Bioorganic & Medicinal Chemistry xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Simplified lipid II-binding antimicrobial peptides: Design, synthesis and antimicrobial activity of bioconjugates of nisin rings A and B with pore-forming peptides

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ARTICLE INFO

Keywords:
Antimicrobial peptide
Lantibiotics
Bioconjugates
Cationic peptide
Peptoid

ABSTRACT

New designs of antimicrobial peptides are urgently needed in order to combat the threat posed by the recent increase of resistance to antibiotics. In this paper, we present a new series of antimicrobial peptides, based on the key structural features of the lantibiotic nisin. We have simplified the structure of nisin by conjugating the lipid II-binding motif at the *N*-terminus of nisin to a series of cationic peptides and peptoids with known antibacterial action and pore-forming properties. Hybrid peptides, where a hydrophilic PEG4 linker was used, showed good antibacterial activity against *Micrococcus luteus*.

1. Introduction

The rise and spread of resistance to antibiotics and other antimicrobials is well established as one of the greatest threats to modern health. The last few years have seen a rise in bacterial infections that are resistant to even last line of defence antibiotics such as vancomycin and carbapenems. Thus, the bleak prospect of the occurrence of bacterial infections that are resistant to every available therapeutic may soon become a reality.[1] One of the tools that is essential to tackling resistance is the discovery and development of new antimicrobials with novel mechanisms of action. Several drug discovery initiatives have been launched for the development of antibiotics, on both a national and international level,[1,2] but in spite of this the number of novel drugs reaching the market each year is decreasing. The pressure for new antibiotics is driving a resurgence of interest in natural products[3] and the re-evaluation of existing classes of compounds.[1] Antimicrobial peptides are one class of natural products that are seeing an increase in interest from pharmaceutical research and development. Many peptides are found to have potent antibacterial activity and a low propensity to select for resistance but they are held back from clinical use by their poor drug-like properties. New developments in peptide chemistry and design are enabling the discovery of a new generation of peptide therapeutics that can be selective, non-toxic and orally bioavailable.[4]

The lantibiotics are a group of antimicrobial peptides that have received increasing attention over the last decade. They are ribosomally synthesised by a wide range of bacteria and have notable structural complexity, owing in part to the extensive range of post-translational modifications that occur during the processing of the prepeptide. The key structural feature of the lantibiotics is the incorporation one or more of the uncommon amino acids, lanthionine (Lan) and methyllanthionine (MeLan) (Fig. 1), which give rise to peptides with multiple, often overlapping, cyclic motifs.[5] A significant number of lantibiotics are reported to have excellent antibacterial activity, notably against multi-drug resistant strains of bacteria, but their development as drugs has been held back by poor drug-likeness and a lack of synthetic methods available to generate or modify their complex structures. The poor drug-likeness of the lantibiotics can be attributed mostly to their chemical instability: sulfur-containing lanthionine residues are prone to oxidation; and unreacted dehydro residues can be easily cleaved in basic conditions, attacked by nucleophiles or undergo hydrolysis in strongly acidic conditions.[6,7] Solubility can also be a problem; notably, nisin is poorly soluble at neutral or high pH[8] and whilst the thioether bridges of lantibiotics provide some protection to proteolysis and thermal degradation[9] they are still prone to enzymatic degradation in the gastrointestinal tract and in the bloodstream.[10] Therefore the current generation of lantibiotics are unlikely to be orally bioavailable. However, as the pressure for new antimicrobials mounts, there are now a small handful of lantibiotics undergoing clinical trials. For example, NVB302 has completed Phase 1 clinical trials and MU1140 is in preclinical development, both for treatment of *C. difficile* infections.[11]

Nisin is the most widely studied member of the lantibiotics (Fig. 1). It has broad spectrum antimicrobial activity, notably against

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https://doi.org/10.1016/j.bmc.2018.10.015
Received 22 July 2018; Received in revised form 16 October 2018; Accepted 18 October 2018

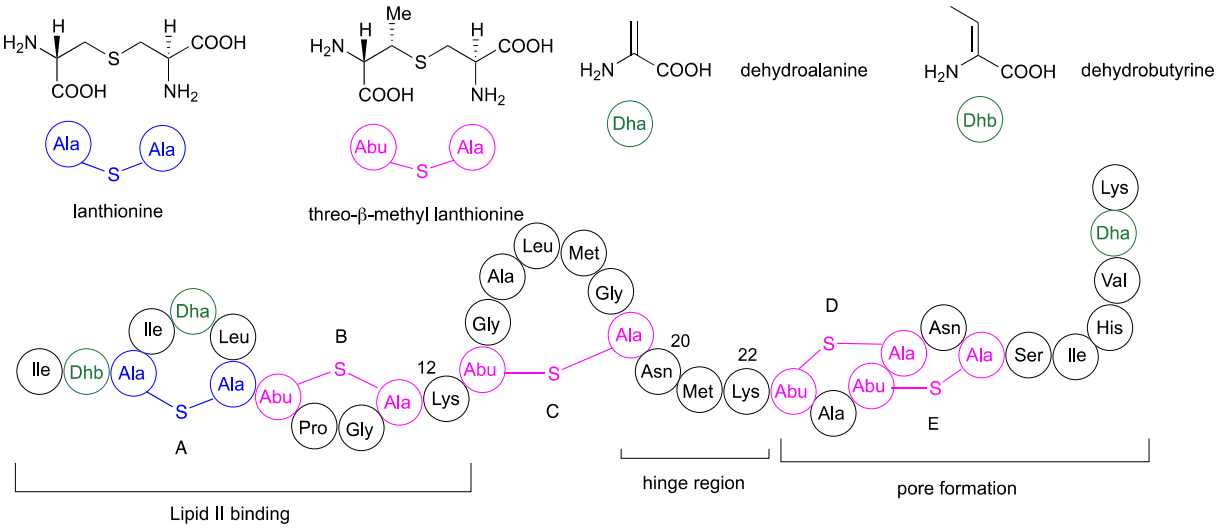


Fig. 1. Structure of nisin Z, highlighting the residues involved in key aspects of the antimicrobial activity.

methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant enterococci (VRE). Despite widespread use as a food preservative[12] over a period of decades few nisin-resistant strains have been reported (via mechanisms such as nisin-degrading enzymes and modification of the cell wall[13]). It binds with high selectivity to lipid II, a key biosynthetic precursor of the bacterial cell wall, with the resulting 8:4 nisin:lipid II complex leading to the formation of pores in the bacterial membrane.[14,15] NMR studies have revealed key interactions between rings A and B of nisin and the pyrophosphate moiety of lipid II.[16] Although the nisin(1–12) fragment, which just comprises rings A and B, has little or no antibacterial activity,[17,18] it is able to antagonise the activity of full-length nisin, suggesting that this fragment recognises and maintains affinity for lipid II.[17] After binding to lipid II, the C-terminal moiety, nisin(23–33), comprising the DE rings, inserts into the bacterial membrane to form the pore structure.[19] The structural role of ring C and the "hinge region" (nisin(20–22)) is not completely understood, but are clearly important for the activity of nisin. Flexibility in this region is one key factor. Mutagenesis studies have shown reduced activity when Pro residues are introduced at positions 20 and 21;[20] although binding to lipid II is still possible, the assembly and membrane insertion of the nisin:lipid II pore complex is prevented by these mutations.[19] In support of this, screening of a library of mutants of all three hinge positions identified sequences such as Ala-Ala-Ala and Ala-Ala-Ser, with small, chiral amino acids, as leading to improved activity.[21] Shorter hinge region mutants showed greatly reduced activity but mutants with two extra amino acids were well tolerated,[22] and mutations in this region were also found to increase activity against significant pathogens such as MRSA and VRE.[23]

The total synthesis of nisin using a segment synthesis approach[24] and the solid-phase peptide synthesis of fragments and analogues of nisin[25–30] as well as of other lantibiotics[31,32] has been reported. Nevertheless, despite the many advances in synthetic methodology reported over the past few decades[33] neither synthetic methodology nor bioengineering approaches have yet developed enough to enable the rapid generation of analogues for antimicrobial screening[34,35] although a range of synthetic analogues of lantibiotics are providing insights into the structural requirements for antibacterial activity and into the mode of action.[36] A semisynthetic approach to lantibiotic design and synthesis, using simpler and more accessible building blocks designed to mimic the key functions of nisin (lipid II binding, flexible hinge and membrane anchoring/pore formation) may be an effective alternative, and may also afford lead structures with better chemical and enzymatic stability, as well as improved pharmacokinetic properties. Several groups have recently reported hybrid bioconjugates with the lipid II binding motif of nisin modified with lipids,[18] vancomycin,[37] magainin,[38] and a cross-stapled synthetic nisin D/E fragment,[39] as well as engineered nisin analogues with antimicrobial peptide sequences fused to the lipid II binding motif.[40] Most of these bioconjugates and engineered analogues had slightly lower antibacterial action than wild type nisin, with some losing the potential for pore formation.[18,37,40] However, promising activities against Gram-negative bacteria,[40] and improved activities compared to nisin in clinically relevant resistant bacteria[18] have been reported. In this work, we aimed to prepare simplified semisynthetic lantibiotics, where nisin(1–12) is conjugated to known pore-forming peptides, mimicking the role of the complex nisin D/E moiety. We have also investigated different synthetic linkers between the pore-forming peptides and nisin(1–12), in order to elucidate whether the hinge region can be drastically simplified in this manner, and whether hydrophobic or hydrophilic linkers are preferred.

2. Results and discussion

2.1. Design of nisin(1–12)-pore forming peptide hybrids

We aimed to prepare simplified semisynthetic lantibiotics, preserving the activity and functionality of nisin, but with improved drug-like properties and via an accessible synthetic route. By replacing the C-terminal rings C, D and E, we removed many of the synthetically challenging lanthionine residues and the unstable dehydro residues (Fig. 2). One peptide/peptoid hybrid was also designed to minimize or eliminate amino acid sequences that would be prone to enzymatic degradation. The peptides were also designed with cationic sequences to mimic the polarity of nisin and enhance aqueous solubility.

For the lipid II recognition moiety, we used the wild type nisin (1–12) (A + B) **1**, modified with an alkyne "handle" to allow rapid and clean attachment to a spacer unit (mimicking the hinge region) and to the pore forming peptide via click chemistry. As well as the mild and biocompatible reaction conditions used for click chemistry, we also envisaged that the resulting triazole moiety would be a good amide bond mimic and would confer additional stability and resistance to proteases on the resulting semisynthetic lantibiotic.[41] As mimics of the pore-forming region of nisin, we used two peptide sequences with well-characterised antimicrobial and structural properties.

The mastoparan analogue Polybia-MP1 **2** has been shown to have antimicrobial activity against both Gram positive and Gram negative bacteria,[42] and the amphipathic α-helical structure has been demonstrated to form pore structures, depending on the lipid bilayer composition.[43] The cationic peptide Pexiganan **3** was developed through

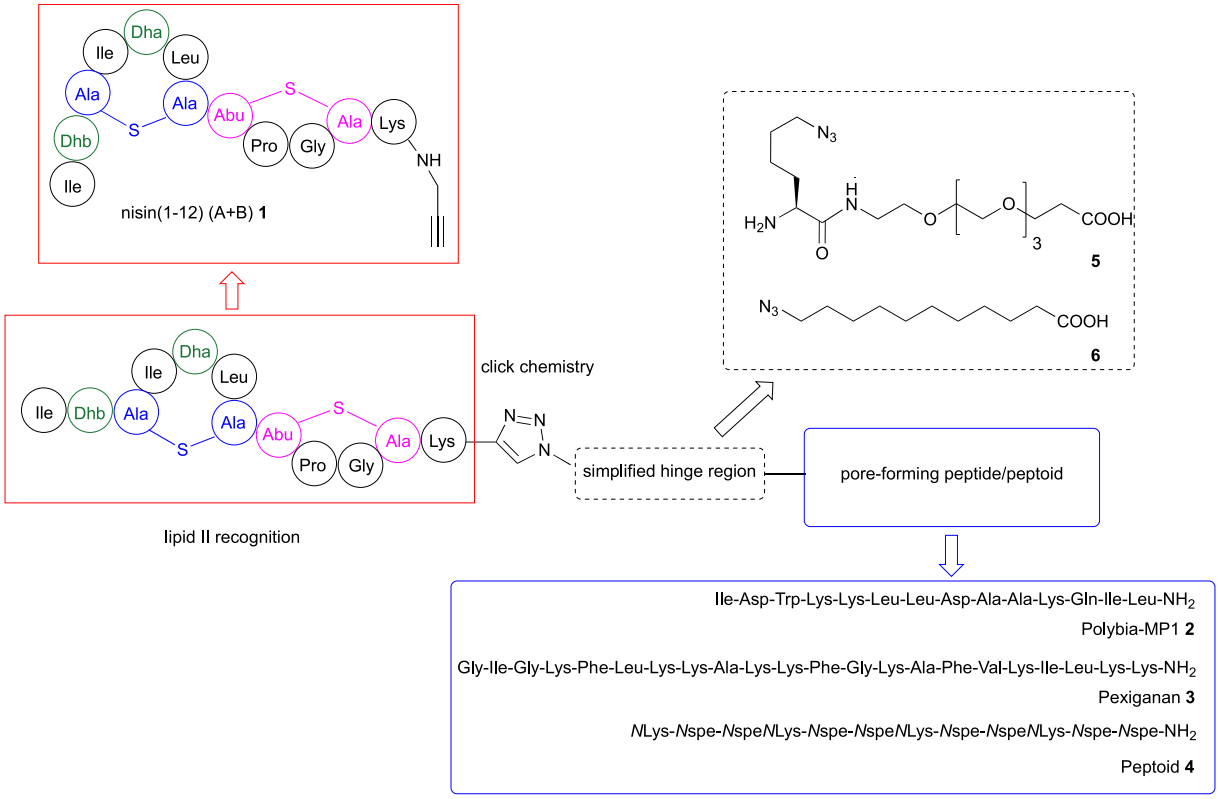


Fig. 2. Modular design of simplified semisynthetic lantibiotics based on nisin.

structure-activity studies of magainin, a well-studied model antimicrobial peptide. It has broad spectrum activity against both Gram positive and Gram negative bacteria,[44] and NMR studies have shown that it adopts a dimeric antiparallel coiled coil structure on binding to lipid bilayers[45] and has the ability to form pores in a toroidal like mechanism.[46] In a bid to further minimize the protease susceptibility of these semisynthetic lantibiotics, we also investigated a peptoid mimic[47] of magainin (**4**) reported to have similar antibacterial activity to pexiganan.[48] For the hinge mimics, we investigated the PEG linker **5**, as PEG is commonly incorporated into peptides to aid solubility, maintain flexibility and reduce immunogenicity.[49] Inspired by the good lipid II binding activity of the recently reported nisin(1–12)-lipid conjugates,[18] we also studied the effects of incorporation of the alkyl spacer **6** as a hinge region substitute.

2.2. Preparation of nisin(1–12) (A + B) 1 for click chemistry

Nisin is susceptible to digestion at Lys(12) by trypsin,[17] facilitating the preparation of the wt lipid II binding moiety. Commercially available preparations typically contain 75% NaCl, 22.5% dairy proteins and only 2.5% of nisin, however Liskamp and co-workers have recently published reliable procedures for the scalable purification of nisin followed by trypsin digestion to give nisin(1–12).[50] Procedures for the conversion of nisin fragments to propargyl amides for click chemistry have also recently been reported.[18,39,51] We therefore carried out the enrichment, purification and trypsin digest as previously described[50] to afford nisin(1–12), which was then coupled to propargylamine to give nisin(1–12) (A + B) **1** (Scheme 1).[18]

2.3. Synthesis of pore-forming peptides and peptoids with hydrophilic and hydrophobic linkers

The tail peptide pexiganan **3** was synthesised via standard Fmoc SPPS methodology, using Rink amide resin.[52] Side-chain deprotection and cleavage of the resin-bound intermediate **7** from the resin (Scheme 2) afforded the desired C-terminal amide peptide **3**, which was used as a control in subsequent biological experiments. To install the PEG hinge mimic **5**, commercially available Fmoc-NH-$(PEG)_4$-COOH was coupled to **7**, followed by Fmoc-Lys(N_3)-OH: deprotection and cleavage from the resin then gave Lys(N_3)-PEG4-pexiganan **8**.

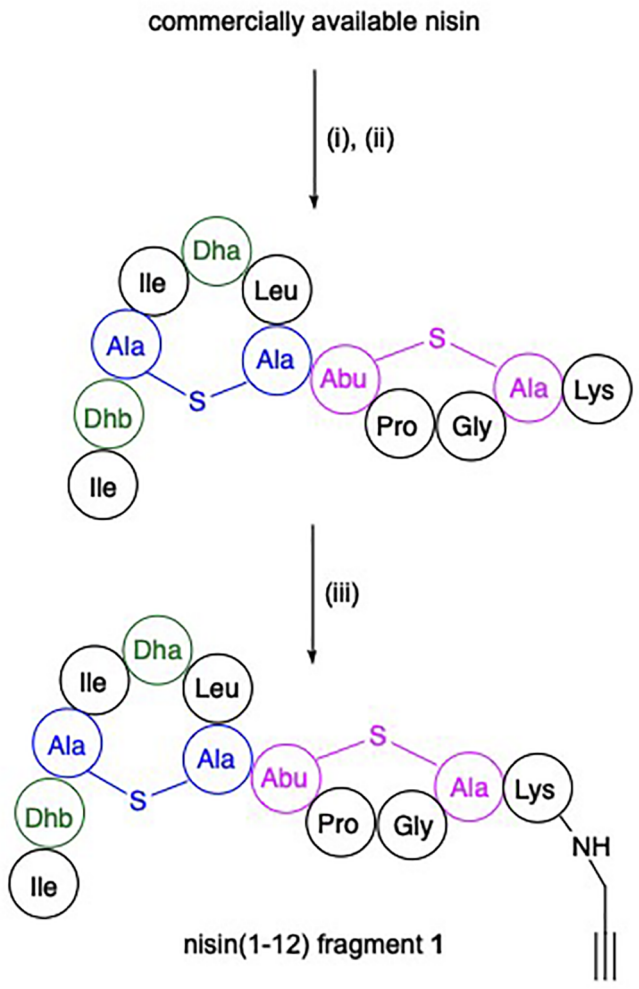
For the Polybia-MP1 based peptides, resin-bound intermediate **9** was also synthesised via standard Fmoc SPPS methodology. Cleavage from the resin gave the desired Polybia-MP1 **2** to be used as a control. A similar synthetic sequence to that described above afforded Lys(N_3)-PEG4-Polybia-MP1 **10** (Scheme 3). To install the hydrophobic linker, 11-azidoundecanoic acid **6** (prepared from the reaction of 11-bromoundecanoic acid with sodium azide) was coupled to **9**, followed by deprotection and cleavage from the resin, to give N_3-C11-Polybia-MP1 **11**.

The peptoid mimic of magainin (**4**) was synthesised on Rink amide resin via the previously reported procedure,[53] using the amine monomers (*R*)-(+)-α-methylbenzylamine (MBA) and *N*-Boc-1,4-butanediamine (Scheme 4). Resin-bound intermediate **12** was also coupled to 11-azidoundecanoic acid **6** to give N_3-C11-peptoid **13**.

2.4. Semisynthetic lantibiotics prepared via click chemistry

With the linker-peptide and linker-peptoid conjugates, and the nisin (1–12) (A + B) **1** in hand, click chemistry was employed to form the semisynthetic lantibiotics. Initially, copper-catalysed click chemistry using conditions similar to those previously reported (2.4 equiv $CuSO_4{\cdot}5H_2O$ and 4.8 equiv sodium ascorbate)[37] was used to couple Lys(N_3)-PEG4-pexiganan **8** to nisin(1–12) (A + B) **1** to afford the semisynthetic lantibiotic **14** (Table 1).

Whilst this procedure could also be used to conjugate N_3-C11-Polybia-MP1 **11** to nisin(1–12) (A + B) **1** to give **16**, in our hands this approach proved capricious and the remaining peptides **15** and **17** could not be synthesised at all using this approach. However, an



Scheme 1. Preparation of nisin(1–12) (A + B) **1**. (i) H_2O/CH_2Cl_2, centrifugation, filtration; (ii) trypsin, Tris buffer, 40 h (11%); (iii) propargylamine (51 equiv.), BOP, DIPEA, 20 min (61%).

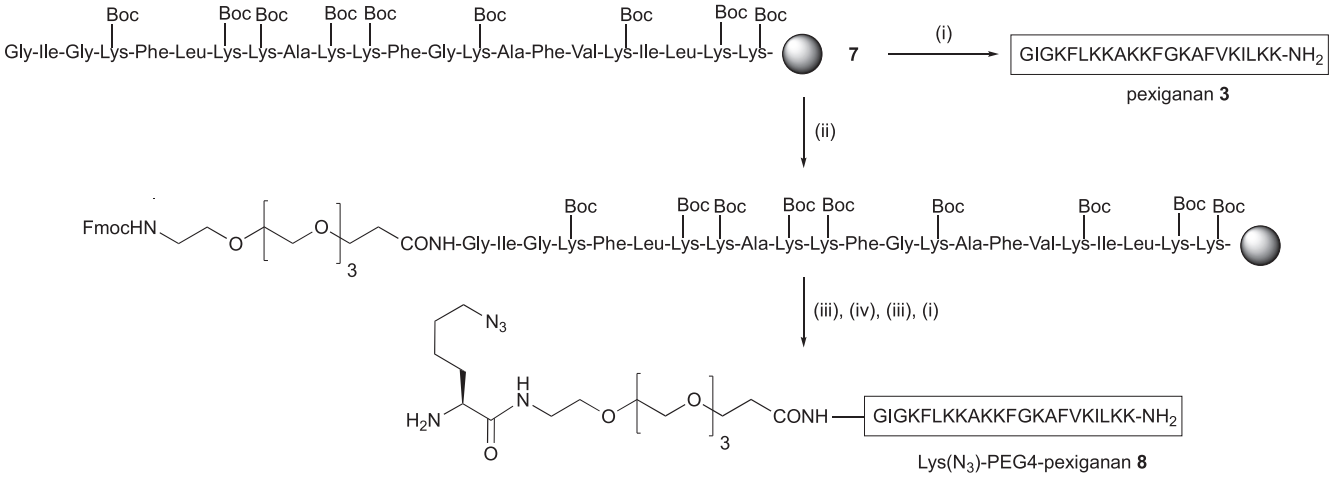
improved procedure utilizing addition of the polytriazole ligand tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA),[17] previously reported to improve copper-catalysed click reactions by stabilising the Cu^I species,[54] enabled all four of the semisynthetic lantibiotics to be successfully synthesised (Scheme 5).

2.5. In vitro biological testing

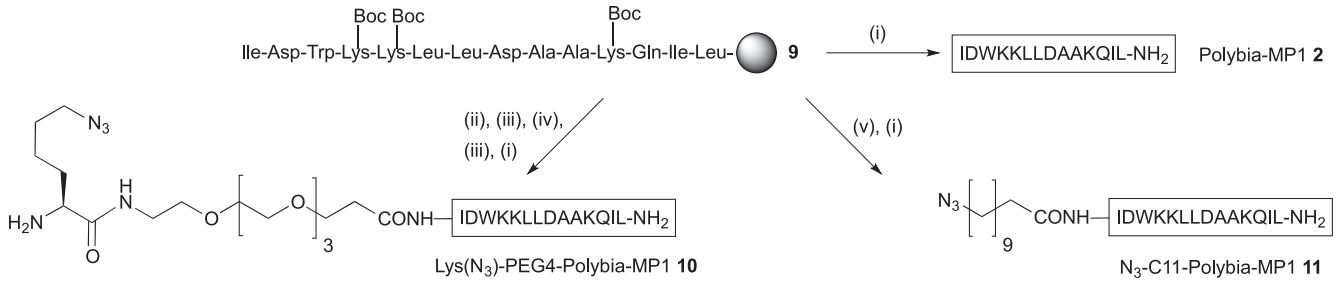
In order to quantify the biological activity of the semisynthetic lantibiotics, two *in vitro* screening methods, agar well diffusion and growth inhibition in broth, were employed.[55] In the initial round of testing, the agar well diffusion method was used for the Polybia-MP1 hybrid peptides **15** and **16**. Four different strains of bacteria were tested, two indicator strains of Gram positive bacteria, *Micrococcus luteus* and *Bacillus subtilis* and two strains of Gram negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa*. A number of controls were also used: kanamycin; Polybia-MP1 **2**; wild type nisin(1–12) (A + B) **1**; and full length nisin. Inhibition zones were visible for all hybrid peptides and controls against *M. luteus*. All peptides performed worse against the Gram-negative bacteria with only kanamycin showing any inhibition against *E. coli* and *P. aeruginosa*. Whilst previous reports suggested that pexiganan **3**[44] and Polybia-MP1 **2**[42] would have significant activity against both of these Gram-negative bacteria, in these papers the microbiological testing was carried out using the broth dilution assay. We hypothesise that the agar well diffusion assay may not be appropriate for peptides such as pexiganan and Polybia-MP1, as they are rich in Lys residues which may bind to the agar and prevent interactions with the bacteria.

To determine the MIC of the hybrid peptides and to compare their activities more accurately, further testing was carried out on *M. luteus* as this appeared to be the most sensitive strain of Gram positive bacteria. The broth micro-dilution method was used as this consumed the minimum amount of material. Peptides (A + B)-Lys-PEG4-pexiganan **14**, (A + B)-Lys-PEG4-Polybia-MP1 **15**, (A + B)-C11-Polybia-MP1 **16** and (A + B)-C11-peptoid **17** were compared with wild-type nisin, nisin (1–12) (A + B) 1, Polybia-MP1 **2**, Pexiganan **3** and peptoid **4** as controls (Table 2, Figs. S1 and S2 (Supporting Information))

Nisin showed greater activity than any of the hybrids or linear peptides. As expected, the nisin(1–12) (A + B) **1** showed very low activity; poor activity against *M. luteus* and *L. lactis*,[17] and also against *B. subtilis, S. aureus*, MRSA and VRE[18] have previously been reported. The absence of antimicrobial activity for nisin(1–12) (A + B) **1** has been attributed to the absence of the pore-forming C-terminal sequence. All but one of the semisynthetic lantibiotics were shown to have greater activity than nisin(1–12) (A + B) **1**, although none performed significantly better than their respective tail peptide controls **2**, **3** and **4**. Clearly the conjugation of the pore-forming antibiotic peptides restores antimicrobial activity to the lipid II binding motif. However, it does not prove that the activity of these semisynthetic lantibiotics is conferred by a lipid II dependent mechanism; their antibacterial properties may be solely due to the activity of the cationic peptide moiety. Conversely, we



Scheme 2. Synthesis of pexiganan **3** and Lys(N_3)-PEG4 pexiganan **8**. (i) 96.5% TFA, 2.5% H_2O, 1% TIPS; (ii) Fmoc-NH-PEG(4)-COOH, HBTU, DIPEA; (iii) piperidine, DMF; (iv) Fmoc-Lys(N_3)-OH, HBTU, DIPEA; (v) piperidine, DMF; (vi) 96.5% TFA, 2.5% H_2O, 1% TIPS.



Scheme 3. Synthesis of Polybia-MP1 peptides. (i) 96.5% TFA, 2.5% H_2O, 1% TIPS; (ii) Fmoc-NH-PEG(4)-COOH, HBTU, DIPEA; (iii) piperidine, DMF; (iv) Fmoc-Lys (N_3)-OH, HBTU, DIPEA; (v) 11-azidoundecanoic acid **6**, HBTU, DIPEA.

cannot conclude that the activity of these semisynthetic lantibiotics is due to pore formation: indeed, for the nisin(1–12)-lipid hybrids previously reported,[18] experiments with model membranes ruled out pore formation. Whilst the Lys-PEG4 linked peptides **14** and **15** showed promising antimicrobial activity, the biological activities of the C11-alkyl linked hybrids **16** and **17** were poor. This could be attributed to a reduction of flexibility in the hinge region, or to disruption by the alkyl linker of the helix-forming propensities of the Polybia-MP1 and peptoid tails.

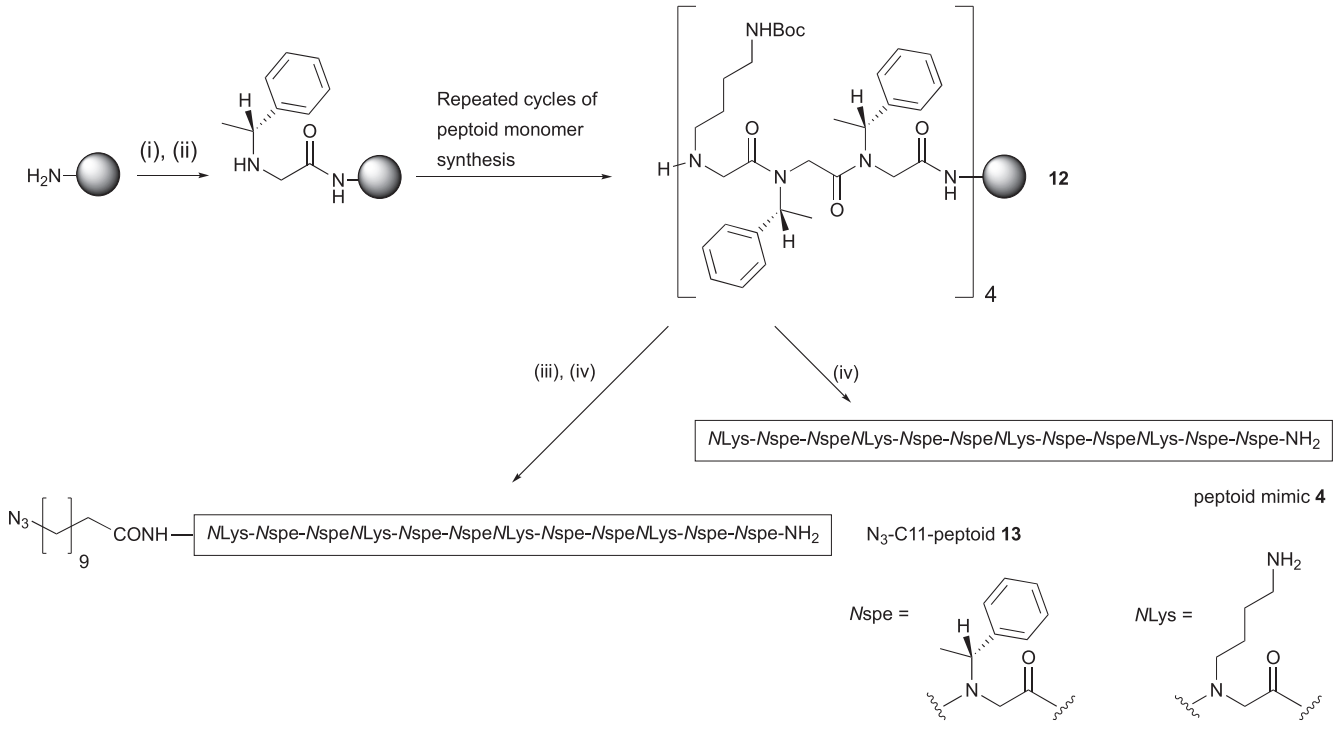
Table 1
Summary of click reactions to give semisynthetic lantibiotics **14–17**.

Tail peptide	Semisynthetic lantibiotic
Lys(N_3)-PEG4-pexiganan **8**	(A + B)-Lys-PEG4-pexiganan **14**
Lys(N_3)-PEG4-Polybia-MP1 **10**	(A + B)-Lys-PEG4-Polybia-MP1 **15**
N_3-C11-Polybia-MP1 **11**	(A + B)-C11-Polybia-MP1 **16**
N_3-C11-peptoid **13**	(A + B)-C11-peptoid **17**

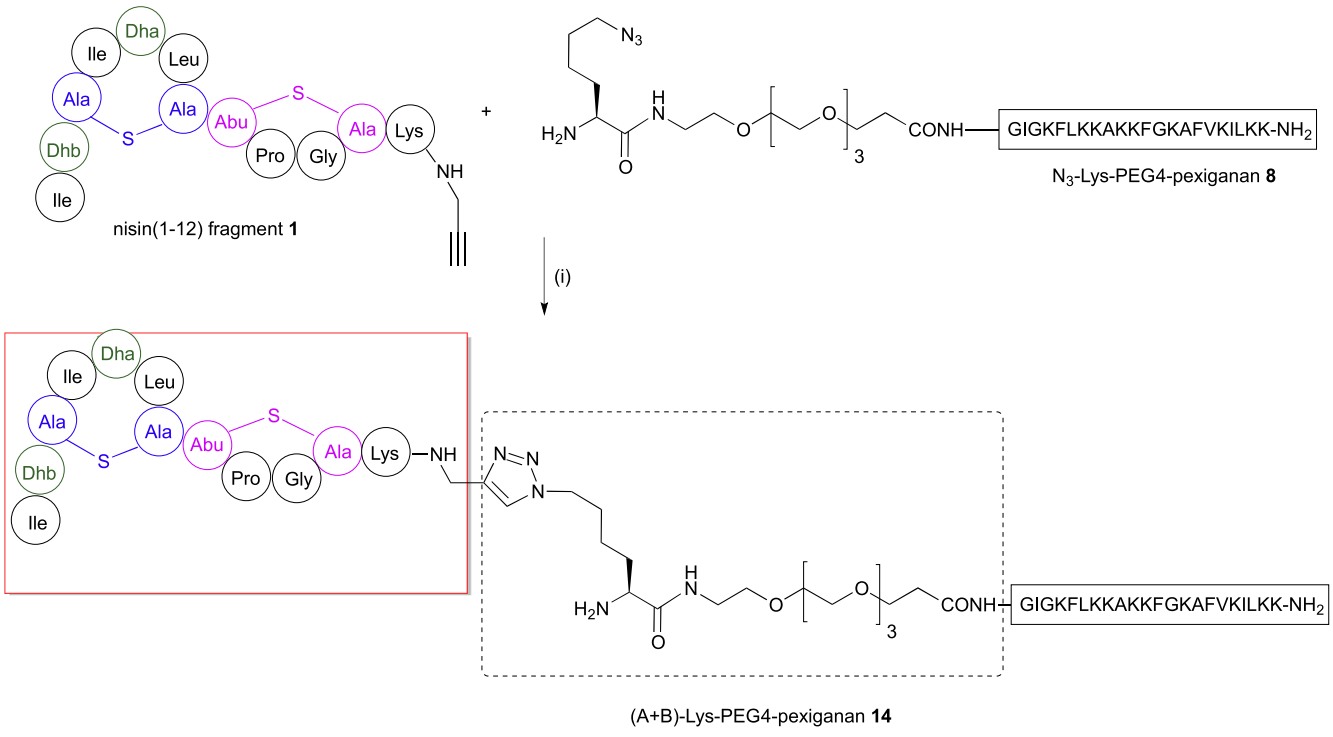
3. Conclusion

Lantibiotics hold considerable promise as leads from which the next generation of antimicrobial therapeutics can be developed, due to their unique mode of action. However, their poor drug-likeness and complex structural features have hindered the realization of this potential. In this paper, we report a new family of simplified semisynthetic lantibiotics. These have been designed to be synthetically accessible, whilst retaining the important features of the lantibiotic nisin – selective binding to lipid II and pore formation – which lead to its biological activity. We have developed a simple approach involving bioconjugation of pore-forming helical peptides to the key lipid II-binding region of nisin, the N-terminal nisin(1–12) fragment, which can be used to rapidly access nisin analogues without the need for complicated synthetic or biotransformation strategies. This has established a methodology that could be used for synthesis of libraries of peptides for screening for antimicrobial activity, in particular against clinically relevant strains of MRSA and VRE. Cobb and Martin have recently also reported a similar strategy to access other nisin(1–12)-peptoid hybrids. They have also observed increased activity compared with truncated nisin(1–12) (A + B), with one bioconjugate having a MIC similar to full-length nisin, although all had a reduction in activity compared to the peptoids alone.[56]

Whilst it is surprising that these hybrid peptides and peptoids did not result in a major improvement in biological activity, this suggests that the structural requirements to bind lipid II and form a nisin



Scheme 4. Synthesis of magainin related peptoids. (i) bromoacetic acid, DIC, NMP; (ii) (*R*)-(+)-MBA, NMP; (iii) 11-azidoundecanoic acid **6**, HBTU, DIPEA; (iv) 96.5% TFA, 2.5% H_2O, 1% TIPS. (Cycles of peptoid monomer synthesis are as follows: (a) bromoacetic acid, DIC, NMP, followed by either (b) (*R*)-(+)-MBA, NMP or (c) Boc-1,4-butanediamine, NMP).



Scheme 5. Representative synthesis of semisynthetic lantibiotic (A + B)-Lys-PEG4-pexiganan **14**. (i) $CuSO_4 \cdot 5H_2O$ (2.4 equiv), sodium ascorbate (4.8 equiv), TBTA (0.6 equiv), μW, 80 °C, 20 min.

analogue:lipid II pore have been over-simplified. Indeed, the structure of the linker between the lipid II-binding and pore forming regions, as a mimic of the "hinge region" of nisin, is known to be critical[19,20,21] and recent high-resolution solid-state NMR studies of a lipid II-nisin complex in DOPC liposomes has indicated the importance of this region for the macromolecular organization of the pore.[57] In this initial proof-of-principle study, we have investigated the biological properties of hydrophilic Lys-PEG4 linked peptides and hydrophobic C11-alkyl linked peptides. The Lys-PEG4 linked peptides had better activity, with the best semisynthetic lantibiotic, (A + B)-Lys-PEG4-pexiganan **14**, matching the activity of its tail peptide, pexiganan **3**. Moreover, the resulting semisynthetic lantibiotics with PEG linkers have much better aqueous solubility at physiological pH than wild type nisin, and are also expected to have greater *in vivo* stability as they lack the amino acid sequences that render nisin vulnerable to proteolysis.[17] The mode of action of these simplified semisynthetic lantibiotics still remains to be established, and will be followed up by circular dichroism and model membrane studies to elucidate the structure, lipid II binding and pore formation properties of these peptides.

Table 2
MICs determined by broth micro-dilution.

Peptide	MIC (μM)
Nisin	0.0234
Nisin(1–12) (A + B) **1**	> 12.0
Polybia-MP1 **2**	1.50
Pexiganan **3**	0.750
Peptoid **4**	1.50
(A + B)-Lys-PEG4-pexiganan **14**	0.750
(A + B)-Lys-PEG4-Polybia-MP1 **15**	3.00
(A + B)-C11-Polybia-MP1 **16**	> 12.0
(A + B)-C11-peptoid **17**	6.00

4. Experimental

4.1. General experimental details for chemical synthesis

Reagents were purchased from chemical suppliers and were used as received without further purification. Dry DMF and THF were purchased as anhydrous solvents in SureSeal bottles from Sigma-Aldrich Co. Ltd. Brine refers to a saturated solution of sodium chloride in water and sodium bicarbonate refers to a saturated solution of sodium hydrogen carbonate in water. Ether refers to diethyl ether and petrol to petroleum ether fractions boiling between 40 and 60 °C. All water used was either distilled using an Elga Purelab Option R7 water purifier or used directly from a bottle of HPLC-grade water. All reactions were carried out in closed systems under Argon.

LRMS refers to low resolution mass spectrometry and HRMS refers to high resolution mass spectrometry. LRMS was performed on a Waters Acquity UPLC attached to a Waters Acquity SQD using HPLC grade water and acetonitrile (both with 0.1% formic acid) as the solvents. HRMS Was performed on a Waters Xevo G2-XS QTof with ESI source attached to a Waters Acquity UPLC system (I class).

^{1}H NMR was performed on a 600 MHz AMX Bruker Spectrometer. The chemical shifts (δ) were given in units of ppm relative to tetramethylsilane (TMS), where δ(TMS) = 0 ppm. Data processing was carried out using ACD/NMR Academic Edition, Advanced Chemistry Development, Inc. The multiplicity used for assignment is indicated by the following abbreviations: s = singlet, d = doublet, t = triplet, q = quadruplet, qn = quintet, m = multiplet, br = broad, app. d. = apparent doublet and the coupling constants (J) were measured in Hertz (Hz). Deuterated chloroform ($CDCl_3$) was used as the solvent for all NMR analysis.

4.2. General experimental details for peptide synthesis

Peptides were synthesised using an automated peptide synthesiser in 5 mL syringes with frits, purchased from Multisyntech GmbH. Microwave couplings and click chemistry were carried out using a Personal Chemistry Smith Creator Microwave Assisted Organic Synthesizer loaded with either 0.5, 2 or 5 mL reaction vials as

purchased from Biotage. HPLC grade DMF purchased from Sigma Aldrich was used as the primary solvent throughout peptide synthesis. Peptides were centrifuged using an Eppendorf Centrifuge 5810 R and were lyophilised using an VirTis Benchtop Pro freeze dryer. Fmoc-protected amino acids were purchased from Novabiochem, except for Fmoc-NH-PEG(4)-COOH which was purchased from Iris Biotech Gmbh.

4.3. Methods for automated peptide synthesis

Peptides were synthesised on a MultiSynTech Syro Peptide Synthesiser (model MP-60). The peptide synthesiser contains an agitation block which held 5 mL syringes with frits connected to a vacuum pump to remove solvents. Pre-loaded Rink amide resins (100–200 mesh) and Fmoc protected amino acids were purchased from Merck KGaA/Novabiochem, and were used in all cases of automated peptide synthesis. The total volume of all reagents in each step was 1.5 mL and all reagents were dissolved in HPLC grade DMF.

Fmoc deprotection: A solution of piperidine in DMF (40% v/v, 1.5 mL) was added to the syringe containing an N-terminal Fmoc-protected peptide. The mixture was agitated for 20 s every minute for a total of 3 min. The reagents were removed by vacuum filtration and the resin was washed with DMF (6 × 1.5 mL). A second portion of piperidine in DMF (40% v/v, 0.75 mL) was added to the syringe followed by DMF (0.75 mL) to give a final solution of 20% v/v of piperidine in DMF. The mixture was agitated for 20 s every minute for a total of 10 min. The reagents were removed by vacuum filtration and the resin washed with DMF (6 × 1.5 mL).

Amino acid coupling: Fmoc protected amino acid (4 eq.), HBTU (4 eq.) and DIPEA (10 eq.) were added to the reaction syringe. The mixture was agitated for 20 s every minute for a total of 40 min. The reagents were removed by vacuum filtration and the resin was washed with DMF (4 × 1.5 mL).

4.4. Methods for automated peptoid synthesis

Peptoids were synthesised in a similar manner to peptides using an automated peptide synthesiser as before. In addition to DMF, reagent grade *N*-methylpyrrolidone (NMP) purchased from Sigma Aldrich was also used as a solvent. The synthesiser was reprogrammed to add the non-amino acid reagents and both NMP and DMF were used as solvents. Bromoacetic acid was used as a 1.2 M solution in DMF and Boc-butanediamine and (*R*)-(+)-α-methylbenzylamine (MBA) were both used as 1 M solutions in NMP. *N,N′*-Diisopropylcarbodiimide (DIC) was used neat.

Fmoc deprotection of resin: The Fmoc deprotection protocol for automated peptide synthesis was used.

Bromoacetic acid coupling: Bromoacetic acid (20 eq.) and DIC (25 eq.) were added to the reaction syringe and the mixture was agitated for 20 s every minute for a total of 1 h. The reagents were removed by vacuum filtration and the resin was washed with DMF (3 × 1.5 mL) and NMP (3 × 1.5 mL).

Amine coupling: The required amine (25 eq.) was added to the reaction syringe and the mixture was agitated for 20 s every minute for a total of 1 h. The reagents were removed by vacuum filtration and the resin was washed with NMP (3 × 1.5 mL) and DMF (3 × 1.5 mL).

4.5. Peptide cleavage and purification

Cleavage and purification procedures were the same for both peptides and peptoids.

Cleavage from the resin: Peptides and peptoids were washed with DMF (5 × 1.5 mL), CH_2Cl_2 (5 × 1.5 mL), methanol (5 × 1.5 mL) and diethyl ether (5 × 1.5 mL) and dried in desiccator for 30 min. A solution of 96.5% TFA, 2.5% water and 1% TIPS (1 mL) was added to the reaction syringe and the mixture was agitated for 40 min on the platform shaker. The cleavage solution was removed by filtration and the filtrate was transferred to a 15 mL Falcon tube containing 13 mL of diethyl ether to give a milky white precipitate. Another aliquot (1 mL) of the cleavage solution was added to the reaction syringe and the mixture was agitated for a further 20 min on the platform shaker. The cleavage solution was again removed by filtration and transferred to the same Falcon tube. The Falcon tube was centrifuged for 10 min at 4000 rpm at 0 °C after which the diethyl ether was carefully decanted. This process was repeated with a further 2 aliquots of fresh diethyl ether (13 mL) after which the peptide was re-dissolved in water and lyophilised.

HPLC purification: Peptides and peptoids were analysed and purified via reverse phase HPLC using a Dionex system with a PDA-100 photodiode array detector, a model ASI-100 automated sample injector and Chromeleon Software version 2.0. Preparative purification was performed using an ACE 5 C8-300 column (150 × 10 mm), a Dr Maisch Reprosil Gold 200 C8 (150 × 10 mm) or a Phenomenex Onyx monolithic C18 column (100 × 3 mm) with UV detection at 214 and 254 nm. Conditions varied between peptides and are reported as such. The mobile phase is quoted as a percentage of solvent B (acetonitrile with 0.1% TFA) in solvent A (water with 0.1% TFA). The fractions containing the correct peak were combined and lyophilised.

4.6. Peptide analysis by HPLC

Purified peptides were analysed using a using the same Dionex system as used for purification. An ACE 5 C18-AR analytical column (150 × 4.6 mm) was used with a flow rate of 1.0 mL min^{-1} and a linear gradient of 5–95% over 60 min (A = water, 0.1% TFA and B = acetonitrile, 0.1% TFA) and with UV detection at 214 and 254 nm unless otherwise stated.

4.7. Peptide and peptoid synthesis

*Pexiganan resin-bound intermediate **7**:* Rink amide resin (50 mg, 0.7 mmol g^{-1}) was added to a reaction syringe, washed with DMF (4 × 1.5 mL) and left to swell for 20 min in 1.5 mL DMF. After this time, the Fmoc group was removed using the standard Fmoc deprotection step as described in Section 4.3. Subsequent amino acid couplings and Fmoc deprotections were carried out as described in Section 4.3 with stock solutions of the following amino acids: Fmoc-Ala-OH; Fmoc-Phe-OH; Fmoc-Gly-OH; Fmoc-Ile-OH; Fmoc-Lys(Boc)-OH; Fmoc-Leu-OH; Fmoc-Val-OH.

*Pexiganan **3**:* Pexiganan resin-bound intermediate **7** was cleaved under standard conditions, washed with ether and lyophilised as described above. The peptide was purified using a gradient of 5–40% over 30 min at 4 mL min^{-1} using a C18 semi-preparative column. The residue was purified a second time using the same conditions to give pexiganan **3** (8.7 mg, 7%).

LRMS: (ES^+) calculated $[C_{122}H_{210}N_{32}O_{22}]^+$ = 2475.6292; found *m/z* = 360.7 $[M+6H+K]^{7+}$, 413.7 $[M+6H]^{6+}$, 420.4 $[M+5H+K]^{6+}$, 496.2 $[M+5H]^{5+}$, 620.2 $[M+4H]^{4+}$, 826.6 $[M+3H]^{3+}$.

*Lys(N_3)-PEG4-pexiganan **8**:* Fmoc-NH-PEG(4)-COOH was coupled to pexiganan resin-bound intermediate **7** using standard amino acid coupling protocol as described in Section 4.3. Fmoc deprotection was carried out as described above. Fmoc-Lys(N_3)-OH was then coupled to the *N*-terminus using the standard amino acid coupling protocol and Fmoc deprotection was carried out as described in Section 4.3. The peptide was cleaved under standard conditions, washed with ether and lyophilised as described above. The peptide was purified using a gradient of 5–40% over 30 min at 4 mL min^{-1} using a C18 semi-preparative column. The residue was purified a second time using the same conditions to give Lys(N_3)-PEG4-pexiganan **8** (10.0 mg, 5%).

HRMS: (ES^+) calculated $[C_{139}H_{242}N_{37}O_{28}]^+$ = 2877.8645; found: *m/z* = 480.6530 $[M+6H]^{6+}$, 576.5814 $[M+5H]^{5+}$, 720.4740 $[M+4H]^{4+}$.

*Polybia-MP1 resin-bound intermediate **9**:* Rink amide resin (50 mg, 0.7 mmol g^{-1}) was added to a reaction syringe, washed with DMF

(4 × 1.5 mL) and left to swell for 20 min in 1.5 mL DMF. After this time, the Fmoc group was removed using the standard Fmoc deprotection step as described in Section 4.3. Subsequent amino acid couplings and Fmoc deprotections were carried out as described above with stock solutions of the following amino acids: Fmoc-Ala-OH; Fmoc-Asp(O^tBu)-OH; Fmoc-Gln-OH; Fmoc-Ile-OH; Fmoc-Lys(Boc)-OH; Fmoc-Leu-OH; Fmoc-Trp-OH. Double coupling was used for all amino acid couplings.

Polybia-MP1 ***2:*** Polybia-MP1 resin-bound intermediate **9** was cleaved under standard conditions, washed with ether and lyophilised as described in Section 4.5. The peptide was purified using a gradient of 5–40% over 30 min at 4 mL min^{-1} using a C18 semi-preparative column. The residue was purified a second time using the same conditions to give Polybia-MP1 2 (4.1 mg, 5% yield).

LRMS: (ES^+) $[C_{78}H_{133}N_{20}O_{19}]^+$ calculated = 1654.0050; found *m/z* = 424.9 $[M+2Na+2H]^{4+}$, 552.2 $[M+3H]^{3+}$, 828.2 $[M+2H]^{2+}$.

Lys(N_3)-PEG4-Polybia-MP1 ***10:*** Fmoc-NH-PEG(4)-COOH was coupled to Polybia-MP1 resin-bound intermediate **9** using standard amino acid coupling protocol as described in Section 4.3. Fmoc deprotection was carried out as described above. Fmoc-Lys(N_3)-OH was then coupled to the *N*-terminus using the standard amino acid coupling protocol and Fmoc deprotection was carried out as described above. The peptide was cleaved under standard conditions, washed with ether and lyophilised as described in Section 4.5. The peptide was purified using a gradient of 5–40% over 30 min at 4 mL min^{-1} using a C18 semi-preparative column. The residue was purified a second time using the same conditions to give Lys(N_3)-PEG4-Polybia-MP1 **10** (6.0 mg, 7%).

HRMS: (ES^+) $[C_{95}H_{163}N_{25}O_{25}]^+$ calculated = 2055.2325; found: *m/z* = 514.8204 $[M+4H]^4$, 686.0886 $[M+3H]^{3+}$, 1028.6212 $[M+2H]^{2+}$.

N_3-C11-Polybia-MP1 ***11:*** 11-azidoundecanoic acid **6** was coupled to Polybia-MP1 resin-bound intermediate **9** using standard amino acid coupling protocol as described in Section 4.3. Fmoc deprotection was carried out as described above. The peptide was cleaved under standard conditions, washed with ether and lyophilised as described in Section 4.5. The peptide was purified using a gradient of 5–40% over 30 min at 4 mL min^{-1} using a C18 semi-preparative column. The residue was purified a second time using the same conditions to give N_3-C11-Polybia-MP1 **11** (6.0 mg, 8%).

HRMS: (ES^+) $[C_{89}H_{152}N_{23}O_{20}]^+$ calculated = 1863.1579; found *m/z* = 622.0614 $[M+3]^{3+}$, 932.5844 $[M+2H]^{2+}$.

Peptoid mimic resin-bound intermediate ***12:*** Rink amide resin (50 mg, 0.7 mmol g^{-1}) was added to a reaction syringe, washed with DMF (4 × 1.5 mL) and left to swell for 20 min in 1.5 mL DMF. After this time, the Fmoc group was removed using the standard Fmoc deprotection step as described in Section 4.4 above. The resin bound amine was then bromoacetylated with the addition of 1.2 M bromoacetic acid in DMF (584 μL) and neat DIC (135 μL) and the coupling was carried out as described above. Bromide substitution was then carried out with the addition of 1 M MBA (875 μL) and the coupling was carried out as described above. These two reactions were repeated followed by a third bromoacetylation and the addition of 1 M *N*-Boc butanediamine in NMP (891 μL). The coupling was carried out as described above. The peptoid chain was built up using this submonomer approach with stock solutions of the reagents as listed below:

Reagent	Mass (g)	Solvent	Volume (mL)
Bromoacetic acid	1.67	DMF	10.0
DIC	2.04	neat	2.50
N-Boc-butanediamine	0.941	NMP	5.00
MBA	1.21	NMP	10.0

Peptoid mimic ***4:*** Peptoid mimic resin-bound intermediate **12** was cleaved under standard conditions, washed with ether and lyophilised as described in Section 4.5. The peptide was purified using a gradient of 5–60% over 30 min at 5 mL min^{-1} using a C18 semi-preparative column. The peptoid was purified a second time using the same conditions to give 4 (4.1 mg, 5% yield).

LRMS: (ES^+) $[C_{104}H_{140}N_{17}O_{12}]^+$ calculated = 1819.0862; found *m/z* = 455.5 $[M+4H]^{4+}$, 607.2 $[M+3H]^{3+}$.

N_3-C11-peptoid ***13:*** 11-azidoundecanoic acid **7** was coupled to Peptoid mimic resin-bound intermediate **12** using standard amino acid coupling protocol as described in Section 4.3. The peptoid was cleaved under standard conditions, washed with ether and lyophilised as described in Section 4.5. The peptoid was purified using a gradient of 5–60% over 30 min at 5 mL min^{-1} using a C18 semi-preparative column. The peptoid was purified a second time using the same conditions to give N_3-C11-peptoid **13** (6.5 mg, 7%).

HRMS: (ES^+) $[C_{115}H_{159}N_{20}O_{13}]^+$ calculated = 2028.2390; found *m/z* = 508.0722 $[M+4H]^{4+}$, 677.0892 $[M+3H]^{3+}$, 1015.1232 $[M+2H]^{2+}$.

4.8. Semisynthetic lantibiotics prepared via click chemistry

(A + B)-Lys-PEG4-pexiganan ***14:*** Nisin(1–12) (A + B) **1** (1.00 mg, 710 nmol) dissolved in 50 μL of DMF and Lys(N_3)-PEG4-pexiganan **8** (2.8 mg, 710 nmol) dissolved in 50 μL of water were transferred to a 250 μL microwave vial. Stock solutions of $CuSO_4{\cdot}5H_2O$ (42.0 mg, 5.00 mL water), sodium ascorbate (67.0 mg, 5.00 mL water) and tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) (2.20 mg, 500 μL DMF) were made up. 50.0 μL of each stock solution was added to the reaction vial. The reaction mixture was then irradiated for 20 min at 80 °C. After this time, the reaction mixture was quenched with buffer (3 mL, 0.1% TFA in water), filtered and purified directly using reverse phase HPLC over a gradient of 5–40% over 30 min at 4 mL min^{-1} using a C18 semi-preparative column. The peptide was purified a second time using the same conditions to give (A + B)-Lys-PEG4-pexiganan **14** (300 μg, 8% yield).

HRMS: (ES^+) $[C_{193}H_{328}N_{51}O_{40}S_2]^+$ calculated = 4064.4636; found *m/z* = 581.7919 $[M+7H]^{7+}$, 678.5875 $[M+6H]^{6+}$, 814.1019 $[M+5H]^{5+}$, 1017.3734 $[M+4H]^{4+}$.

(A + B)-Lys-PEG4-Polybia-MP1 ***15:*** Nisin(1–12) (A + B) **1** (1.00 mg, 710 nmol) dissolved in 50 μL of DMF and Lys(N_3)-PEG4-Polybia-MP1 **10** (2.37 mg, 710 nmol) dissolved in 50 μL of water were transferred to a 250 μL microwave vial. Stock solutions of $CuSO_4{\cdot}5H_2O$ (42.0 mg, 5.00 mL water), sodium ascorbate (67.0 mg, 5.00 mL water) and TBTA (2.20 mg, 500 μL DMF) were made up. 50.0 μL of each stock solution was added to the reaction vial. The reaction mixture was then irradiated for 20 min at 80 °C. After this time, the reaction mixture was quenched with buffer (3 mL, 0.1% TFA in water), filtered and purified directly using reverse phase HPLC over a gradient of 5–40% over 30 min at 4 mL min^{-1} using a C18 semi-preparative column. The peptide was purified a second time using the same conditions to give (A + B)-Lys-PEG4-Polybia-MP1 **15** (300 μg, 9% yield).

HRMS: (ES^+) $[C_{149}H_{250}N_{39}O_{37}S_2]^+$ calculated = 3241.8316; found *m/z* = 649.3778, 811.7181 $[M+4H]^{4+}$, 1081.5 $[M+3H]^{3+}$.

(A + B)-C11-Polybia-MP1 ***16:*** Nisin(1–12) (A + B) **1** (1.00 mg, 710 nmol) dissolved in 50 μL of DMF and N_3-C11-Polybia-MP1 **11** (1.57 mg, 710 nmol) dissolved in 50 μL of water were transferred to a 250 μL microwave vial. Stock solutions of $CuSO_4{\cdot}5H_2O$ (42.0 mg, 5.00 mL water), sodium ascorbate (67.0 mg, 5.00 mL water) and TBTA (2.20 mg, 500 μL DMF) were made up. 50.0 μL of each stock solution were added to the reaction vial. The reaction mixture was then irradiated for 20 min at 80 °C. After this time, the reaction mixture was quenched with buffer (3 mL, 0.1% TFA in water), filtered and purified directly using reverse phase HPLC over a gradient of 5–50% over 40 min at 5 mL min^{-1} using a C18 semi-preparative column. The peptide was purified a second time to give (A + B)-C11-Polybia-MP1 **16** (200 μg, 8% yield).

HRMS: (ES^+) $[C_{143}H_{238}N_{37}O_{32}S_2]^+$ calculated = 3049.7570; found *m/z* = 610.9606 $[M+5H]^{4+}$, 763.4 $[M+4H]^{4+}$, 1017.5 $[M+3H]^{3+}$.

(A + B)-C11-peptoid ***17:*** Nisin(1–12) (A + B) 1 (1.00 mg, 710 nmol)

dissolved in 50 μL of DMF and N_3-C11-peptoid **13** (1.76 mg, 710 nmol) dissolved in 50 μL of water were transferred to a 250 μL microwave vial. Stock solutions of $CuSO_4{\cdot}5H_2O$ (42.0 mg, 5.00 mL water), sodium ascorbate (67.0 mg, 5.00 mL water) and TBTA (2.20 mg, 500 μL DMF) were made up. 50.0 μL of each stock solution were added to the reaction vial. The reaction mixture was then irradiated for 20 min at 80 °C. After this time, the reaction mixture was quenched with buffer (3 mL, 0.1% TFA in water), filtered and purified directly using reverse phase HPLC over a gradient of 5–60% over 30 min at 5 mL min^{-1} using a C18 semi-preparative column. The peptide was purified a second time using the same conditions to give (A + B)-C11-peptoid **17** (250 μg, 9% yield).

HRMS: (ES^+) $[C_{169}H_{245}N_{34}O_{25}S_2]^+$ calculated = 3214.8381; found m/z = 536.9815 $[M+6H]^{6+}$, 644.1757 $[M+5H]^{5+}$, 804.9666 $[M+4H]^{4+}$, 1072.9497 $[M+3H]^{4+}$.

4.9. In vitro screening

General methods: In vitro work was carried out under sterile conditions in a category 2 safety hood. Oxoid Nutrient Broth No 2 was used purchased from ThermoFisher Scientific as a dehydrated powder and was made up as a solution according to the manufacturer's instructions. OD600 readings of bacteria stock solutions were taken using a Nanodrop 2000c Spectrophotometer. Polypropylene and polystyrene 96-well plates were purchased from Greiner Bio One. Aeraseal Sterile gas permeable seals were purchased from Excel Scientific. 96-well plates were incubated overnight in Eppendorf Thermomixer comfort/C incubators. OD625 readings of 96-well plates were measured using a Tecan infinite M200 Pro plate reader with i-control 1.11 software.

All of the peptides and peptoids synthesised were cleaved in TFA and purified using a solvent system with 0.1% TFA. It was assumed that all of the cationic residues exist as TFA salts. Due to the high number of cationic residues, the TFA content makes up a significant proportion of the isolated mass. It has previously been reported that the presence of TFA can inhibit bacterial growth in assays[58] so to check if it would have an effect in the assays, a control solution of sodium acetate TFA salt was tested for antibacterial activity. It was made up to the same concentration as the sample with the highest concentration of TFA salt. No inhibitory activity was observed in agar well diffusion plates at the maximum amount tested, 25 μL of 0.4 mg mL^{-1}.

Agar well diffusion method screening: All compounds to be tested were made up to a concentration of 1 mg mL^{-1} in sterile water except sodium trifluoroacetate which was made up to 0.4 mg mL^{-1}. LB agar plates were inoculated with 100 μL of an overnight culture of either *Micrococcus luteus, Bacillus subtilis*, *Escherichia coli* or *Pseudomonas aeruginosa* and spread evenly across the surface of the plate. The overnight cultures of bacteria were grown at 37 °C in Oxoid Nutrient Broth No. 2 with shaking. Four wells of 8 mm diameter were punched into each plate. 25 μL of the stock solution was spotted into a well for each strain of bacteria. Plates were incubated for 48 h at 37 °C except for those inoculated with *M. luteus* which were incubated at 30 °C. Later work with *M. luteus* was carried out at 37 °C. After 48 h, antibacterial activity was evaluated by the appearance of a zone of growth inhibition.

Broth micro-dilution method screening: Oxoid Nutrient Broth No 2 was used with 1% DMSO to aid solubilisation. All compounds to be tested were made up to 0.60 mM stock solutions in sterile water with 1% DMSO. To ensure peptides were fully dissolved stock solutions were placed in a bath sonicator for 30 s. 10 μL of peptide stock solution was added to 90 μL of nutrient broth with 1% DMSO. This was then transferred to the first column of a 96-well polypropylene plate. A two-fold serial dilution was carried out as described by R. Hancock and coworkers:[59] 50 μL of nutrient broth was added to columns 2–10. 50 μL was taken from column 1 and added to column 2 and mixed well by pipetting up and down 8 times. 50 μL was taken from column 2 and added to column 3 and again mixed well. This process was repeated through to column 10. The 50 μL taken from 10 was discarded. 250 μL of nutrient broth was added to the sterility control column, 12, and 240 μL was added to the growth control column, 11.

10 μL of an overnight stock of bacteria adjusted to have an OD 0.817 (of a 1 in 10 dilution) was added to each well except from the final column which was reserved as a sterility control containing only nutrient broth. A further portion of Oxoid Nutrient Broth No 2 was added to make each well up to 250 μL total volume. A gas permeable adhesive seal was applied to each plate and they were incubated for 20 h at 37 °C. After this time broth and bacteria were re-suspended and transferred to clear, flat-bottomed, polystyrene 96-well plates and the OD was measured. The MIC is defined as the lowest concentration of the antimicrobial agent that inhibits visible growth of the tested isolate as observed with the unaided eye. The error of the MIC results is estimated as one half of the interval between the MIC dilution and the next lowest concentration (i.e. the highest concentration that resulted in growth of bacteria).

Acknowledgements

We thank the Engineering and Physical Sciences Research Council UK (EPSRC) for the award of a PhD Studentship (to RD, grant EP/L504889/1) to the UCL Department of Chemistry for the award of a PhD studentship (to SAM) and for the award of grant EP/M013219/1 (to FT).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmc.2018.10.015.

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