Semisynthetic glycopeptide antibiotics

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Chapter 4 |

Vancomyxins: Vancomycin-Polymyxin Nonapeptide Conjugates

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4.1 Introduction

Vancomycin (**Fig. 1**) is the most prominent clinically-used glycopeptide antibiotic and exhibits potent activity against Gram-positive bacteria. It functions by inhibiting cell-wall biosynthesis by targeting the peptidoglycan precursor lipid II, and specifically by binding the D-Ala-D-Ala terminus of the lipid II pentapeptide via a network of five hydrogen bonds. In binding to this peptidoglycan precursor, vancomycin prevents cell-wall polymerization by bacterial transpeptidases and transglycosylases which leads to decreased bacterial cell-wall integrity, eventually resulting in lysis of the bacterial cell.[1,2] Furthermore, binding of vancomycin to lipid II is enhanced by cooperative dimerization which increases the binding affinity of vancomycin to lipid II and enhances its antimicrobial activity.[3–5] While lipid II is also present in Gram-negative bacteria, vancomycin is unable to access it due to the presence of the additional outer membrane (OM) found in Gram-negatives.[6] The OM is characterized by an inner leaflet of phospholipids and an outer leaflet decorated by lipopolysaccharide (LPS).[7] Notably, the ability of vancomycin to also bind to Gram-negative lipid II from *E. coli* was confirmed by the group of Shlaes. Furthermore, this study suggested that defects in the LPS core can revert resistance of Gram-negative strains to large hydrophilic molecules such as vancomycin.[6] Additionally, in a recent investigation Bardoel and coworkers showed that serum can sensitize multi-drug resistant (MDR) *K. pneumonia* to vancomycin, a process facilitated by the membrane attack complex (MAC) of the complement system found in human serum. The MAC forms pores in the OM causing disruption, allowing otherwise Gram-positive specific antimicrobials to also exert their action against Gram-negative strains.[8] These studies, and others carried out in the same area, highlight the potential of vancomycin to be effective against Gram-negative bacteria when the integrity of the OM is compromised.

Different strategies to sensitize Gram-negative bacteria to antibiotics which typically only work against Gram-positive pathogens have been explored in the literature.[9–11] The two main approaches used most often rely on: a) covalent attachment of OM-disrupting or OM-bypassing moieties and b) co-administration with "adjuvants'', which can either affect the OM integrity or impair the bacteria's efflux system.[7,12,13] Previously described covalent conjugates include those reported by Miller and coworkers wherein vancomycin was linked to an iron sequestering siderophore mimetic to yield hybrids with slightly reduced activity against Gram-positive strains but with enhanced activity towards a hypersensitive strain of *Pseudomonas aeruginosa* under iron depleted conditions.[14] More recently, the group of Haldar reported a lipophilic cationic vancomycin analogue, VanQAmC$_{10}$, which was shown to be bactericidal against MDR *A. baumannii*.[15] Another recent vancomycin derivative, developed by the groups of Wender and Cegelski, involves the introduction of an arginine-amide moiety at the

vancomycin C-terminus, significantly enhancing activity against *E. coli* (MIC 8-16 μM) including resistant strains.[16,17] Notably, this arginine-vancomycin conjugate was demonstrated to successfully reduce bacterial burden >6-log fold compared to vehicle and vancomycin in a murine thigh *E. coli* infection model.[16] In addition to such covalent approaches to enhance anti-Gram-negative activity, agents capable of potentiating or synergizing with Gram-positive-specific antibiotics also present an attractive option. In this regard, many OM-disrupting cyclic or linear cationic peptides have been reported to sensitize Gram-negative pathogens to anti-Gram-positive antibiotics including vancomycin.[10,18,19]

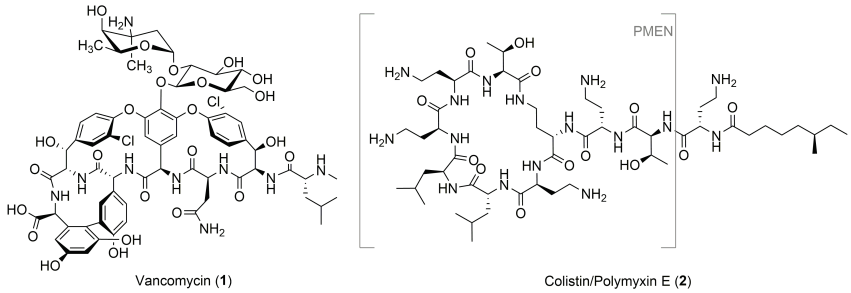


Fig. 1. Structure of vancomycin and colistin.

Among the most notable OM disrupting agents are the so-called polymyxin nonapeptides which are derived from the clinically used antibiotics polymyxin B and polymyxin E (colistin). Readily obtained by enzymatic degradation of the full-length antibiotic, the cyclic nonapeptides lack the fatty acyl tail and *N*-terminal Dab residue present in the parent polymyxins (**Fig. 1**). Due to its associated (nephro)toxicity when administered systemically, colistin has traditionally only been used as an antibiotic of last resort.[20] However, given increasing rates of resistance, the use of colistin is now on the rise.[20] By comparison, the polymyxin nonapeptides are significantly less toxic than the parent compounds,[21,22] but still maintain the capacity to bind to Gram-negative bacteria by recognition of the Lipid A unit of LPS.[23,24] Given their high positive charge, polymyxin nonapeptides displace the divalent cations responsible for stabilizing membrane packing in the Gram-negative OM[25] resulting in disruption of the OM.[20] Notably, while polymyxin nonapeptides retain little-to-none of the activity of the parent antibiotic,[26] they function effectively as synergistic agents and can improve the activity of otherwise Gram-positive specific antibiotics including vancomycin.[18,19]

To date, a small number of studies have explored the effect of conjugating polymyxins to antibiotic agents with the aim of using the covalently attached OM disruptor as an adjuvant for the antimicrobial agent. Generally speaking, these studies have focused on conjugation with Gram-negative active antibiotics. In a recently

example, Schweizer and coworkers described the ligation of full-length polymyxin B to the aminoglycoside tobramycin.[27] The resulting hybrid did not outperform either polymyxin or tobramycin in direct activity, but interestingly did potentiate other antibiotics towards several *P. aeruginosa* strains, including MDR isolates.[27] In another even newer development, researchers at Polyphor described bicyclic hybrids comprising a monocyclic β-hairpin peptidomimetic of protegrin I and PMEN.[28] While neither monocyclic peptide exhibits significant activity on its own, the bicyclic hybrids demonstrated extremely potent activity both *in vitro* and in mouse models of infection with a range of Gram-negative pathogens. Notably, these bicyclic constructs are proposed to target the extracellular part of the OM protein BamA, thereby avoiding the need to pass the OM.[28]

Given previous reports showing that covalent attachment of siderophores, LPS binding moieties, or positively charged moieties to vancomycin can lead to improved antimicrobial activity against Gram-negative strains,[15–17,29,30] we hypothesized that conjugation of vancomycin to the highly positively charged OM disruptor PMEN could sensitize Gram-negative strains. Our interest in exploring vancomycin-PMEN conjugates was further spurred given that OM disruption has also previously been demonstrated to enable anti-Gram-negative activity for vancomycin.[6,8,10,18,19] Also of note are recent reports showing that the covalent attachment of cationic moieties to vancomycin is also an effective means to resensitize clinically relevant vancomycin-resistant Gram-positive strains.[15,31–35] For these reasons we anticipated that vancomycin-PMEN conjugates might exhibit enhanced activity towards drug-resistant Gram-positive strains as well. Here we report the synthesis and evaluation of the vancomyxins, a new class of vancomycin-PMEN hybrid antibiotics. The antimicrobial activities of the vancomyxins against Gram-positive and Gram-negative bacteria (including drug-resistant clinical isolates) as well as an assessment of their toxicity towards eukaryotic cells is here reported.

4.2 Results and Discussion

4.2.1 Development and initial activity assessment of the vancomyxins

As a strategy for preparing the vancomycin/PMEN conjugates we envisioned the use of so-called “click chemistry”, wherein complementary azide and alkyne containing precursors are covalently linked by means of the well-established copper-catalyzed azide-alkyne cycloaddition reaction.[36–38] In pursuing this approach we opted to add the required azide handle to the N-terminus of the PMEN moiety while the alkyne functionality was incorporated into the vancomycin structure at two different locations (**Fig. 2**). The azido-modified PMEN building blocks were obtained via a convenient semisynthetic approach starting from colistin. In short, degradation of colistin using the readily available enzyme

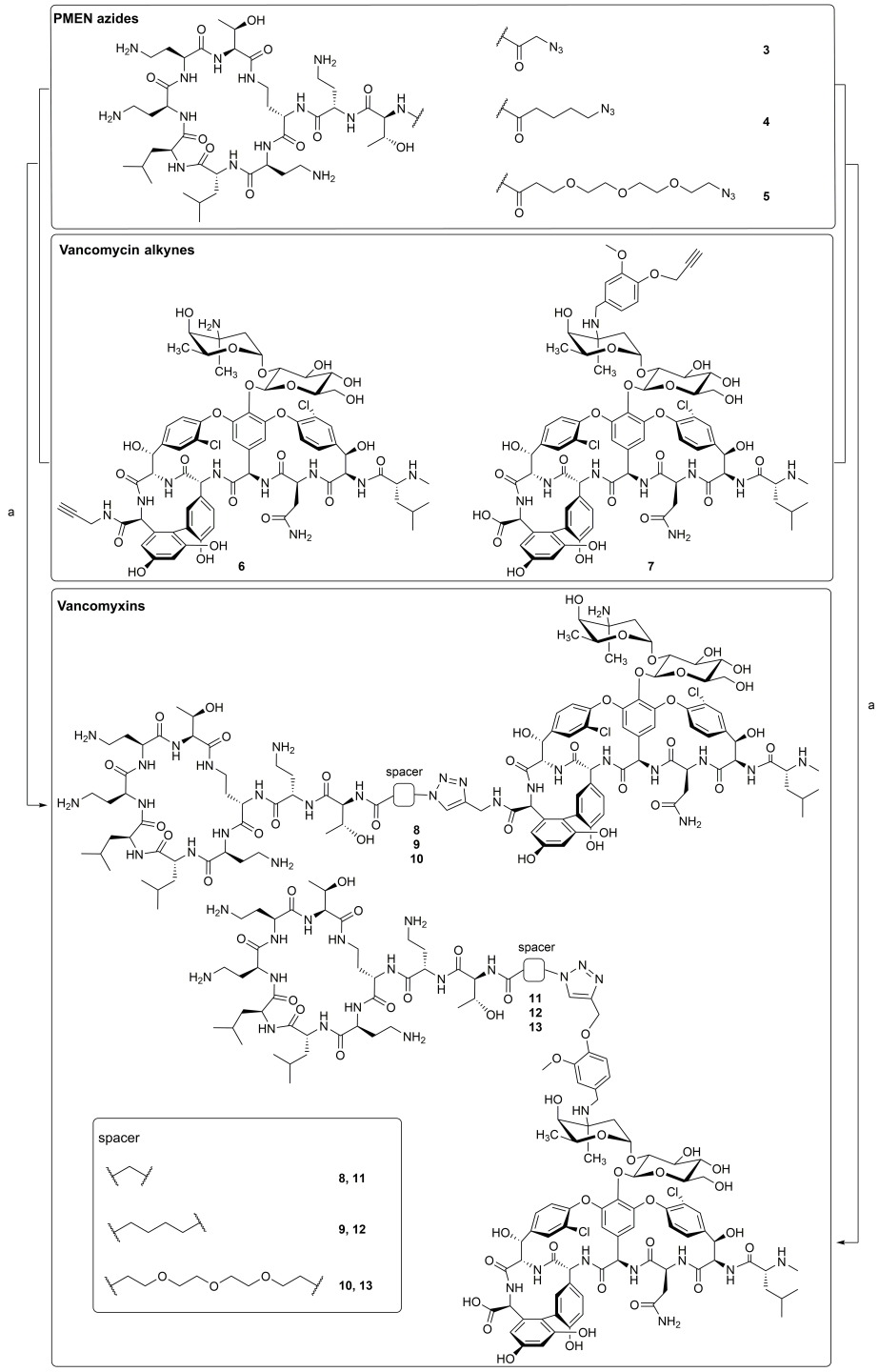
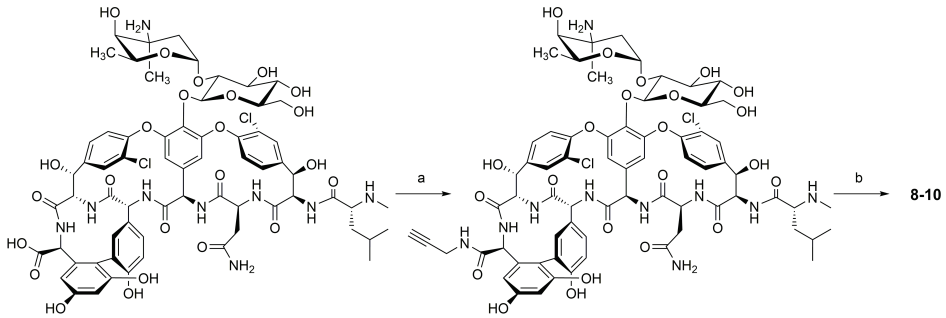


Fig. 2. Structure of vancomyxins 8-13. a) $CuSO_4$ pentahydrate, sodium ascorbate, H_2O.

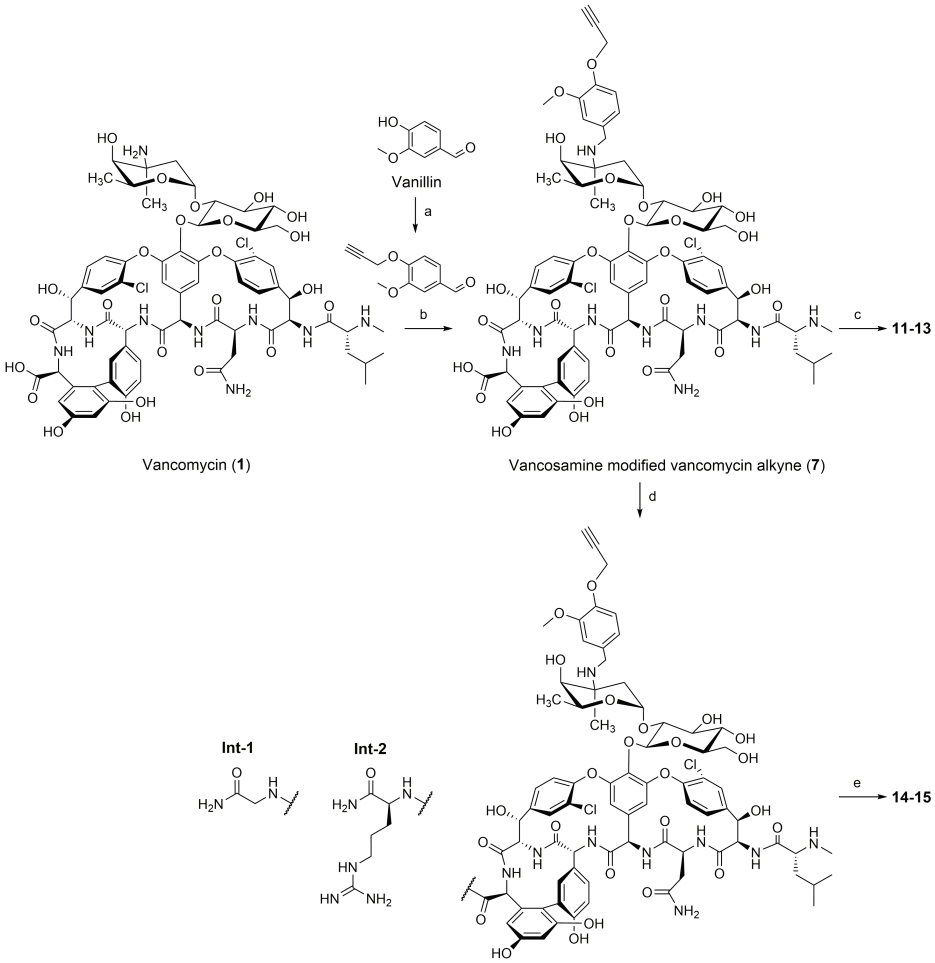
ficin yielded PMEN which was subsequently converted to PMEN-Boc$_4$ wherein the four Dab side chains are selectively protected and the *N*-terminus remains free.[39] Subsequently, azido-carboxylic acids of varying lengths were coupled to the *N*-terminus of PMEN-Boc$_4$ using BOP/DIPEA. Following Boc-deprotection and HPLC purification, the azide-modified PMEN building blocks **3-5** where obtained (**Fig. 2**). In the case of the alkyne-modified vancomycin partners, we followed a previously described protocol reported by Sharpless and coworkers who used click chemistry approaches in preparing various dimers of vancomycin.[38] To this end, an alkyne handle was incorporated at either the vancomycin C-terminus or at the vancosamine moiety. Given that neither of these modifications impacts the lipid II binding core of vancomycin, it was expected that structural alterations at these sites would not impair the ability of vancomycin to recognize its target. To install the alkyne at the C-terminus, vancomycin was treated with propargyl amine and HBTU/DIPEA resulting in building block **6**. For the preparation of building block **7**, installation of the alkyne handle at the vancosamine moiety was achieved via reductive amination using a known alkyne-containing aromatic aldehyde (see **Fig. 2**, **Scheme 1, Scheme 2**).[31,38] With the required PMEN-azides and vancomycin-alkynes in hand, the conjugation step involving triazole formation was achieved by means of copper catalysis.[36–38] In all cases the ligation reactions proceeded cleanly and rapidly to yield the expected vancomyxins (**8-13**). As indicated in **Fig. 2**, compounds **8** and **11** feature the shortest spacer deriving from the azido glycine modified PMEN, compounds **9** and **12** contain a 5-carbon moiety while compounds **10** and **13** include a longer and more hydrophilic PEG$_3$ spacer.



Scheme 1. Synthesis route of vancomyxin 8-10. a) propargylamine, HBTU, DIPEA, DMF/DMSO, RT; b) PMEN-azide **3**, **4**, or **5**, $CuSO_4$, sodium ascorbate, H_2O, RT

The antibacterial activities of PMEN azides **3-5**, the alkyne modified vancomycins **6** and **7,** and the resulting hybrid vancomyxins **8-13** were all assessed for antibacterial activity. As expected, compounds **3-5** were significantly less active than colistin while compounds **6** and **7** showed activity comparable to vancomycin (see supporting information **Table S1**). The vancomyxins **8-13** were initially assessed against two Gram-negative and two Gram-positive strains (**Table 1**). Notably, against the Gram-

negative *E. coli* ATCC25922 strain used, the vancomyxins displayed improved antimicrobial activity, with a >8-fold reduction in MIC compared to vancomycin observed for compounds **8, 9, 11, 12** (MIC going from >128 to 16 μg/mL). The decrease in MIC values was less pronounced for the compounds with PEG_3-based spacers **10** and **13**. Against the *K. pneumonia* ATCC27736 strain used, vancomyxin **11** was found to have an MIC of 8 μg/mL, a >16-fold improvement with respect to vancomycin, while the other vancomyxins were also found to have increased potencies in the range of >4- to >8-fold (**Table 1**). In the case of the Gram-positive *B. subtilis* and *S. aureus* ATCC29213 strains used in this preliminary screen, it was found that conjugation of PMEN to vancomycin did not significantly impair anti-Gram-positive activity relative to the parent vancomycin.



Scheme 2. Synthesis route of vancomyxin 11-15. a) propargylbromide, K_2CO_3, DMF, RT; b) $NaBH_3CN$, DIPEA, DMF/MeOH, 70 °C then 50 °C; c) PMEN-azide **3**, **4**, or **5**, $CuSO_4$, sodium ascorbate, H_2O, RT; d) L-glycine amide or L-arginine amide, PyBOP, DIPEA, DMF/DMSO, RT; e) **3**, $CuSO_4$, sodium ascorbate, H_2O, RT.

Table 1. Preliminary antibacterial activity assessment of vancomyxins 8-13.

	MIC (µg/mL)						
Strain ID	**Vancomycin**	**8**	**9**	**10**	**11**	**12**	**13**
Gram-negative bacteria							
E. coli ATCC25922	>128	16	16	32	16	16	32
K. pneumonia ATCC27736	>128	32	16	64	8	16	32
Gram-positive bacteria							
B. subtilis 168	0.25	0.5	0.25	0.5	0.25	0.25	1
S. aureus ATCC29213	0.125	0.25	0.25	0.25	0.25	0.25	0.5

MIC = Minimum inhibitory concentration

In assessing these preliminary results, it was notable that compounds **10** and **13** consistently exhibited higher MIC values compared to the other vancomyxins, indicating that shorter spacers are preferable. In addition, the location used for attachment of the PMEN moiety to vancomycin (C-terminus or vancosamine) was found to have minimal impact on antibacterial activity, with MIC values differing by no more than 2-fold for the same spacers. Compound **11**, wherein the PMEN motif is connected to the vancosamine functionality, performed particularly well. On the basis of these results against Gram-negative and Gram-positive strains, conjugates **8**, **9**, **11,** and **12** were selected for further assessment and vancomyxin **11** for further modification.

As noted above, recent studies by the groups of Wender and Cegelski have shown that C-terminal modification of vancomycin with a positively charged amino acid (arginine-amide) leads to a significant improvement of anti-Gram-negative activity. These reports prompted us to synthesize two additional compounds building upon vancomyxin **11** wherein either a glycine amide or arginine amide was coupled to the C-terminus of **11**. In doing so it is possible to probe the influence of charge at the C-terminus: whereas the parent vancomyxin **11** contains a negative charge, analogues **14** and **15** are neutral and positively charged respectively (**Fig. 3**). Vancomyxins **14** and **15** were prepared by coupling either glycine amide or arginine amide to the C-terminus of vancomycin alkyne building block **7** (see supporting information **Scheme 2**). Subsequent copper-catalyzed click ligation to azido-PMEN building block **3** yielded analogues **14** and **15**. An expanded antibacterial activity assessment using a variety of Gram-negative and Gram-positive strains was then performed for vancomyxins **14** and **15** along with compounds **8**, **9 11,** and **12**, which were identified as most promising in the initial screen.

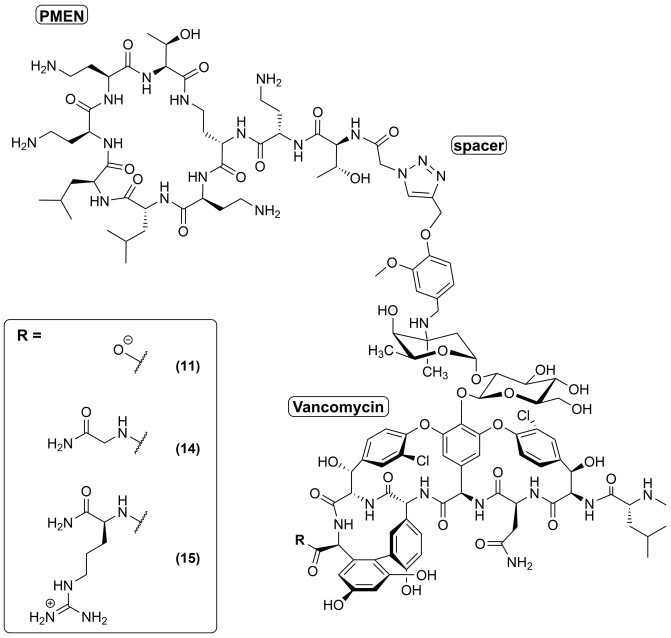


Fig 3. Structure of vancomyxin 11, 14 and 15 with different charges at the C-terminus at neutral pH.

4.2.2 *In vitro* antibacterial activity against Gram-negative bacteria

As anticipated, neither vancomycin nor PMEN showed any activity against the Gram-negative strains tested when administered on their own. However, the addition of 8 μg/mL PMEN to vancomycin resulted in increased activity against *E. coli* (≥4-fold), *K. pneumonia* and *A. baumannii* (both ≥2-fold), as well as *P. aeruginosa* (≥8/16-fold). This synergistic effect of PMEN with vancomycin is in accordance with previous studies.[18,19] In the case of vancomyxins, **8**, **9**, **11**, **12**, **14**, and **15**, however, the covalent linking of the vancomycin and PMEN units was found to enhance the activity against most of the strains tested. Against the two *E. coli* strains used, all six vancomyxins showed improved activity relative to the vancomycin/PMEN combination. Notably, the C-terminally-modified **14** and **15** exhibited the greatest enhancement with a 4-fold improvement in activity against *E. coli* ATCC35218, reaching MICs as low as 8 μg/mL. When tested against *K. pneumonia*, the vancomyxins typically displayed a 4- to 8-fold improved activity compared with the vancomycin/PMEN combination, which itself showed little effect. In this case, **11** was found to be the most active compound with an MIC of 8 μg/mL against both *Klebsiella* strains tested and **12** with an MIC of 8 μg/mL against *K. pneumonia* ATCC13883 (a 16-fold enhancement relative vancomycin + 8 μg/mL PMEN). Similarly, in assays with *A. baumannii*, the vancomycin/PMEN combination was only moderately

active (MIC 128 µg/mL) while the vancomyxins showed 2- to 8-fold improvements in antibacterial activity. Interestingly, while the covalently linked vancomyxins show enhanced activity compared to vancomycin supplemented with PMEN against *E. coli*, *K. pneumonia*, and *A. baumannii*, the opposite pattern was observed in the case of *P.*

Table 2. *In vitro* antibacterial activity assessment of the vancomyxins against Gram negative strains.

Bacterial strain	MIC in µg/mL										
Strain ID	**Vanco mycin**	**Colistin**	**PMEN**	**Vanco mycin + 8 µg/mL PMEN**	**8**	**9**	**11**	**12**	**14**	**15**
E. coli ATCC25922	>128	0.5	>128	32	16	16	16	16	16	16
E. coli ATCC35218	128	0.125	>128	32	16	16	16	16	8	8
K. pneumoniae ATCC13883	>128	0.5	>128	128	32	32	8	8	16	32
K. pneumoniae ATCC27736	>128	0.25	>128	128	32	16	8	16	16	16
A. baumannii ATCC17978	>128	0.25	>128	128	128	64	32	32	128	128
A. baumannii BAA-747	>128	0.125	>128	128	32	64	32	32	16	32
P. aeruginosa ATCC10145	>128	1	>128	16	>128	>128	64	64	32	32
P. aeruginosa ATCC27853	>128	0.5	>128	4	16	32	16	16	16	16

MIC = Minimum inhibitory concentration, nd = not determined, PMEN = Polymyxin E nonapeptide

aeruginosa. Against *P. aeruginosa* ATCC10145 vancomycin supplemented with 8 μg/mL PMEN exhibited an MIC of 16 μg/mL (an >8-fold enhancement) while the vancomyxyins showed little to no improvement relative to vancomycin. A similar trend was observed with *P. aeruginosa* ATCC27853. While the vancomyxins in this case generally displayed an >8-fold reduction in MIC compared to vancomycin alone (16 μg/mL vs >128 μg/mL), PMEN supplementation strongly synergized with vancomycin reducing its MIC to 4 μg/mL (**Table 2**). The greater sensitivity of the *P. aeruginosa* strains to the vancomycin/PMEN combination versus the larger covalently linked vancomyxins may be attributable to the known low-permeability of the *P. aeruginosa* outer membrane.[40] Furthermore, previous investigations have established that *Pseudomonas* strains are particularly sensitive to the polymyxin antibiotics as well as the synergistic effects of the corresponding nonapeptides when co-administered with other Gram-positive specific antibiotics.[41,42]

From a mechanistic perspective, we hypothesized that the enhanced anti-Gram-negative activity of the vancomyxins compared to vancomycin might be due to the ability of PMEN to bind LPS and disrupt the outer membrane.[23,24] To investigate this, we performed an LPS antagonization assay which indeed points to an interaction of compound **11** with LPS, as its MIC against *E. coli* ATCC25922 increased significantly from 16 μg/mL to >128 μg/mL when incubated with exogenous LPS (**Table 3**).

Table 3. LPS antagonization of compound 11 and colistin.

	MIC in μg/mL (MIC in μM shown in brackets)	
	No LPS	**1 mg/mL LPS**
Colistin	0.5 (0.428)	>16 (>14)
Vancomyxin **11**	16 (6)	>128 (>86)

MIC = Minimum inhibitory concentration. Strain used: *E. coli* ATCC25922.

4.2.3 *In vitro* antibacterial activity against Gram-positive bacteria

In assessing the activity of the vancomyxins against Gram-positive bacteria, a number of vancomycin-sensitive (MIC ≤2 μg/mL), vancomycin-intermediate (MIC 4-8 μg/mL) and vancomycin-resistant (≥16 μg/mL) strains were selected (**Table 4**). For the vancomycin-intermediate and vancomycin-resistant strains the effect of PMEN addition at 8 μg/mL was also investigated which, not surprisingly, had no impact on the reduced potency of vancomycin. This lack of synergy was expected given that PMEN serves as an OM disruptor, a barrier only present in Gram-negative strains. Among the vancomycin-sensitive strains tested (MSSA, MRSA and VSE) the vancomyxins were found to exhibit a similar or slightly enhanced activity relative to vancomycin. Notably, when assessed against *B. subtilis*, compounds **8**, **9**, **11** and **12** were found to be as active as vancomycin, while **14** and **15** were found to be extremely potent with activities below 0.008 μg/mL, a

>32-fold improvement. Similarly, all the vancomyxins showed significantly increased potency compared to vancomycin against the strain of *S. simulans* tested. When tested

Table 4. *In vitro* antibacterial activity assessment of the vancomyxins against Gram-positive strains.

Bacterial strain	MIC in µg/mL										
Strain ID	**Vancomycin**	**Colistin**	**PMEN**	**Vancomycin + 8 µg/mL PMEN**	**8**	**9**	**11**	**12**	**14**	**15**
B. subtilis 168	0.25	8	>128	nd	0.5	0.25	0.25	0.25	≤0.008	≤0.008
*S. simulans*22	0.125	4	>128	nd	≤0.008	0.031	0.031	0.016	≤0.008	≤0.008
MSSA ATCC29213	0.125	>128	>128	nd	0.25	0.25	0.25	0.25	0.25	1
MRSA USA300	0.25	>128	>128	nd	0.5	0.25	0.25	0.25	0.25	1
VISA LIM-2	4	>128	>128	4	2	2	2	4	2	8
VISA NRS402	8	>128	>128	8	16	8	8	2	8	4
VRSA 2 (vanA)	128	>128	>128	128	32	64	64	128	>128	>128
VRSA 3b (vanA)	>128	>128	>128	>128	32	>128	32	>128	>128	>128
VRE E1246 (vanA)	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128
VRE E7406 (vanB)	32	>128	>128	32	8	64	64	128	128	128
VSE E980	0.5	>128	>128	nd	0.25	0.25	0.25	0.25	0.125	0.125
VRE E155 (vanA)	>128	>128	>128	>128	64	64	64	64	64	8
VRE E7314 (vanB)	128	>128	>128	128	2	8	2	8	0.5	0.031

MIC = Minimum inhibitory concentration, nd = not determined, PMEN = Polymyxin E nonapeptide, MSSA = Methicillin-sensitive *S. aureus*, MRSA = Methicillin-resistant *S. aureus*, VISA = Vancomycin-intermediate *S. aureus*, VRSA = Vancomycin-resistant *S. aureus*, VSE = Vancomycin-sensitive *Enterococci*, VRE = Vancomycin-resistant *Enterococci*. E1246 and E7406 are *E. faecalis*, E980, E155 and E7315 are *E. faecium* strains.

against VISA, the vancomyxins demonstrated potencies similar or slightly enhanced relative to vancomycin. Interestingly however, against VRSA, compounds **9**, **12**, **14** and **15** were found to show no enhancement of activity while compounds **8** and **11** displayed improved antibacterial potencies with a ≥4-fold reduction of MIC values compared to vancomycin. Among the VRE strains tested, the activities of the vancomyxins relative to vancomycin were found to be highly variable. Against vancomycin-resistant *E. faecalis*, the vancomyxins showed little-to-no enhancement whereas against vancomycin-resistant *E. faecium*, particularly the VanB type, the vancomyxins exhibited potent antibacterial activity. Specifically, for the VanB type *E. faecium* strain tested, vancomyxins **9** and **12** have an MIC of 8 μg/mL and vancomyxins **8** and **11** have MIC values of 2 μg/mL, while **14** and **15** demonstrated even more impressive potencies with MICs of 0.5 μg/mL and 0.031 μg/mL respectively (**Table 4**).

The enhanced antimicrobial activities observed for the vancomyxins against vancomycin-resistant strains suggest that our novel compounds are able to (partially) compensate for the reduced binding of the vancomycin core to the D-Ala-D-Lac lipid II motif common to VREs containing either *vanA* or *vanB* resistance genes. It is well established that mutation of the D-Ala-D-Ala unit found in wild type lipid II to the D-Ala-D-Lac unit found in VanA or VanB positive strains results in a >1000-fold reduction in binding affinity for vancomycin and loss of antimicrobial activity.[1,2,4,43] The finding that the vancomyxins overcome this resistance, especially in the case of VanB positive *E. faecium*, indicates that other structural features are contributing to their enhanced activity. Specifically, the large positive net charge introduced by conjugating the PMEN motif to vancomycin may facilitate electrostatic interactions with the negatively charged bacterial membrane. This in turn may lead to increased membrane anchoring and lipid II engagement thereby interfering with cell wall synthesis and lowering the MIC of the vancomyxins towards otherwise vancomycin-resistant strains. In this regard it is noteworthy that previous reports have also described the addition of positively charged moieties to vancomycin as means of overcoming vancomycin resistance.[4,15,31–35] Furthermore, conjugation of vancomycin to bacteriocin nisin(1-12) has previously shown to reduce the MIC against VRE.[44]

As described above, vancomyxins **14** and **15** were synthesized to assess if a difference in charge at the C-terminus (neutral and positive) compared to **11** (negative) would enhance activity. Against Gram-negative strains this did not appear to have a large effect on activity as the MICs of vancomyxins **11**, **14** and **15** are generally similar, differing by no more than 2-fold. Against Gram-positive strains, however, there are notable species-dependent differences; against VRSA the negatively charged C-terminus of **11** results in activity superior to that of the neutral **14** and positive-charged **15**, while

against vancomycin-resistant *E. faecium* the positively charged **15** exhibits enhanced activity.

4.2.4 Cell-based toxicity studies

In parallel to the antibacterial activity assays, the hemolytic properties of the vancomyxins were also assessed, revealing them to be non-hemolytic up to the highest concentration tested (512 µg/mL). These findings are in line with our expectations given the non-hemolytic nature of both vancomycin and colistin (**Fig. 4A**). We next turned our attention to evaluating the nephrotoxicity of the vancomyxins. Reports in the literature indicate that both polymyxins and vancomycin can impair kidney function.[45,46] In particular, proximal tubule epithelial cells are known to be sensitive to the polymyxins as a result of extensive reabsorption and intracellular accumulation.[46] In addition, proximal tubule cells have been previously used to characterize the cytotoxic effects of vancomycin.[47] To compare the hybrid vancomyxins with the corresponding parent compounds, their nephrotoxicity was assessed by means of a viability assay using conditionally immortalized proximal tubule epithelial cells (ciPTECs), with relative mitochondrial activity after 24 hours as the endpoint measurement. In these assays, polymyxin B was found to exhibit relatively high nephrotoxicity (TC_{50} = 0.07 mM) while vancomycin and PMEN were significantly less toxic (>50% viability at a concentration of 1 mM for both). By comparison, the vancomyxins were found to exhibit intermediate toxicity towards ciPTECs with TC_{50} values ranging from 0.11 mM for compound **15** to 0.37 mM for compound **8** (**Table 5** and **Fig. 4B**), concentrations generally multiple orders of magnitude higher than the corresponding MIC values.

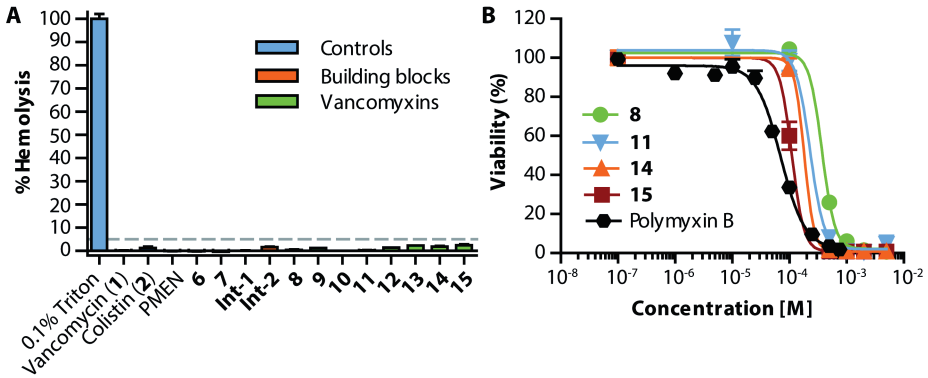


Fig. 4. Toxicity assessment of the vancomyxins. (**A**) Hemolytic activity of the vancomyxins and their intermediates. (**B**) Cytotoxicity of the vancomyxins against conditionally immortalized proximal tubule epithelial cells. *Experiment (**B**) performed by Jaco Slingerland.*

Table 5. TC_{50} values for the vancomyxins.

Sample Id	TC_{50} (mM)
Vancomycin (**1**)	> 1
PMEN	> 1
8	0.37
11	0.23
14	0.18
15	0.11
Polymyxin B	0.07

Experiment performed by Jaco Slingerland.

4.3 Conclusions

While vancomycin is an important antibiotic for the treatment of hospitalized patients with Gram-positive infections, it has little activity against Gram-negative bacteria due to the inaccessibility of its target. Given that vancomycin can bind to the Gram-negative form of lipid II,[6] OM disruptors present a possible means for enhancing the activity of vancomycin against Gram-negative pathogens. [8,10,18,19] To this end, combination strategies involving polymyxin nonapeptide or other OM disruptors have been explored.[10,18,19] However, the covalent conjugation of vancomycin to the OM disrupting PMEN motif has not been previously described. In this study we report a new class of vancomycin-PMEN hybrids, the vancomyxins. The vancomyxins maintain the activity of vancomycin against vancomycin-sensitive strains and in some cases also overcome vancomycin-resistance in Gram-positive organisms. Against Gram-negative organisms the vancomyxins also show enhanced activity that was generally superior to that observed with a simple combination of vancomycin with PMEN. It is worth noting that when comparing the activities of the hybrid vancomyxins to vancomycin or the combination of vancomycin/PMEN, the conventional concentration units of μg/mL was used. However, given that the molecular weights of the vancomyxins are approximately double that of vancomycin, the differences in MIC are even more pronounced when comparing the appropriate molar concentrations (see supporting information **Tables S1 and S2**). In addition, LPS binding of the vancomyxins is confirmed. Furthermore, the vancomyxins are not hemolytic and exhibit lower toxicity against kidney cells compared to the clinically used polymyxin B. In summary, these findings indicate that the covalent attachment of an OM disrupting PMEN motif to vancomycin is a viable strategy for enhancing its anti-Gram-negative activity.

4.4 Experimental Methods

General procedures. All reagents were commercially available, American Chemical Society (ACS) grade or finer and used without further purification unless stated otherwise. For characterization of new compounds high resolution mass spectrometry (HRMS) was performed on a Shimadzu Nexera X2 UHPLC system with a Waters Acquity HSS C_{18} column (2.1 × 100 mm, 1.8 μm) at 30 °C and equipped with a diode array detector. At a flow rate of 0.5 mL/min, a solvent system with solvent A, 0.1% formic acid in H_2O, and solvent B, 0.1% formic acid in CH_3CN, was used. Gradient elution was as follows: 95:5 (A/B) for 1 min, 95:5 to 15:85 (A/B) over 6 min, 15:85 to 0:100 (A/B) over 1 min, 0:100 (A/B) for 3 min, then reversion back to 95:5 (A/B) for 3 min. This system was connected to a Shimadzu 9030 QTOF mass spectrometer (ESI ionization) calibrated internally with Agilent's API-TOF reference mass solution kit (5.0 mM purine, 100.0 mM ammonium trifluoroacetate and 2.5 mM hexakis(1*H*,1*H*,3*H*-tetrafluoropropoxy)phosphazine) diluted to achieve a mass count of 10000. Purity and confirmation of the synthesis of small molecule building blocks, although previously reported in the literature, was assessed with nuclear magnetic resonance (NMR). Spectra were obtained from a Bruker DPX-300, super conducting magnet with a field strength of 7.0 Tesla, equipped with 5 mm BBO, Broadband Observe probe head, high resolution with Z- Gradient, and a 5 mm 19F / 1H dual high-resolution probe.

Compounds were purified using preparative high performance liquid chromatography (HPLC) using a BESTA-Technik system with a Dr. Maisch Reprosil Gold 120 C_{18} column (25 × 250 mm, 10 μm) and equipped with a ECOM Flash UV detector monitoring at 214 nm. All compounds were ran at a flow rate of 12 mL/min. For the vancomyxins and PMEN a solvent system with solvent A, 0.1% TFA in H_2O/CH_3CN 95:5, and solvent B, 0.1% TFA in H_2O/CH_3CN 5:95, was used. For the vancomyxins (**8-15**) the gradient elution was as follows: 95:5 (A/B) for 5 min, 95:5 to 40:60 (A/B) over 50 min, 40:60 to 0:100 (A/B) for 1 min, 0:100 (A/B) for 2 min, then reversion back to 95:5 (A/B) over 1 min, 95:5 (A/B) for 2 min. For PMEN the gradient elution was as follows: 100:0 (A:B) for 5 min, 100:0 to 70:30 (A:B) over 50 min, 70:30 to 0:100 (A:B) for 1 min, 0:100 (A:B) for 2 min, then reversion back to 100:0 (A:B) over 1 min, 100:0 (A:B) for 2 min. The vancomycin building blocks (**7**, **Int-1, Int-2**) had an alternative solvent system of solvent A, 50 mM ammonium acetate, and solvent B, H_2O/CH_3CN 5/95. Gradient elution was as follows: 95:5 (A/B) for 2 min, 95:5 to 80:20 (A/B) for 5 min, 80:20 to 40:60 (A/B) over 40 min, 40:60 to 0:100 (A/B) for 1 min, 0:100 (A/B) for 2 min, then reversion back to 95:5 (A/B) over 1 min, 95:5 (A/B) for 2 min.

Purity of the vancomyxins was assessed by integration and confirmed to be >95% unless stated otherwise (see supporting information **Fig. S1**), using analytical

reverse phase HPLC (RP-HPLC) using a Shimadzu Prominence-i LC-2030 system with a Dr. Maisch ReproSil Gold 120 C_{18} column (4.6×250 mm, 5 µm) at 30 °C and equipped with a UV detector monitoring at 214 nm. At a flow rate of 1 mL/min, a solvent system with solvent A, 0.1% TFA in H_2O/CH_3CN 95:5, and solvent B, 0.1% TFA in H_2O/CH_3CN 5:95, was used. Gradient elution was as follows: 95:5 (A/B) for 2 min, 95:5 to 0:100 (A/B) over 55 min, 0:100 (A/B) for 2 min, then reversion back to 95:5 (A/B) over 1 min, 95:5 (A/B) for 2 min.

Bacterial strains used for MIC assays. The following strains were obtained from BEI Resources, NIAID, NIH: *Staphylococcus aureus*, Strain HIP12864, NR-46074. *Staphylococcus aureus*, Strain LIM 2, NR-45881. *Staphylococcus aureus*, Strain HIP11983, NR-46411. *Staphylococcus aureus*, Strain HIP13419, NR-46413.

Synthesis of PMEN. PMEN was obtained by enzymatic digestion of colistin by ficin, by modification of a previously reported method.[48] Colistin sulphate (4.8 mmol, 1 eq) was dissolved in H_2O (180 mL). To the solution were added dithiothreitol (1.3 mmol, 0.3 eq) and ficin (~0.06 mmol, 0.01 eq). Enzymatic cleavage was run at 37 °C under nitrogen atmosphere overnight. Additional dithiothreitol (0.3 mmol 0.06 eq) and ficin (~0.01 mmol, 0.002 eq) were added, followed by incubation overnight. Once complete, the solution was heated to reflux for 20 min, cooled down and filtered. The filtrate was adjusted to pH 2 with 5 M HCl. Sample was extracted by *n*-butanol (5 x 50 mL). The pH of the aqueous layer was neutralized with 6 M NaOH. The resulting sample was lyophilized after addition of *t*-BuOH. Pure PMEN was obtained by reverse phase HPLC purification. *Experiment performed by Jaco Slingerland.*

Synthesis of PMEN-Boc$_4$. PMEN-Boc_4 was prepared as previously described.[39] PMEN (semi-pure after extraction, 2.0 g, 2.2 mmol) was dissolved in H_2O (13 mL). Triethylamine (13 mL) was added to it and the mixture was stirred for 5 min. 2-(Boc-oxyimino)-2-phenylacetonitrile (Boc-ON) was dissolved in dioxane (13 mL) and added to the PMEN. Reaction was run at RT for 25 min. Reaction was quenched by the addition of methanolic NH_3 (7 M, 8 mL). The resulting mixture was concentrated on the rotavap. The residue was dissolved in MeOH (200 mL) and filtered. The filtrate was collected, concentrated and subjected to flash column chromatography (5% MeOH/DCM – 10% MeOH/DCM/0.5% Et_3N). Relevant fractions were combined and solvent was evaporated. Yield: 1.9 g (1.4 mmol, 65% (~ 90% pure)). *Experiment performed by Jaco Slingerland.*

Synthesis of PMEN azides ***3-5***

*PMEN-C_2-N_3 (**3**).* PMEN-Boc_4 (0.45 g, 0.34 mmol) was dissolved in DCM and DMF (8:2 v:v, 10 mL). In a separate flask, 2-azidoacetic acid (68 mg, 0.68 mmol) and BOP (0.30 g, 0.68 mmol) were dissolved in DCM (8 mL). The mixture of 2-azidoacetic acid and BOP

was then added to the PMEN-Boc_4, followed by addition of DIPEA (0.24 mL, 1.4 mmol). The reaction was left to stir overnight at RT under N_2 atmosphere. After completion, the solvent was evaporated and the residue treated with TFA/TIPS/H_2O (95:2.5:2.5, 8 mL) for 1.5 h. The reaction mixture was added to ice-cold MTBE/PE (2/1, 120 mL). The resulting precipitate was washed with MTBE/PE (2/1). Crude peptide was lyophilized from t-BuOH/H_2O and HPLC purified. Yield: 130 mg, 0.13 mmol, 39%. *Experiment performed by Jaco Slingerland.*

*PMEN-C_5-N_3 (**4**).* Compound was prepared as PMEN-C_2-N_3 (**3**), starting from PMEN-Boc_4 and 5-azidopentanoic. Yield: 85 mg, 0.08 mmol, 36%. *Experiment performed by Jaco Slingerland.*

*PMEN-$(PEG)_3$-N_3 (**5**).* Compound was prepared as PMEN-C_2-N_3 (**3**), starting from PMEN-Boc_4 and 3-(2-(2-(2-azidoethoxy)-ethoxy)ethoxy)propanoic acid. Yield: 120 mg, 0.11 mmol, 43%. *Experiment performed by Jaco Slingerland.*

*Synthesis of vancomycin alkynes **6-7**.* Vancomycin alkyne building block **6** was synthesized as previously described[3] and used without any further purification. Vancomycin alkyne building block **7** was synthesized according to procedures described previously with minor alterations.[31,38] In short, 3-methoxy-4-(prop-2-yn-1-yloxy)benzaldehyde was synthesized starting from vanillin as described in the literature.[49] Subsequently, amine-derivatized vancomycin **7** was prepared by dissolving vancomycin HCl (1.3 mmol, 1 eq) in 1/1 DMF/MeOH (40 mL). 3-methoxy-4-(prop-2-yn-1-yloxy)benzaldehyde (2.6 mmol, 2 eq) and DIPEA (6.5 mmol, 5 eq) were added and the reaction was stirred at 70 °C for 2 h. Next the mixture was cooled to 50 °C and $NaBH_3CN$ (13 mmol, 10 eq) was added. After 5 h another 10 eq of $NaBH_3CN$ were added and after 16 h again 1 eq of 3-methoxy-4-(prop-2-yn-1-yloxy)benzaldehyde and 10 eq of $NaBH_3CN$ were added. The reaction was stirred for another 24 h before a few mL of H_2O were added. Solvent was evaporated and the mixture was dissolved in a minimum amount of DMF. Product was precipitated in cold Et_2O twice (2 x 600 mL). The precipitate was redissolved in HPLC buffer and purified using preparative HPLC. Fractions were analyzed using analytical HPLC and pure fractions were pooled and lyophilized.

*Synthesis of C-terminally modified vancomycin alkynes **Int-1** and **Int-2**.* **Int-1** and **Int-2** were synthesized according to a previous procedure.[17] In short, **7** (62 µmol, 1 eq) was dissolved in 1/1 DMF/DMSO (5 mL). Glycine amide HCl or arginine amide HCl (124 µmol, 2 eq) was added and the mixture was cooled to 0 °C. DIPEA (310 µmol, 5 eq) and PyBOP (93 µmol, 1.5 eq) were added. The mixture was allowed to warm to RT and stirred for 16 h. Additional equivalents of glycine/arginine amide HCl, DIPEA, and PyBOP were added at 0 °C and the reaction was further stirred at RT until LCMS showed complete

disappearance of starting material **7**. DMF was evaporated and 600 mL CH_3CN was added to precipitate the product. The mixture was passed over a filter and washed with 600 mL CH_3CN and 600 mL Et_2O. **Int-1** was used in the next reaction without intermediate purification. **Int-2** was dissolved in HPLC buffer and purified using preparative HPLC. Fractions were analyzed using analytical HPLC and pure fractions were pooled and lyophilized.

*Copper-catalyzed azide-alkyne cycloaddition to synthesize vancomyxins (**8-15**)*. The ligation protocol used generally followed that previously described by Silverman *et al*.[38] In short, to a solution of the vancomycin alkyne (0.03 mmol, 1 eq) in H_2O (1.5 mL), the PMEN azide (0.03 mmol, 1 eq) in H_2O (1.5 mL) was added. Subsequently, sodium ascorbate (0.008 mmol, 0.25 eq) and $CuSO_4 \cdot 5H_2O$ (0.003 mmol, 0.1 eq) were added and the mixture was allowed to stir at RT for 16 h. The reaction mixture was directly purified using preparative high performance liquid chromatography (HPLC) using a C_{18} column (25 × 250 mm, 10 µm) with UV detection at 214 nm. The following method was used: Flow rate = 12 mL/min; solvent A, 0.1% TFA in H_2O/CH_3CN 95:5, and solvent B, 0.1% TFA in H_2O/CH_3CN 5:95. The gradient elution was as follows: 95:5 (A/B) for 5 min, 95:5 to 40:60 (A/B) over 50 min, 40:60 to 0:100 (A/B) for 1 min, 0:100 (A/B) for 2 min, then reversion back to 95:5 (A/B) over 1 min, 95:5 (A/B) for 2 min. Fractions were immediately freeze dried and subsequently analyzed by LCMS. Pure product containing fractions were redissolved, pooled, and lyophilized to yield the vancomyxins as white powders (for yields and HRMS characterization see **Table 6**).

Table 6. HRMS analysis and yields.

Sample ID	Chemical formula	Calculated M + H	Calculated (M+2H)/2	Measured	Yield
3	$C_{42}H_{77}N_{17}O_{12}$	1012.6016	506.8047	506.8043	39%
4	$C_{45}H_{83}N_{17}O_{12}$	1054.6485	527.8282	527.8278	36%
5	$C_{49}H_{91}N_{17}O_{15}$	1158.6959	579.8519	579.8515	43%
6	$C_{69}H_{78}Cl_2N_{10}O_{23}$	1485.4696	743.2387	743.2379	65%
7	$C_{77}H_{85}Cl_2N_9O_{26}$	1622.5061	811.7570	811.7562	52%
8	$C_{111}H_{155}Cl_2N_{27}O_{35}$	2497.0634	1249.0356	1249.0345	54%
9	$C_{114}H_{161}Cl_2N_{27}O_{35}$	2539.1103	1270.0591	1270.0581	74%
10	$C_{118}H_{169}Cl_2N_{27}O_{38}$	2643.1577	1322.0828	1322.0821	55%
11	$C_{119}H_{162}Cl_2N_{26}O_{38}$	2634.0998	1317.5538	1317.5525	53%
12	$C_{122}H_{168}Cl_2N_{26}O_{38}$	2676.1468	1338.5773	1338.5762	38%
13	$C_{126}H_{176}Cl_2N_{26}O_{41}$	2780.1941	1390.6010	1390.6000	18%
Int-1	$C_{79}H_{89}Cl_2N_{11}O_{26}$	1678.5435	839.7757	839.7769	78%
Int-2	$C_{83}H_{98}Cl_2N_{14}O_{26}$	1777.6232	889.3155	889.3165	29%
14	$C_{121}H_{166}Cl_2N_{28}O_{38}$	2690.1373	1345.5726	1345.5742	44%
15	$C_{125}H_{175}Cl_2N_{31}O_{38}$	2789.2169	1395.1124	1395.1142	51%

Broth microdilution assays. From glycerol stocks bacteria were plated out on blood agar plates overnight at 37°C. One colony was transferred to growth media and grown at 37°C at 200 rpm to exponential growth phase as determined by OD_{600}. The growth media used for most strains was cation adjusted Mueller Hinton Broth (0.5 mM Mg^{2+} and Ca^{2+}) + 0.002% of polysorbate 80 (p80), except for all *Enterococci* and *VISA/VRSA* strains, for which the positive control was unable to grow well in this media, therefore TSB + 0.002% p80 was used in these cases. For VRSA the media was supplemented with 6 µg/mL vancomycin at this stage. At OD_{600} = 0.5 the bacteria were diluted 100-fold in media (not supplementing vancomycin from here on for VRSA) and 50 µL was added to a 2-fold serial dilution series of test compound (50 µL) to reach a total volume of 100 µL per well. The 96-well polypropylene plates were incubated at 37°C at 600 rpm overnight (18-20 h for Gram-negative strains, 20-24 h for Gram-positive strains) and plates were inspected for visual bacterial growth. Synergy experiments were performed in a similar manner as the MIC assay, except 8 µg/mL PMEN final concentration was added to the wells. LPS antagonization assays were performed in a similar manner except that a final concentration of 1 mg/mL LPS was added to the wells. In this case colistin was used as a control. MICs are reported as the median of triplicates.

Hemolysis assays. Defibrinated whole sheep blood was centrifuged for 15 minutes at 4°C (400 g). The top layer was discarded and the bottom layer was washed with phosphate buffered saline (PBS) and centrifuged for 15 minutes at 4°C (400 g). Washing cycles were repeated at least three times. In polypropylene 96-well microtiter plates 10-fold serial dilutions of antibiotics in PBS with 0.002% p80 in biological triplicates were added (75 µL) and an equal volume of packed blood cells diluted 25x in PBS with 0.002% p80 (75 µL) was added to all wells. Plates were incubated for 20 h at 37°C with continuous shaking (500 rpm). After incubation, plates were centrifuged for 5 min (800 g) and 25 µL of supernatant was transferred to a clear UV-star flat-bottom polystyrene 96-well plate already containing 100 µL H_2O per well. Absorption was measured at 415 nm. Data were corrected by subtraction of the background response of 1% DMSO in the presence of cells with no antibiotic and normalized using the absorbance of 0.1% Triton X-100 with blood cells as 100% hemolysis control.

PTECs assay – Cell culture. ciPTECs overexpressing organic anion transporter 1 (OAT-1)[50,51] were cultured in DMEM/F12 medium, supplemented with fetal calf serum (10%), insulin (5 µg/mL), transferrin (5 µg/mL), selenium (5 µg/mL), hydrocortisone (35 ng/mL), Epidermal Growth Factor (10 ng/mL) and tri-iodothyronine (40 pg/mL). Cells were cultured at 33°C for sustained proliferation. For the experiment, cells were washed with HBSS and detached by incubating them with Accutase® solution for 5 minutes at 37°C. Density was adjusted to $2.0x10^5$ cells/mL of which 100 µL was added to each well of a 96 well plate. Seeded cells were incubated for 24 h at 33°C, followed by 6 days

incubation at 37°C to allow them to fully differentiate. Medium was refreshed every second or third day. All cells were grown in a humidified atmosphere containing 5% (v/v) CO_2.

PTECs assay – Cell viability assay. Cytotoxicity was assessed using PrestoBlue™ cell viability reagent. Compounds were dissolved and diluted in serum free medium. Differentiated ciPTECs were washed once with HBSS and exposed to the compounds for 24 h at 37°C. Afterwards, cells were washed with HBSS, and incubated with 10% PrestoBlue™ reagent in HBSS at 37°C for 1 h in the dark. Fluorescence was recorded using excitation wavelength of 530 nm and emission wavelength of 590 nm. Raw data were corrected for PrestoBlue™ background fluorescence and reported relative to the no-treatment control (cells with medium only). Data were fitted with Graphpad Prism software by non-linear regression with 0 as constraint to obtain TC_{50} values. Presented data are based on triplicates and presented as mean ± S.E.M (or SD).

Table S1. *In vitro* antibacterial activity assessment (in µM) of the vancomyxins.

Bacterial strain	MIC in µM									
Strain ID	**Vancomycin**	**Colistin**	**PMEN**	**Vancomycin + 8 µg/mL PMEN**	**8**	**9**	**11**	**12**	**14**	**15**
E. coli ATCC25922	>86	0.428	>138	22	6	6	6	6	6	6
E. coli ATCC35218	86	0.107	>138	22	6	6	6	6	3	3
K. pneumoniae ATCC13883	>86	0.428	>138	86	13	13	3	3	6	11
K. pneumoniae ATCC27736	>86	0.214	>138	86	13	6	3	6	6	6
A. baumannii ATCC17978	>86	0.214	>138	86	51	25	12	12	48	46
A. baumannii BAA-747	>86	0.107	>138	86	13	25	12	12	6	11
P. aeruginosa ATCC10145	>86	0.855	>138	11	>51	>50	24	24	12	11
*P. aeruginosa*ATCC27853	>86	0.428	>138	3	6	13	6	6	6	6
B. subtilis 168	0.168	7	>138	nd	0.2	0.098	0.095	0.093	≤0.003	≤0.003
S. simulans 22	0.084	3	>138	nd	≤0.003	0.012	0.012	0.006	≤0.003	≤0.003
MSSA ATCC29213	0.084	>109	>138	nd	0.1	0.098	0.095	0.093	0.093	0.358
MRSA USA300	0.168	>109	>138	nd	0.2	0.098	0.095	0.093	0.093	0.358
VISA LIM-2	3	>109	>138	3	0.8	0.79	0.76	1	0.74	3
VISA NRS402	5	>109	>138	5	6	3	3	0.75	3	1
VRSA 2 (vanA)	86	>109	>138	86	13	25	24	48	>48	>46
VRSA 3b (vanA)	>86	>109	>138	>86	13	>50	12	>48	>48	>46
VRE E1246 (vanA)	>86	>109	>138	>86	>51	>50	>49	>48	>48	>46
VRE E7406 (vanB)	22	>109	>138	22	3	25	24	48	48	46
VSE E980	0.337	>109	>138	nd	0.1	0.098	0.095	0.093	0.046	0.045
VRE E155 (vanA)	>86	>109	>138	>86	26	25	24	24	24	3
VRE E7314 (vanB)	86	>109	>138	86	0.8	3	0.76	3	0.19	0.01

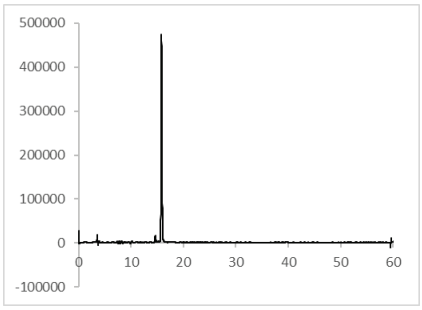
MIC = Minimum inhibitory concentration, nd = not determined, PMEN = Polymyxin E nonapeptide, MSSA = Methicillin-sensitive *S. aureus*, MRSA = Methicillin-resistant *S. aureus*, VISA = Vancomycin-intermediate *S. aureus*, VRSA = Vancomycin-resistant *S. aureus*, VSE = Vancomycin-sensitive *Enterococci*, VRE = Vancomycin-resistant *Enterococci*. E1246 and E7406 are *E. faecalis*, E980, E155 and E7315 are *E. faecium* strains.

Table S2. *In vitro* antibacterial activity assessment of the PMEN-azide and vancomycin-alkyne building blocks.

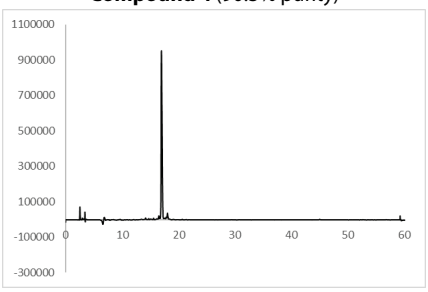
Bacterial strain	**MIC in µg/mL** (MIC in µM shown in brackets)						
Strain ID	**3**	**4**	**5**	**6**	**7**	**Int-1**	**Int-2**
*E. coli*ATCC25922	64 (63)	8 (8)	128 (111)	64 (43)	>128 (>79)	64 (38)	16 (9)
E. coli ATCC35218	64 (63)	8 (8)	32 (28)	32 (22)	>128 (>79)	32 (19)	8 (4)
K. pneumoniae ATCC13883	32 (32)	4 (4)	32 (28)	>128 (>86)	>128 (>79)	>128 (>76)	128 (72)
K. pneumoniae ATCC27736	32 (32)	8 (8)	64 (55)	>128 (>86)	>128 (>79)	>128 (>76)	128 (72)
A. baumannii ATCC17978	nd	nd	nd	128 (86)	>128 (>79)	128 (76)	128 (72)
A. baumannii BAA-747	>128 (>126)	>128 (>121)	>128 (>111)	128 (86)	>128 (>79)	128 (76)	128 (72)
P. aeruginosa ATCC10145	8 (8)	4 (4)	16 (14)	>128 (>86)	>128 (>79)	>128 (>76)	>128 (>72)
P. aeruginosa ATCC27853	nd	nd	nd	>128 (>86)	>128 (>79)	>128 (>76)	>128 (>72)
B. subtilis 168	0.25 (0.168)	>128 (>138)	8 (7)	0.125 (0.084)	0.5 (0.308)	0.125 (0.074)	0.016 (0.009)
S. simulans 22	0.125 (0.084)	>128 (>138)	4 (3)	0.25 (0.168)	1 (0.616)	0.25 (0.149)	0.125 (0.07)
MSSA ATCC29213	>16 (>16)	>16 (>15)	>16 (>14)	0.5 (0.336)	2 (1)	0.5 (0.298)	0.5 (0.281)
MRSA USA300	>16 (>16)	>16 (>15)	>16 (>14)	0.5 (0.336)	2 (1)	0.5 (0.298)	0.5 (0.281)
VISA LIM-2	nd	nd	nd	2 (1)	16 (10)	4 (2)	2 (1)
VISA NRS402	nd	nd	nd	4 (3)	16 (10)	4 (2)	4 (2)
VRSA 2 (vanA)	nd	nd	nd	>128 (>86)	>128 (>79)	>128 (>76)	>128 (>72)
VRSA 3b (vanA)	nd	nd	nd	>128 (>86)	>128 (>79)	>128 (>76)	>128 (>72)
*E.*VRE 1246 (vanA)	nd	nd	nd	>128 (>86)	>128 (>79)	>128 (>76)	>128 (>72)
VRE 74064 (vanB)	nd	nd	nd	16 (11)	32 (20)	64 (38)	32 (18)
VSE E980	nd	nd	nd	1 (0.673)	1 (0.616)	0.5 (0.298)	0.25 (0.141)
VRE 155 (vanA)	nd	nd	nd	>128 (>86)	>128 (>79)	>128 (>76)	64 (36)
VRE 7314 (vanB)	nd	nd	nd	128 (86)	64 (39)	32 (19)	4 (2)

MIC = Minimum inhibitory concentration, nd = not determined, PMEN = Polymyxin E nonapeptide, MSSA = Methicillin-sensitive *S. aureus*, MRSA = Methicillin-resistant *S. aureus*, VISA = Vancomycin-intermediate *S. aureus*, VRSA = Vancomycin-resistant *S. aureus*, VSE = Vancomycin-sensitive *Enterococci*, VRE = Vancomycin-resistant *Enterococci.* E1246 and E7406 are *E. faecalis*, E980, E155 and E7315 are *E. faecium* strains.

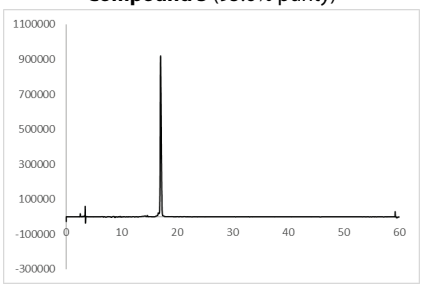
Compound 3 (90.8% purity)



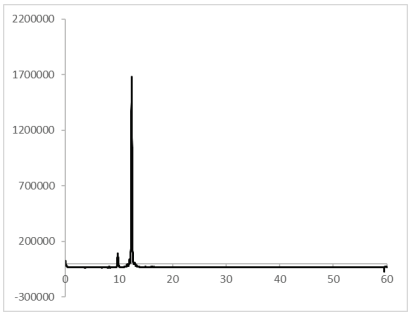
Compound 4 (90.3% purity)



Compound 5 (95.6% purity)



Compound 6 (85.4% purity)



Compound 7 (90.1% purity)

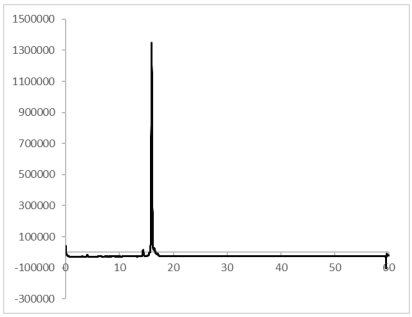
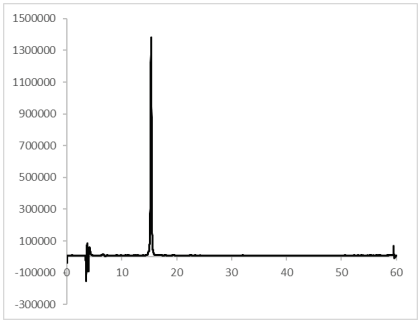
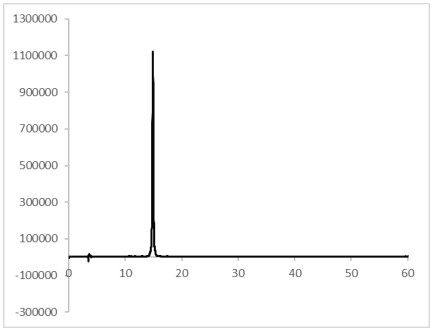


Fig. S1. Purity of intermediates and vancomyxins determined by analytical HPLC.

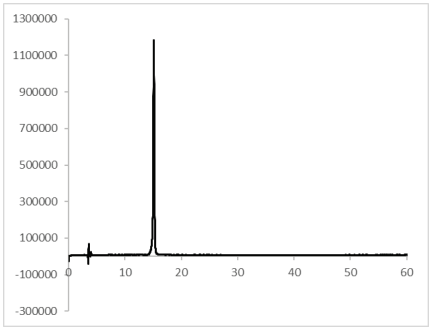
Vancomyxin 8 (98.6% purity)



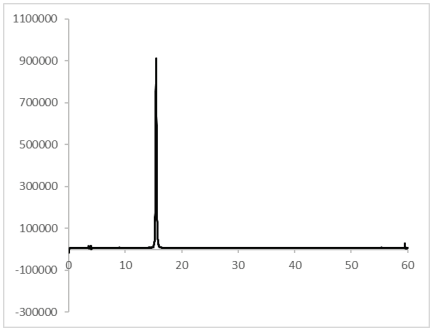
Vancomyxin 9 (97.0% purity)



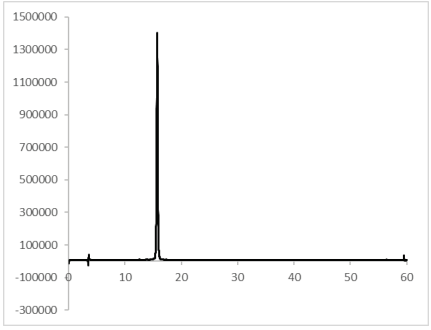
Vancomyxin 10 (98.2% purity)



Vancomyxin 11 (99.0% purity)



Vancomyxin 12 (99.9% purity)



Vancomyxin 13 (96.4% purity)

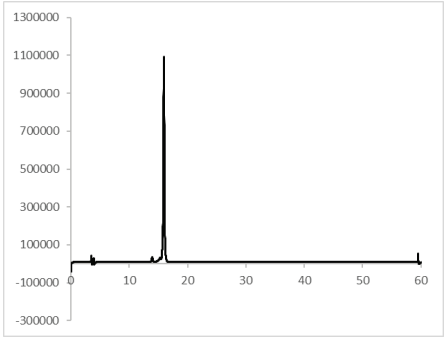


Fig. S1. Purity of intermediates and vancomyxins determined by analytical HPLC (continued).

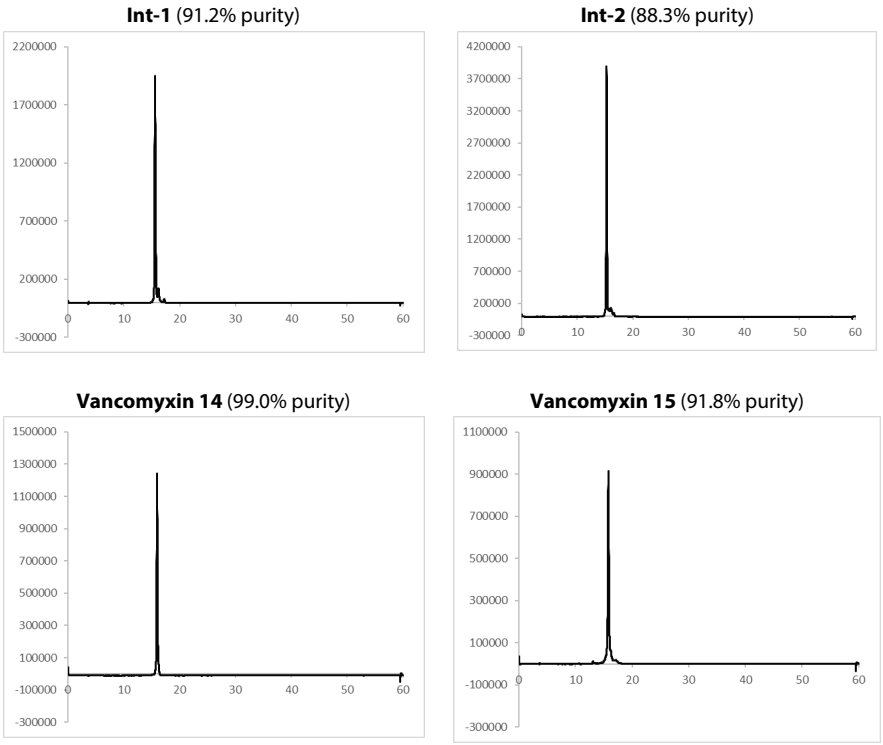


Fig. S1. Purity of intermediates and vancomyxins determined by analytical HPLC (continued).

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