text{sheet}}(q) = \phi \cdot V_p \cdot \Delta \rho^2 \cdot N_p \cdot \bar{P}_{\text{sheet}}(q)$$  \hspace{1cm} (3)

Under the assumption that the length of the peptide sheets are much greater than the lateral dimension, i.e., $c \gg a, b$, the form factor $\bar{P}_{\text{sheet}}(q)$ is given by

$$\bar{P}_{\text{sheet}}(q) = F_c(q) \int_0^{2\pi} A_{\text{sheet}}(q, \alpha)^2 d\alpha$$  \hspace{1cm} (4)

where the amplitude is given by

$$A_{\text{sheet}}(q, \alpha) = \frac{\sin(qb \cos(\alpha)/2)}{qb \cos(\alpha)/2} \cdot \frac{\sin(qa \sin(\alpha)/2)}{qa \sin(\alpha)/2}$$  \hspace{1cm} (5)

and

$$F_c(q) = \frac{2 \text{Si}(qc)/(qc) - 4 \sin^2(qc/2)/(qc)^2}{2 \text{Si}(qc)/(qc) - 4 \sin^2(qc/2)/(qc)^2}$$  \hspace{1cm} (6)

where $\text{Si}(x) = \int_0^x t^{-1} \sin t \, dt$.

Nanotube model:
The expression for the absolute scattering intensity, from this structure can be written as [34]:

$$I_{\text{nanotube}}(q) = \phi \cdot P \cdot V_{\text{tot}} \cdot \Delta \rho^2 \cdot P(q) \cdot A(q)^2 \cdot \bar{D}W(q)$$  \hspace{1cm} (7)

where $\phi$ is the volume fraction of the peptide, $P$ is the aggregation number given by $P = \pi (R_o^2 - R_i^2) L / V_p$, where $V_p$ is the volume of a peptide chain, $R_o$ is the outer radius and $R_i$ is the inner radius of the tubes, and $L$ is the length. $A(q)^2$ is given by:

$$A(q)^2 = \frac{R_o^2 A(q, R_e) - R_i^2 A(q, R_i)}{(R_o^2 - R_i^2)}$$  \hspace{1cm} (8)

where $A(q, x) = 2 J_1(q x) / (q x)$ and $J_1(x)$ is the first order Bessel function.

The longitudinal form factor $P(q)_L$ is given by:

$$P(q)_L = \frac{2 \text{Si}(Q L) / Q L - 4 \sin^2(Q L/2) / (Q L)^2}{2 \text{Si}(Q L) / Q L - 4 \sin^2(Q L/2) / (Q L)^2}$$  \hspace{1cm} (9)
where $L$ is the length of the nanotubes and $\text{Si}(x)$ is the Sine-function.

In addition, we introduced a slight distribution in the inner radius for possible shape fluctuations, and a Debye Waller factor, $\text{DW}(q) = \exp(-q^2 \sigma^2)$, describing possible surface roughness of the inner and outer wall.

Synchrotron-small angle x-ray scattering on peptide-lipid interaction.

For preparation of unilamellar lipid vesicles, synthetic DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine), DMPG (1,2-dimyristoyl-sn-glycero-3-phospho-(10-rac-glycerol)), and DMPE-PEG (1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-$N$-[methoxy(polyethylene glycol)-2000]) (from Avanti Polar Lipids) were used in the molar ratio 9:7.5:2.5. The lipids were first dissolved in a 1:3 methanol:chloroform solution, and then the organic solvents were removed completely under vacuum using a Heidolph rotary evaporator with a Vacuubrand vacuum pump. The resulting lipid film was hydrated with 50 mM Tris buffer, pH 7.4, for at least one hour at 35°C. After sonication for 10 min, the lipid dispersions were extruded through a 100 nm pore diameter polycarbonate filter (21 times) using an Avanti mini-extruder fitted with two 1 mL airtight syringes. Immediately before the SAXS experiment, a peptide solution with the adequate concentration for the target lipid:peptide ratio was mixed 1:1 with the lipid solution (1:1) using a micropipette.

SAXS experiments of the peptide-lipid mixes were performed at the automated BM29 bioSAXS beamline at the European Synchrotron Radiation Facility (ESRF) in Grenoble, France [35]. The data was obtained using an energy of 12.5 keV and a detector distance of 2.87 m, covering a $q$ range of about $0.0047 \text{ Å}^{-1}$ to $0.5 \text{ Å}^{-1}$. The data set was calibrated to an absolute intensity scale using water as a primary standard. 40 µL samples were run through a capillary using the flow mode of the automated sample changer [36]. SAXS data was collected in ten successive frames of 0.5 s each to monitor radiation damage and the data reduction was done using the standard tool at BM29 [37].

The SAXS results of the lipid-peptide mixes were analysed using the theoretical model described in detail in [29]. In short, the model provides a detailed description of the membrane by dividing into probability functions for each component (lipid sub-units/peptide) across the bilayer.

4. Conclusions

We presented a structure-activity relationship study of the small antimicrobial all-$d$ octapeptide amide $H$-kk(1-nal)fk(1-nal)k(nle)-NH$_2$ (D2D) based on 36 analogues, which reveals several interesting results. $d$-alanine-scanning of D2D revealed that the hydrophobic residues in position 3 (1-nal), 4 (phe), 6 (1-nal) and 8 (nle) are important for antimicrobial activity. Furthermore, lysine could be replaced by alanine in either position 1, 2, 5 or 7 without losing antimicrobial activity. Varying hydrophobicity at position 5 (lys) or 7 (lys) didn’t lead to overall more potent and less toxic compounds. Exchanging 1-nal$^6$ with lys$^7$ and lys$^7$ with nle$^8$ led to loss of activity. The N- and C-terminus truncations indicated that the full octapeptide is required for as potent activity as D2D, however [des k$^1$]-D2D retained some antibacterial activity. Substitutions with arginine did not lead to overall more potent and less toxic compounds.

Our best candidate 5, showed MICs of 4 µg/mL against A. baumannii, E. coli, P. aeruginosa and S. aureus with a hemolytic activity of 47 % against red blood cells. Time-kill kinetics showed that compound 5 kill bacteria in a concentration-dependent manner. The peptide showed a $>3$-log reduction in CFU/mL except for P. aeruginosa.

Analysis of CD spectra of D2D and compounds 1–8 were complicated by spectral contributions from naphthylalanine in position 3 and 6. The far-UV band shape could be consistent with $\alpha$-helical secondary structure for D2D and 1–8 in a trifluoroethanol-containing aqueous solvent.

Small angle X-ray scattering (SAXS) experiments showed that D2D and compound 1, 3, 4, 6 and 8 can be described as random unstructured polymer-like chains model. Compound 5 can be described with a nanotube structure model. Compound 2 can be described both with a nanotube structure and a filament-like structure model, while compound 7 only can be explained by larger filament-like structures.
Lipid interaction probed by SAXS showed that a higher amount of compound 5 (~50–60%) inserts into the bilayer compared to D2D (~30–50%).

D2D still remains the lead compound, however compound 5 is also an interesting antimicrobial peptide that needs further investigation mainly due to its formation of nanotube structures and minor increase in antimicrobial activity compared to D2D.

Supplementary Materials: The following are available online, Peptide structures, Analytical HPLC chromatograms, MALDI-TOF-MS spectra. Figure S1: SAXS data for peptide 5; Figure S2: SAXS data for peptide 2; Figure S3: SAXS data for peptide 1, 3, 4, 6 and 8. Table S1: Peptide mass, HPLC retention time and purity; Table S2: Important fit parameters from the analysis of liposomes-peptide mixes


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Conflicts of Interest: The authors declare no conflict of interest.

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**Sample Availability:** Not available.

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